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Dialdehyde-containing nucleic acids and their components: synthesis, properties and affinity modification of proteins

O M Gritsenko, E S Gromova

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Abstract. Synthesis, structure and chemical properties of nucleoside dialdehyde derivatives are described. The introduction of aldehyde groups into oligonucleotides is discussed. The methods of affinity modification of proteins with periodate-oxidised nucleic acid derivatives are considered. The bibliography includes 48 references.

I. Introduction

The study of the nature of specific interactions of proteins with nucleic acids (NA) is an important problem of modern molecular biology. Relatively few methods allowing the identification of functional groups of proteins involved in protein – NA recognition are known. Covalent binding of proteins to NA is an efficient approach.[1] It consists in specific covalent attachment of a protein to NA by modification of one of the components of the protein — NA complex or by addition of a cross-linking reagent. The covalently bound protein is subjected to hydrolysis with proteases and then the amino acid composition of the peptide attached to NA, which is the region of the active site or a recognition domain of the protein, is determined. The method of covalent 'cross-linking' also makes it possible to study the topography of the protein – NA complexes and to establish their stoichiometry.

Elaboration of optimal conditions for protein – NA recognition is an essential feature required for this method. In this case, the modification must not induce significant distortions in the structure of the components of the complex and not interfere with the recognition of NA analogues by specific proteins. Affinity modification of non-modified proteins with NA upon UV irradiation is a way to perform covalent addition of proteins to NA.[1] Another approach consists in the introduction of chemically or photo-activated groups into a protein or NA followed by their activation. Reactive groups are introduced into heterocyclic bases of NA, into phosphate groups or into sugar residues. The first two types of NA activation and the formation of covalently bound products based on these derivatives have been described in several reviews.[1–3] In the present work, we shall consider NA derivatives containing two aldehyde groups in the sugar residue with regard to their synthesis, properties and affinity modification of proteins.

Earlier, dialdehyde derivatives of NA were extensively used for establishing the structure of monomeric components of nucleic acids and for the analysis and separation of oligonucleotide mixtures.[4] The nucleotide sequences adjacent to the 3′-end of RNA were determined using oxidation of the 3′-terminal nucleotide unit of RNA with subsequent cleavage of the adjacent phosphodiester bond and identification of a heterocyclic base.[4] At present, dialdehyde derivatives of NA are most often used for introduction of various functional groups into NA and affinity modification of proteins.

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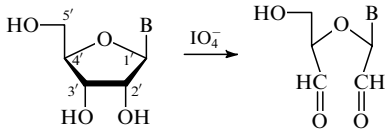
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II. Introduction of aldehyde groups into the sugar residue of nucleic acids

The methods used for the introduction of two aldehyde groups into nucleosides and mononucleotides as well as into 3′- and 5′-terminal residues of oligonucleotides are well known. A procedure for introduction of aldehyde groups into any position of the oligonucleotide chain has been developed recently.

1. Synthesis and structure of dialdehyde derivatives of nucleosides and nucleotides

The synthesis of dialdehyde derivatives of nucleosides is based on periodate oxidation of ribonucleosides and their derivatives:[4]



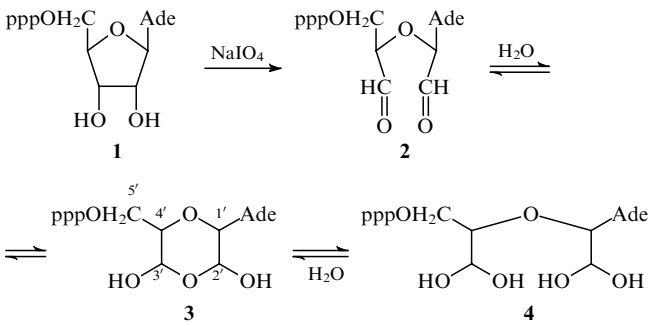
Hereinafter B is a heterocyclic base.

The reaction of adenosine 5′-triphosphate (**1**) with sodium periodate is of the second order (the rate constant k_2 is equal to 1.06×10^3 litre min^{-1} mol^{-1}).[5] The oxidation was carried out with an equimolar amount or a small excess of sodium periodate for 1 h at 4 °C.[5] In another study,[6] mono- and dinucleotides were oxidised with a 20-fold excess of sodium periodate for 1 h at 0 °C. The excess of sodium periodate was removed with ethylene glycol,[6] a small excess of rhamnose[6] or a 4-fold molar excess of $NaAsO_2$.[7] The resulting solutions of oxidised nucleoside derivatives were used as such; the products could be purified by gel chromatography.

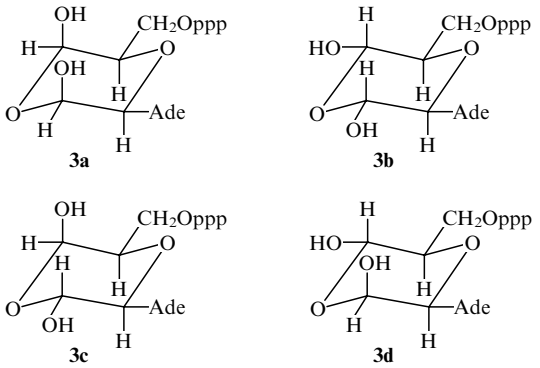
The aldehyde groups in various periodate-oxidised ribonucleotides are hydrated;[8–10] thus the oxidation product of adeno-

sine 5′-triphosphate (oATP) exists as an equilibrium mixture of dialdehyde hydrates (structures **3** and **4**, Scheme 1).[5,9]

Scheme 1



The ^1H and ^{13}C NMR spectra of oATP do not contain resonance signals typical of free aldehyde groups; the polymeric form of oATP was not detected in solution. The cyclic hemiacetal **3** may exist as a mixture of four diastereomers **3a**–**d**.



These conformers are preferred, since the adenine moieties and CH_2Oppp groups are equatorial.[9] The coupling constants correspond to the structures **3b**–**d** (Table 1). Because of steric constrains due to the axial position of two hydroxy groups, the structure **3a** is probably not realised. The structures **3c** and **3d** are more preferable, since the hemiacetal hydroxy groups in 1,4-dioxanes and sugars tend to occupy the axial position (the so-called anomeric effect).[11] The ^1H and ^{13}C NMR spectra also contained signals corresponding to the dialdehyde dihydrate **4**. Thus, oATP exists in solution as a mixture of four compounds, *viz*., three diastereomers of the cyclic hemiacetal **3b**–**d** and the dihydrate **4**, which are in equilibrium with one another, and a small amount of the free dialdehyde **2**.

In aqueous solution at pH 7.0, the UV spectrum of oATP is similar to that of ATP.[5]

The circular dichroism (CD) spectra of dialdehyde derivatives of adenosine, uridine and cytidine revealed strongly reduced Cotton effects in comparison with those observed for the corresponding nucleosides.[12] This points to enhanced free rotation of the heterocyclic base around the glycosidic bond. On the contrary, in the case of the dialdehyde derivative of guanosine the amplitude of the CD maximum increased, which was ascribed [12] to the self-assembly resulting in the formation of oligomers.

In the solid state, the dialdehyde derivatives of adenosine, guanosine, cytidine and uridine are totally polymerised.[12]

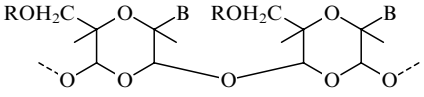


Table 1. Conformational analysis of hydrated forms of oATP (predicted and experimental values of coupling constants).[9]

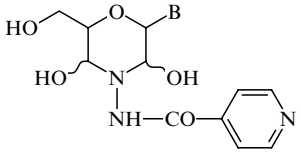
Compound	Position of the OH-groups[a]		Predicted coupling constants (J/ Hz)		Experimental coupling constants (J/ Hz)	
	2′-OH	3′-OH	$J_{1',2'}$	$J_{3',4'}$	$J_{1',2'}$	$J_{3',4'}$
3a	*a*	*a*	< 4	< 4	not detected	
3b	*e*	*e*	> 6	> 6	7.5	8.0
3c	*e*	*a*	> 6	< 4	8.0	1.7
3d	*a*	*e*	< 4	> 6	1.8	8.1
4	–	–	3–6	3–6	4.1	5.0

[a] *a* — Axial, *e* — equatorial.

2. Some chemical properties of periodate-oxidised nucleoside derivatives

The chemical properties of periodate-oxidised nucleoside derivatives (*e.g.*, interaction with hydrazines and amines, degradation in alkaline media and reduction of aldehyde groups) are well known.[4,13] Here, we consider only some model reactions which present interest from the standpoint of affinity modification of proteins.

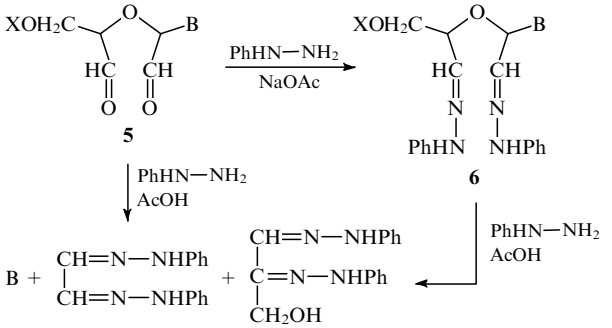
Dialdehyde derivatives of nucleosides and nucleotides readily react with hydrazides, the structure of the reaction products being dependent on the nature of the starting reagents and reaction conditions. The reaction of a nucleoside dialdehyde derivative with isonicotinohydrazide results in the addition of one hydrazide molecule to the nucleoside to give a morpholine derivative.[4]



An analogous product was obtained in the reaction of the above nucleoside derivatives with thiosemicarbazide.[4]

Dialdehyde derivatives of nucleosides and nucleotides **5** react with phenylhydrazine [13,14] to give bisphenylhydrazones. Addition of an excess of phenylhydrazine results in the degradation of dialdehyde derivatives of ribonucleosides or ribonucleotides (Scheme 2).[14]

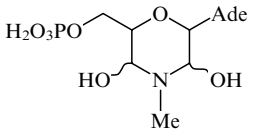
Scheme 2



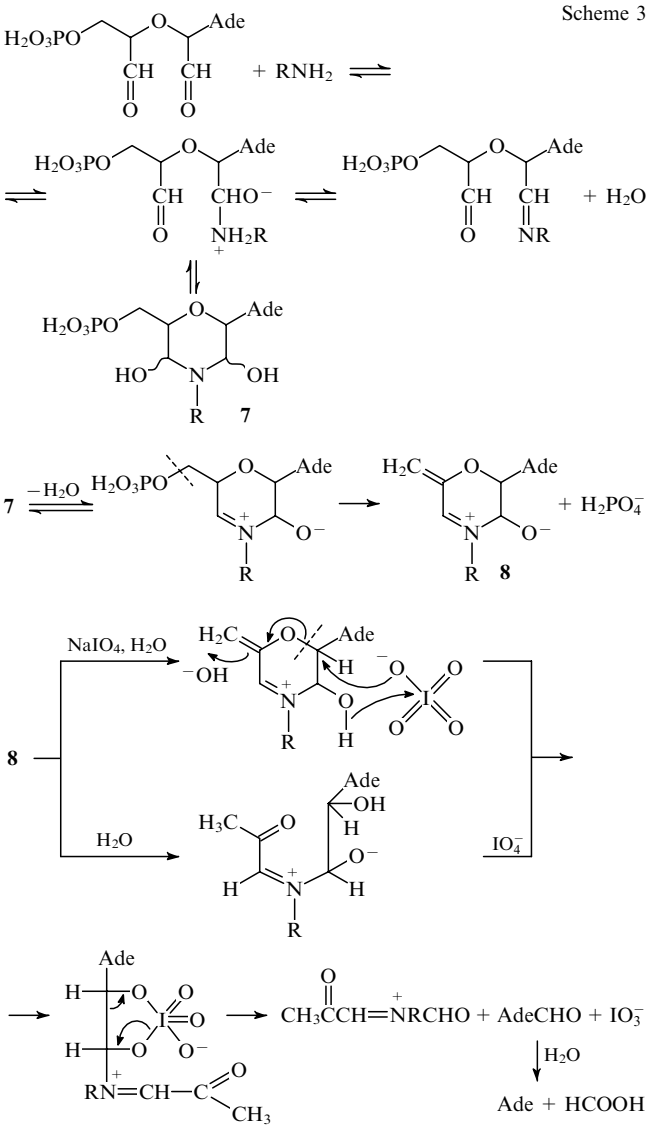
X is the phosphate or H.

In the same way derivatives **5** react with semicarbazide.[4]

The products of periodate oxidation of ribonucleosides and ribonucleotides react readily with amines. The reaction of oxidised adenosine 5′-phosphate with methylamine yields a morpholine derivative of adenosine 5′-phosphate.[4]



Scheme 3



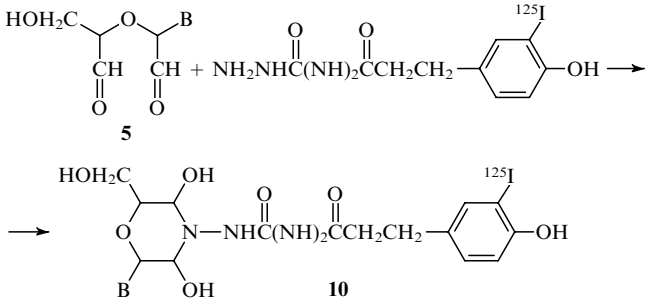
R = Alk.

Combined treatment of ribonucleotide dialdehyde derivative with primary amines and an excess of sodium periodate results in its degradation with elimination of the free base (Scheme 3).[15]

The products of the reaction of periodate-oxidised adenosine with glycine in the presence and absence of sodium cyanoborohydride ($NaBH_3CN$) were identified by spectral methods.[16] Putative condensation products are shown in Scheme 4. It was found that 2-adeninyl-4-carboxymethyl-6-hydroxymethylmorpholine (**9**) is the main reaction product.

The hydrolytic stability of the condensation products of oxidised adenosine or oATP with diaminohexane following reduction with $NaBH_3CN$ has been studied.[16] It was found that stable reaction products are formed only after reduction with $NaBH_3CN$.

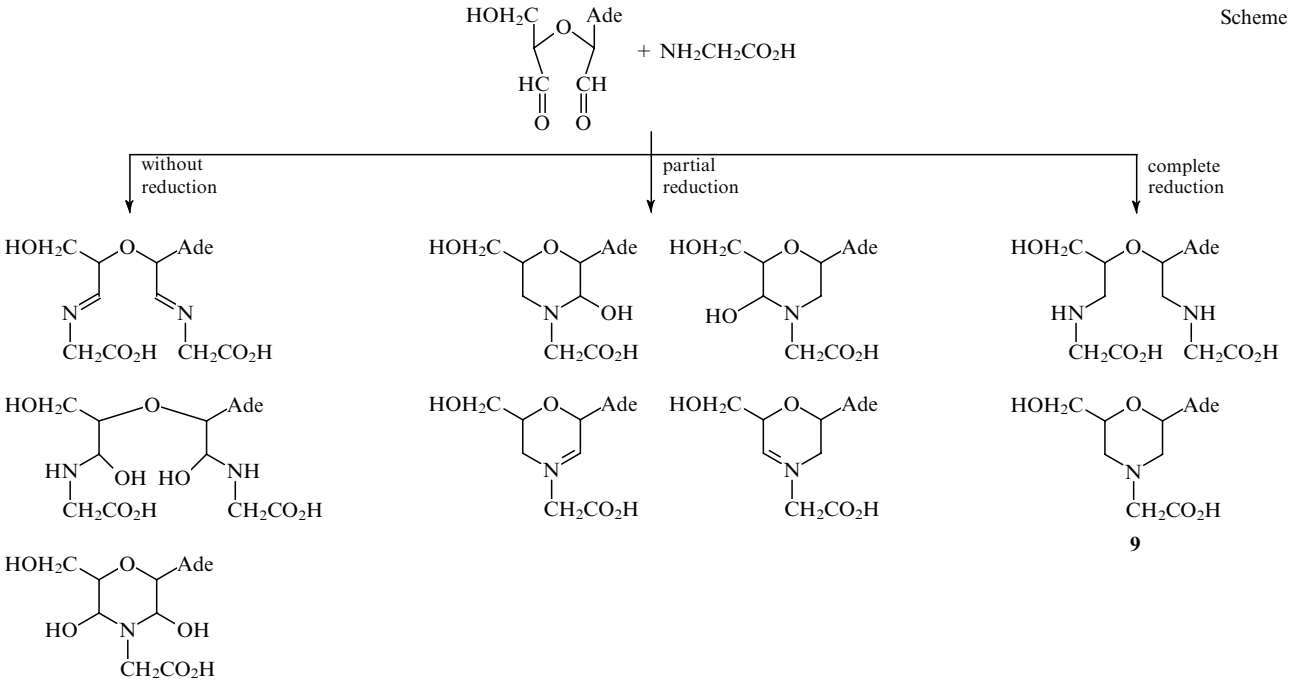
An ^{125}I-containing reagent, *viz*., 3-(3-iodo-4-hydroxyphenyl)-propionylcarbohydrazide, has been devised for identification and quantitative determination of periodate-oxidised ribonucleosides and RNA.[7] Reaction of ribonucleoside-derived dialdehydes with this reagent gives the morpholine derivative **10**, which is stable in neutral and weakly acidic media, but is subjected to rapid degradation in alkaline media and in the presence of ammonia or primary amines.



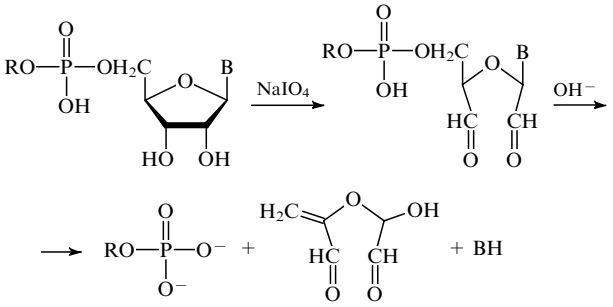
Dialdehydes from nucleoside 5′-phosphates and their derivatives are readily cleaved in alkaline media (pH > 7) with simultaneous scission of the glycosidic bond and β-elimination of the RPO_4^{2-} residue (Scheme 5).[4,13] Primary amines also efficiently catalyse this reaction.

The aldehyde groups of nucleoside and nucleotide dialdehyde derivatives can be completely or partially reduced depending on

Scheme 4

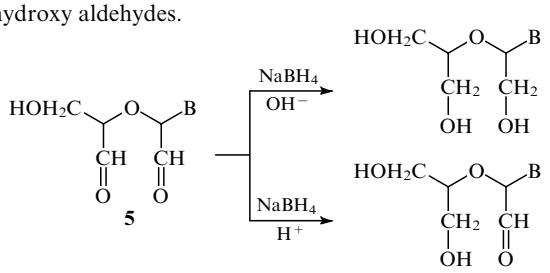


Scheme 5



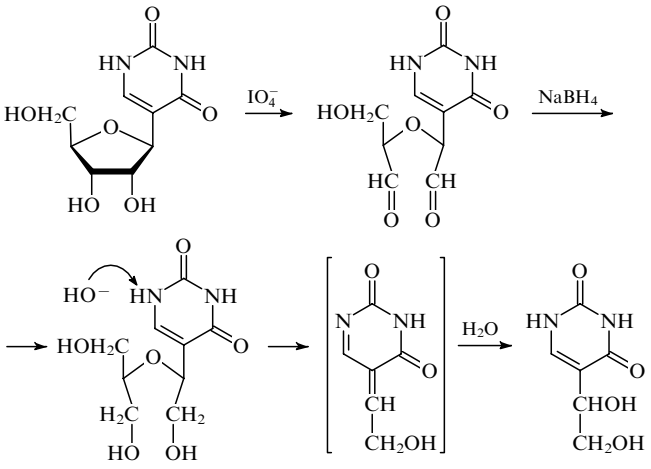
R = H or an oligoribonucleotide.

pH.[14] This results in the formation of the corresponding triols or dihydroxy aldehydes.



Periodate oxidation of pseudouridine gives the corresponding dialdehyde and two unidentified products.[4] Treatment of the dialdehyde with sodium borohydride and alkali results in 5-(α,β-dihydroxyethyl)uracil (Scheme 6).

Scheme 6

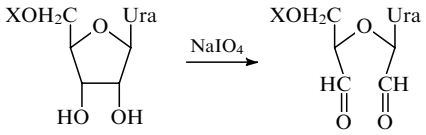


3. Introduction of the dialdehyde fragment into oligonucleotides

a. Introduction of the aldehyde groups into terminal nucleotide residues

The introduction of the dialdehyde fragment into 3′-terminus of RNA or oligoribonucleotides is effected by periodate oxidation. To introduce such a fragment into 3′-terminus of oligodeoxyribonucleotides or 5′-terminus of oligoribo- or oligodeoxyribo-nucleotides, one should first elongate them by attaching a ribonucleotide residue and then subject it to periodate oxidation. The conditions for oxidation of RNA do not practically differ from those for ribonucleosides and their derivatives.[17]

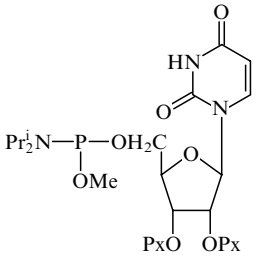
Thus the introduction of a dialdehyde fragment into the 3′-terminus of an oligodeoxyribonucleotide was preceded by attachment of a ribonucleotide.[18] To this end, the oligodeoxyribonucleotide was incubated with ribonucleoside 5′-triphosphate in the presence of terminal deoxyribonucleotidyl transferase. In another study,[19] cytidine 3′,5′-diphosphate (pCp) was attached to an oligodeoxyribonucleotide using T4 RNA-ligase. Then the 3′-terminal phosphate group of the resulting 5′-(oligodeoxyribonucleotide)-pCp3′ was eliminated with alkaline phosphatase after which the 2′,3′-*cis*-diol group was oxidised with periodate. The reaction was carried out with oligodeoxyribonucleotides having cytidine [18–20] or uridine[18] residues at the 3′-end.



X is 5′-(oligodeoxyribonucleotide)-3′.

An equimolar amount or a small excess of sodium periodate was used for the oxidation. The reaction was completed within 30 min at room temperature [18] or within 2 h at 0–4 °C.[19] The excess of sodium periodate was destroyed with ethylene glycol.[18]

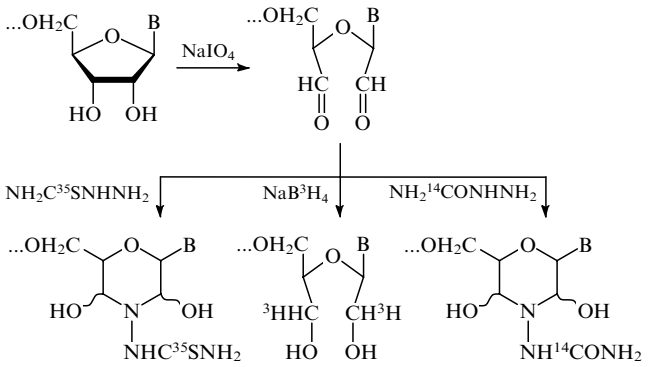
The dialdehyde fragment was also introduced into the 5′-end of an oligodeoxyribonucleotide.[21] The uridine residue was attached to the oligodeoxyribonucleotide by automated solid-phase, phosphoramidite synthesis using a derivative with protected 2′-OH and 3′-OH groups.



Px is 9-phenylxanthen-9-yl (pixyl).

In this case, the 5′-end of the ribonucleoside was attached to the 5′-end of the oligodeoxyribonucleotide. After deprotection, the ribose residue was oxidised with periodate to give a dialdehyde derivative.

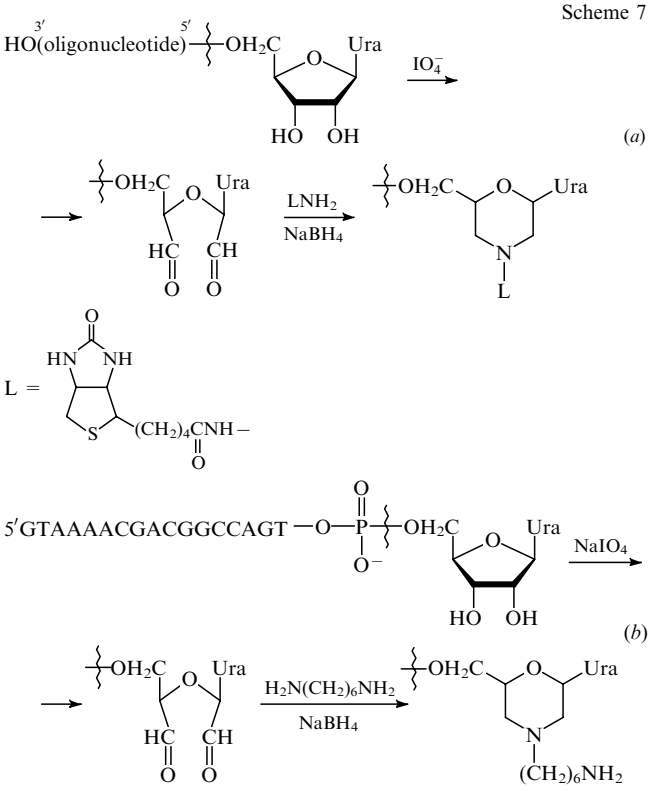
Oligodeoxy- and oligoribo-nucleotides containing oxidised ribose residues have found practical applications. Modified oligodeoxyribonucleotides with a 3′-terminal dialdehyde fragment were used for the introduction of the phosphate group into the 3′-end of the oligodeoxyribonucleotide. To this aim, the oligodeoxyribonucleotide with an oxidised 3′-terminal ribonucleoside residue was treated with a primary amine to give oligonucleotide 3′-phosphate, a dialdehyde derivative of ribose and a heterocyclic base.[18,20] Radioactive labelling of the 3′-terminal nucleoside residue of RNA was based on the reaction of the aldehyde groups with [^{35}S]-thiosemicarbazide, [^{14}C]-semicarbazide, [^{3}H]-isonicotinohydrazide or [^{3}H]-sodium borohydride.[4]



Dialdehyde derivatives of oligodeoxyribo- or oligoribonucleotides can be used for the introduction of a non-radioactive label by treating oligonucleotides with a compound containing an amino group and a non-radioactive label (*e.g.*, with biotin hydrazide, LNH_2) (Scheme 7 *a*) [21] or by preliminary introduction

of a primary amino group into the oligonucleotide with subsequent addition of a non-radioactive label (Scheme 7 *b*).[22]

Scheme 7



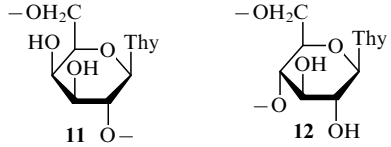
Ribonucleic acids (and oligoribonucleotides) can be cleaved at the internucleotide bond from the 3′-end of the polyribonucleotide chain by combined treatment with periodate and primary amines or by treatment with sodium periodate in an alkaline medium (see Scheme 5).[4,13] Such a treatment yields an oligo- or a polynucleotide 'shortened' by one nucleotide unit from the 3′-end. The whole cycle of conversions can be repeated. A base identified by conventional analytical methods is split off from the product of conversion of the original terminal nucleotide unit by treatment with an acid (pH < 3) or an alkali (pH > 11). This property of dialdehyde derivatives of RNA is the basis for a method for determination of nucleotide sequences in short oligoribonucleotides. Stepwise degradation from the 3′-end was used for the analysis of terminal sequences in various tRNA and tobacco mozaic virus RNA.[4]

The effects of several basic amino acids and primary amines, pH and temperature on the rate of hydrolysis of the phosphodiester bond in the dialdehyde derivatives of some oligoribonucleotides have been studied.[4] It was found that the cleavage of the required phosphodiester bond in the presence of lysine, cyclohexylamine or methylamine occurs most efficiently at elevated temperature (45 °C), while that in the presence of aniline is the most efficient at 24 °C (at pH 6–9).[4] No side reactions were observed at room temperature. A rise in temperature may favour side reactions, such as cleavage of ψU units and heterocyclic nuclei of other nucleosides.[4]

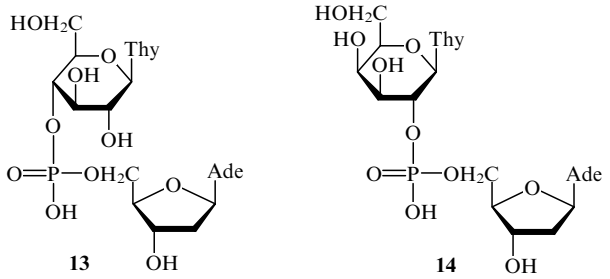
b. Introduction of the aldehyde groups into any position of the oligonucleotide strand

It is not until very recently that the methods for the introduction of aldehyde groups into any position of the oligodeoxyribonucleotide have been developed. Two types of oligonucleotides were used: (1) oligonucleotides containing a hexopyranosyl residue in the oligonucleotide strand[23–25] instead of a deoxyribose residue and (2) oligonucleotides with an additional ribose residue in the 2′-position of ribose.[26] The simplest approach is based on the introduction of galactose residues into the oligonucleotide strand.[23] Prior to the oligonucleotide synthesis, the hydroxy groups designed for subsequent oxidation are protected with appropriate protective groups. It was suggested[24] to introduce both the *cis*-glycol group as a D-galactose residue and the *trans*-glycol group (as a D-glucose residue) into the oligonucleotide.

Preparative syntheses of 1-(3,4-*O*-isopropylidene-β-D-galactopyranosyl)thymine[23] and 1-(2,3-di-*O*-acyl-β-D-glucopyranosyl)thymine[24] from D-galactose and D-glucose, respectively, have been carried out. Then, 14–21-membered oligodeoxyribonucleotides containing a 1-(β-D-glucopyranosyl)thymine (**11**)[23,25] or a 1-(β-D-galactopyranosyl)thymine (**12**)[24] residue at a definite position of the oligonucleotide strand were obtained using standard phosphoramidite procedures.



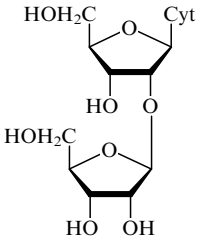
The 1-(β-D-galactopyranosyl)thymine residue was introduced into the oligonucleotide strand through the 2′- and 6′-OH groups, while the 1-(β-D-glucopyranosyl)thymine residue, through the 4′- and 6′-OH groups. The model dinucleoside phosphates, *viz*., 1-(β-D-glucopyranosyl)thymine-4′-*O*-phosphoryl-(4′-5′)-2′-deoxyadenosine (**13**)[24] and 1-(β-D-galactopyranosyl)thymine-2′-*O*-phosphoryl-(2′-5′)-2′-deoxyadenosine (**14**)[23] were synthesised for detailed studies of periodate oxidation.



The oxidation of the *trans*-diol group in compound **13** with a 10-fold excess of sodium periodate at 20 °C proceeded very slowly; the half-reaction time was 9–10 h.[24] The oxidation of compound **14** containing a *cis*-diol group was completed within 1.5 h under these conditions.[23] This circumstance determined the use of oligonucleotides containing a galactopyranose residue for the synthesis of reagents carrying dialdehyde fragments and designed for affinity modification of proteins.

The oxidation of 14-membered galactose-containing oligonucleotides was carried out in 20–200 mM $NaIO_4$ (1.5–2.5 h, 25–37 °C).[25] The yields of the oxidation products (65%–75%) were determined by cleavage of the phosphodiester bond from the 5′-end at the oxidised galactose residue, which was carried out in 10% piperidine (25 min, 95 °C). Under these conditions, oligonucleotides containing a non-oxidised galactose residue remained intact. The structure of oligonucleotides containing an oxidised galactose residue was confirmed by selective cleavage of the phosphodiester bond of the oxidised galactose residue (Fig. 1). This was carried out under conditions of β-elimination analogous to those used for chemical sequencing of RNA.[27] The reaction was conducted in 1 M aniline–acetate buffer (pH 4.5) for 15 min at 95 °C.[25] In contrast with the oligonucleotides **15**–**17** containing a galactose residue, the 14-membered oligonucleotides containing an oxidised galactose residue (**15***–**17***) in different positions of the oligonucleotide chain were cleaved. Their cleavage gave a ^{32}P-labelled tetranucleotide (from **15***), a heptanucleotide (from **16***) and an undecanucleotide (from **17***) containing a 3′-phosphate group (see Fig. 1). In control experiments, insignificant degradation of periodate-oxidised oligonucleotides during gel electrophoresis took place.[25]

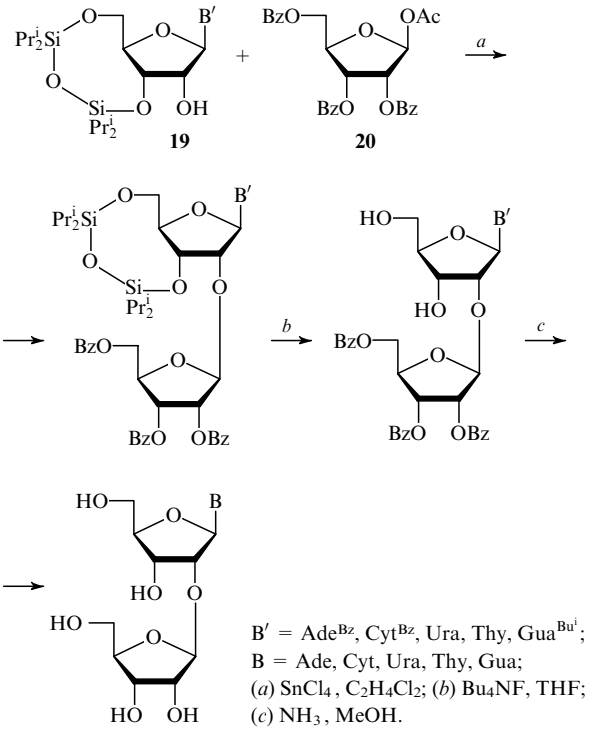
Another approach to regiospecific introduction of additional *cis*-diol groups into oligonucleotides is based on the use of nucleoside disaccharide derivatives **18**.[26, 28, 29]



18

In these compounds, the *cis*-diol groups are converted into aldehyde groups upon oxidation. Disaccharide nucleosides have been identified in tRNAs as minor components; some antibiotics also involve disaccharide fragments. Synthesis of 2′-*O*-ribofuranosylnucleosides **18** was carried out in three steps by condensation of 3′,5′-*O*-blocked *N*-acylribonucleoside (**19**) and a small excess of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**20**) in the presence of tin tetrachloride [28, 29] followed by a two-step deprotection. The glycosylation reaction was stereospecific and resulted in the formation of a β-D-glycosidic bond (Scheme 8).

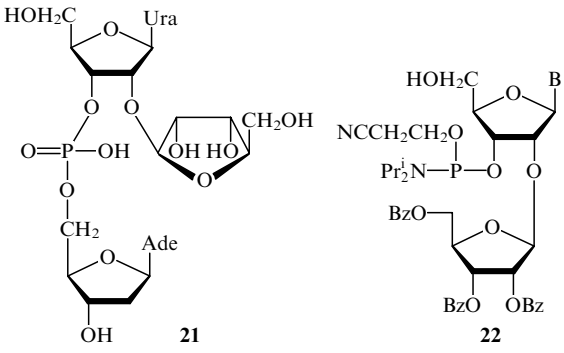
Scheme 8



19 **20**

B′ = Ade^{Bz}, Cyt^{Bz}, Ura, Thy, Gua$^{Bu^i}$;
B = Ade, Cyt, Ura, Thy, Gua;
(*a*) $SnCl_4$, $C_2H_4Cl_2$; (*b*) Bu_4NF, THF;
(*c*) NH_3, MeOH.

The periodate oxidation has been studied using a model dinucleoside phosphate **21** containing the disaccharide residue.[29]



21 **22**

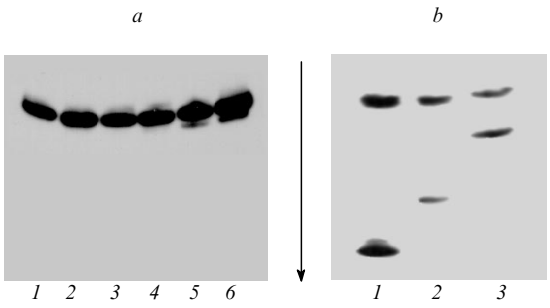


Figure 1. Gel-electrophoretic analysis of stability of the oligonucleotides 5′GCCAXCCTGGCTCT3′ (**15**, **15***), 5′GCCAACCXGGCTCT3′ (**16**, **16***) and 5′GCCAACCTGGCXCT3′ (**17**, **17***) containing a nonoxidised (**15**–**17**) or oxidised (**15***–**17***) 1-(β-D-galactopyranosyl)thymine residue (X).
(*a*) Incubation of the oligonucleotides containing a galactose residue: (*1*, *3*, *5*) — in aniline–acetate buffer, pH 4.5, 95 °C, 15 min; (*2*, *4*, *6*) — in 40 mM Tris-HCl buffer, pH 7.9, 5 mM dithiothreitol, 50 mM NaCl, 15 mM $MgCl_2$, 95 °C, 25 min; (*1*, *2*) oligonucleotide **15**; (*3*, *4*) oligonucleotide **16**; (*5*, *6*) oligonucleotide **17**.
(*b*) Incubation of the oligonucleotides containing an oxidised galactose residue in aniline–acetate buffer, pH 4.5, 95 °C, 15 min; (*1*) oligonucleotide **15***; (*2*) oligonucleotide **16***; (*3*) oligonucleotide **17***.

The model dinucleoside phosphate was oxidised with sodium periodate within 10 min at 20 °C. 14–22-Membered oligonucleotides containing 2′-*O*-ribofuranosylcytidine residues were synthesised using the phosphoramidite method with modified phosphoroamidite (compound **22**).[26]

DNA duplexes used for affinity modification of some DNA-binding proteins have been formed on the basis of oligonucleotides containing dialdehyde fragments at definite positions of the nucleotide strand.

III. Affinity modification of proteins and polypeptides with periodate-oxidised derivatives of nucleic acids

Periodate-oxidised derivatives of nucleotides containing dialdehyde fragments were used for affinity modification of enzymes that use nucleoside triphosphates as cofactors or substrates and transcription factors. In addition, conjugates of 13- and 15-membered oligodeoxyribonucleotides containing 3′-terminal aldehyde groups with poly(L-Lys) have been synthesised to study the regulation of gene expression.[19] 14- and 22-Membered DNA-duplexes with internal dialdehyde fragments were used for covalent cross-linking with restriction endonucleases and methylases (*Eco*RII and *Mva*I [25]) and RNA polymerase of bacteriophage T7.[30]

1. Covalent binding of proteins with nucleotide dialdehyde derivatives

The fundamental process that satisfies the bioenergetic requirements of living organisms is versatile for practically all the objects of the living nature. It consists in the hydrolysis of one of pyrophosphate bonds in adenosine 5′-triphosphate (ATP) or, less often, guanosine 5′-triphosphate (GTP). Numerous biochemical processes occur by virtue of energy released upon hydrolysis of nucleoside triphosphates.

2′,3′-Dialdehyde derivatives of ATP, ADP, GTP and GDP (oATP, oADP, oGTP and oGDP, respectively) were used for affinity modification of some enzymes, mainly for the study of their cofactor-binding or active sites.

The ATP dialdehyde derivative was cross-linked to mitochondrial ATPase and histone kinase in order to study the active sites of these enzymes.[31, 32] Pyruvate carboxylase, acetyl-CoA carbox-

ylase and NAD-dependent isocitrate dehydrogenase require ATP for their cofactor function (the catalytic reaction utilises the energy of ATP hydrolysis). These enzymes have several substrates and cofactors. oATP and oADP were used for covalent binding of these enzymes with the allosteric site as well as for the analysis of interaction of this allosteric site with other sites.[8,33,34] The phosphate group of ATP is added to the 5-ribulose phosphate fragment by phosphoribulokinase to give ribulose-1,5-diphosphate. oATP was used for affinity modification of the binding site in one of phosphoribulokinase substrates.[35]

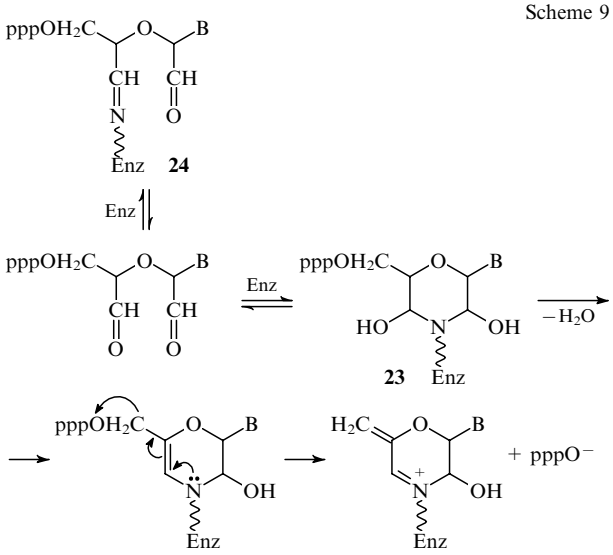
Binding of aminoacyl-tRNA with ribosomes requires the elongation factor EF-Tu. This reaction is accompanied by hydrolysis of GTP. GTP 2′,3′-dialdehyde derivative was covalently linked to the elongation factor EF-Tu in order to identify the binding site for the cofactor.[36] Functioning of a human protein c-H-ras p21 also is accompanied by GTP hydrolysis. This protein, which is localised in the cytoplasmic membrane, accepts external signals stimulating cell growth and transmits them to target proteins localised inside the cell, thus promoting its growth and division. In order to investigate the interaction of GTP and GDP with this protein in more detail the latter was subjected to affinity modification with periodate-oxidised GTP and GDP derivatives.[37] A periodate-oxidised derivative of 1,N^6-ethenoadenosine-5′-monophosphate (oεAMP) was used for covalent addition to alkaline phosphatase from human placenta.[38]

Depending on the object of investigation, proteins and periodate-oxidised components of NA are used for cross-linking in various ratios (Table 2). The time of incubation of proteins with the monomeric analogues of NA containing aldehyde groups varied, on the average, from 5 min to 2 h. In kinetic studies of the oADP interaction with isocitrate dehydrogenase, the incubation time varied from 1 to 8 h.[34] The kinetic curve of inactivation of the tetrameric enzyme was biphasic: the fast phase is coupled with covalent binding of oADP with one enzyme subunit, and subsequent slow phase consists in covalent binding of oADP with the remaining three subunits of the enzyme and results in its complete inactivation.

Traditionally, NA components that contain a radioactive label (^{14}C, ^{3}H or ^{32}P) were used for covalent binding with proteins. The degree of affinity modification was estimated from the radioactivity after electrophoresis in 8%–10% polyacrylamide gel containing 0.1% SDS,[31] gel filtration [19,32] or washing of filters containing reaction mixtures.[36] The degree of affinity modification of pyruvate carboxylase with various concentrations of oATP was determined from the residual activity of the enzyme.[8] The conditions for the preparation of the covalent complexes and subsequent specific cleavage of the covalently bound proteins are listed in Table 2.

Let us consider the mechanism of reaction of the NH_2 groups of proteins with dialdehyde derivatives of monomeric components of NA proposed by Gregory and Kaiser [39] (Scheme 9). As can be seen from Scheme 9, the reaction of the NH_2 group of a protein with a periodate-oxidised nucleotide derivative yields either a dihydroxymorpholine derivative **23** or a Schiff's base **24**.

Scheme 9



Enz is enzyme.

It was pointed out [31,33–35] that stable morpholine derivatives of enzymes with oATP or oADP are formed without reduction. Following addition of an oxidised derivative of a nucleotide to the enzyme, splitting of the tri- or di-phosphate anion upon β-elimination occurs faster than in the absence of the enzyme due to the involvement of the electron pair of the nitrogen atom of the

Table 2. Conditions for the preparation of covalent complexes of proteins with periodate-oxidised derivatives of mononucleotides and for specific cleavage of cross-linked proteins.

Protein or polypeptide (concentration)	Modified analogue of NA (concentration)	Reducing agent	Amount of the reducing agent	Duration of reduction / min	Enzymes or chemical reagents for specific cleavage of the protein fragment	Ref.
Mitochondrial adenosine triphosphatase (100 μg ml^{-1})	Mg-oATP (9.4 or 2.5 μmol)	–	–	–	–	31
NAD-dependent isocitrate dehydrogenase (0.4–0.5 mg ml^{-1})	oADP in the presence of $MnSO_4$ (0.2 mmol $litre^{-1}$)	–	–	–	pronase, carboxypeptidase A, carboxypeptidase B, aminopeptidase	34
Pyruvate carboxylase (10–20 un ml^{-1} or 13.3 un mg^{-1})	Mg-oATP (2 mmol $litre^{-1}$)	$NaBH_4$	10-fold excess with respect to oATP	35	trypsin, chymotrypsin, pronase, α-aminopeptidase	8
Histone kinase	20-fold excess of oATP relative to the enzyme	$NaBH_4$	20-fold excess with respect to oATP	60	trypsin	32
Elongation factor from *T.thermophilus* (20 mmol $litre^{-1}$)	ATP [a] (10 mmol $litre^{-1}$)	$NaBH_3CN$	20 mmol $litre^{-1}$	1	BrCN	36

[a] The oxidation with sodium periodate was carried out after incubation of the elongation factor EF-Tu and ATP (incubation time 10 min, oxidation time 1 min).

morpholine derivative in the splitting of the tri(di)phosphate anion (see Scheme 9).

According to other reports,[8, 32, 36] the reduction of intermediate products (a dihydroxymorpholine derivative or a Schiff's base) with borohydrides ($NaBH_4$ or $NaBH_3CN$) is necessary for stable covalent binding of the enzyme with the nucleotide.

So what is the information that one may derive from cross-linking of proteins with periodate-oxidised mononucleotides? In the first place, specific cleavage of a protein covalently bound with a nucleotide by enzymes or by chemical reagents provides information about the location of amino acids or a protein fragment that form the active or cofactor-binding site of the enzyme. Enzymic hydrolysis is conventionally carried out with trypsin or other specific proteases, while chemical cleavage of proteins at the methionine residues is performed with BrCN (see Table 2).

Enzymic hydrolysis of a modified and reduced conjugate yields a small peptide covalently bound with the nucleotide and then the amino acid sequence of the peptide is determined. To this end, the peptide cross-linked to a radiolabelled oATP is subjected to acid hydrolysis, dansylation and separation by two-dimensional gel electrophoresis followed by identification of dansyl amino acids.[32] The mobility of the radiolabelled dansylated derivative coincided with that of the synthetic dansylated derivative of the covalent adduct of lysine with oATP. The amino acid sequence of the peptide cross-linked with oGDP or oGTP was determined using the Edman degradation.[37]

The lysine residues involved in the covalent binding with periodate-oxidised nucleotide derivatives were identified after proteolysis of products of cross-linking of oATP to histone kinase[32] and pyruvate carboxylase[8] as well as of oGDP and oGTP to the protein c-H-ras p21.[37] It was suggested that the lysine residues present in the active or cofactor-binding sites of enzymes are responsible for the binding of the phosphate groups of ATP due to the basic properties of the ε-amino group of lysine.

As it has been noted above, treatment of the protein–nucleotide conjugate with BrCN results in the cleavage of the covalently bound protein at the methionine residues (Met). Since these residues are rare in occurrence, large protein fragments are usually formed. This method allowed one to demonstrate that the GTP-binding domain of the elongation factor Tu is localised in the N-terminal part of the protein.[36]

Affinity modification of active or cofactor-binding sites of pyruvate carboxylase,[8] mitochondrial ATPase,[31] isocitrate dehydrogenase,[34] phosphoribulokinase[35] and alkaline phosphatase from human placenta[38] with ATP, ADP or εAMP 2′,3′-dialdehyde derivatives led to the inhibition of these enzymes. There are several nucleotide analogues that contain a reactive group and can be used for covalent linking to nucleotide-binding sites of proteins. However, only some of them are inhibitors that can specifically bind with the active site. Site-directed modification should meet two criteria. Let us consider these criteria with oATP as an example. First, ATP must protect the protein from inactivation with oATP. Second, oATP must serve as a substrate for this enzyme. Wherever these two criteria were met, it was demonstrated that oATP is a site-directed inhibitor of mitochondrial ATPase[31] and phosphoribulokinase,[34] whereas oεAMP is a site-directed inhibitor of alkaline phosphatase from human placenta.[38] The dependence of inactivation of multisubunit proteins, *e.g.*, pyruvate carboxylase[8] and NAD-dependent isocitrate dehydrogenase,[35] on the concentration of oATP or oADP and the effects of other cofactors and substrates of enzymes (multisubunit proteins have several substrates and cofactors) on the affinity modification of these proteins with oATP or oADP have been studied in a number of papers. The affinity modification of these proteins with dialdehyde derivatives of nucleotides allowed one to suggest the relative arrangement of the active and cofactor-binding sites of the enzymes.[8, 35]

Thus, in some cases affinity modification of proteins with periodate-oxidised derivatives of nucleotides has made it possible to establish the active and cofactor-binding sites of enzymes that are covalently bound with periodate-oxidised derivatives of mononucleotides and to suggest the location of the oATP-(cofactor-) binding sites in multisubunit proteins.

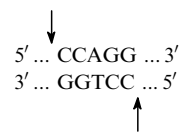
2. Affinity modification of proteins and polypeptides with oligonucleotides containing dialdehyde fragments

Methods for cross-linking of histones to DNA containing randomly introduced aldehyde groups were developed in the 1980's by Mirzabekov *et al.* in structural studies of chromatin.[40–45] The aldehyde groups could be introduced into DNA in several ways. One of them is based on acid treatment of DNA, which results in its depurination and concomitant liberation of the aldehyde group in the sugar moiety. However, this approach can hardly be applied to covalent cross-linking of DNA to proteins, because low pH values prevent the formation of DNA–protein complexes. Yet another method for the random introduction of the aldehyde groups into DNA strands is based on methylation of DNA purines with dimethyl sulfate and subsequent depurination.[40] In order to identify contacts of DNA with histones, cell nuclei were treated with dimethyl sulfate, then depurination was carried out in a neutral medium followed by reduction with sodium borohydride.[41–43] Such a treatment resulted in the covalent binding of the aldehyde groups of DNA with the histone amino acid residues interacting with DNA. The same approach was used to establish DNA–protein contacts in complexes of RNA polymerase with the promoter *lac*UV5,[46] of *lac* repressor with DNA[47] and in the cowpox virus.[48]

The interaction of DNA duplexes and oligonucleotides containing dialdehyde fragments with proteins and polypeptides has been studied recently.[19, 25, 30] The aldehyde groups were introduced regiospecifically both into terminal and nonterminal positions of the oligonucleotide strand. The possibility to introduce aldehyde groups into any position of the oligonucleotide strand has made it possible to investigate template-driven enzymes (RNA polymerases) and enzymes recognising specific sequences in DNA (DNA restriction–modification enzymes). The obvious advantage of dialdehyde reagents is their specificity with respect to adjacent lysine ε-amino groups. Special mention should be also made of the ability of these reagents to form stable covalent complexes with the protein after their reduction. The proximity of definite sugar residues of DNA to the lysine residues of the active or DNA-binding sites of proteins can be established by means of introduction of the aldehyde groups into a definite sugar residue of the DNA duplex and subsequent cross-linking of the modified duplex to the proteins.

The specificity of the dialdehyde fragment with respect to the lysine amino group was used for efficient covalent binding of poly(L-Lys) with two aldehyde groups l in the 3′-terminus of an oligonucleotide.[19]

Probing of the functional sites of the DNA restriction-modification enzymes *Eco*RII and *Mva*I which recognise the sequence

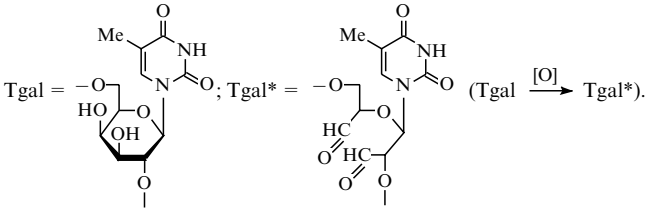


in DNA, was carried out.[25] The restriction endonuclease *Eco*RII (R.*Eco*RII) cleaves the phosphodiester bonds at the positions indicated by the arrows. The DNA-methyltransferases *Eco*RII (M.*Eco*RII) and *Mva*I (M.*Mva*I) methylate the inner cytosine residues at C(5) (M.*Eco*RII) or at N(4) (M.*Mva*I). The cross-linking of these enzymes to DNA duplexes (substrate analogues) that contain an oxidised 1-(β-D-galactopyranosyl)thymine residue was carried out.[25] The modified sugar residue was introduced either into the recognition site (duplex **25***) or into the flanking nucleotide sequences (duplexes **26*** and **27***).

5′ GCCAA**CC**X**GG**CTCT
3′ CGGTT**GGACC**GAGA — **25** (X = Tgal), **25*** (X = Tgal*)

5′ GCCAX**CCTGG**CTCT
3′ CGGTA**GGACC**GAGA — **26** (X = Tgal), **26*** (X = Tgal*)

5′ GCCAA**CCTGG**CX**CT**
3′ CGGTT**GGACC**GAGA — **27** (X = Tgal), **27*** (X = Tgal*)



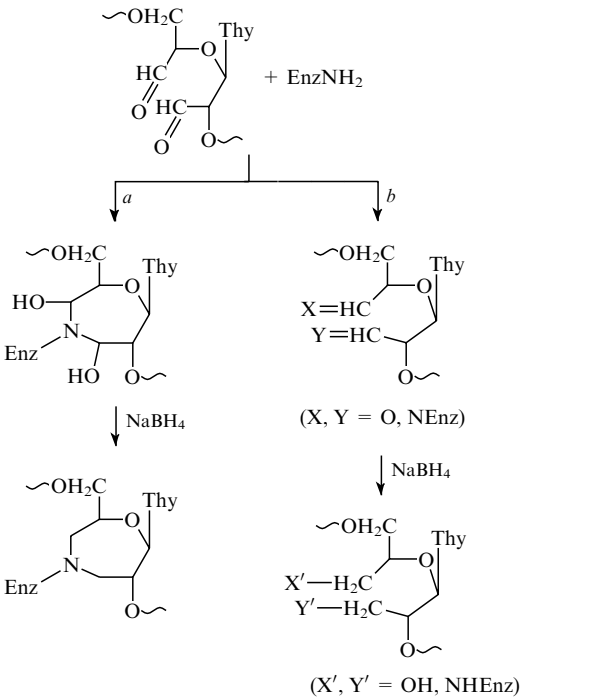
The dialdehyde fragment present in the oxidised 2′-*O*-ribofuranosylcytidine was introduced into DNA duplexes at definite positions (−11, +1, +2) of the coding strand of the T7-consensus promoter in order to investigate the active and DNA-binding sites of RNA polymerase of bacteriophage T7.[30]

5′ TAATACGACTCACTATAGGACT 3′
3′ ATTATGCTGAGTGATATCCTGA 5′ — coding strand
−11 +1 +2

This was followed by cross-linking of these duplexes to T7 RNA polymerase and to its mutant form in which one amino acid residue (Tyr639) apparently located in close proximity to the initiation site was replaced by lysine.

Based on the mechanism of binding of aldehyde groups of modified monomeric components of NA with the lysine residues of proteins, the following scheme has been proposed for the interaction of DNA duplexes (substrate analogues) with DNA restriction-modification enzymes (Scheme 10).[25]

Scheme 10



The formation of a cyclic derivative (route *a*) or a Schiff's base (route *b*) is possible in the first stage. The formation of a 7-membered cyclic derivative is less likely. The next step is the reduction of the DNA–protein conjugate with $NaBH_4$ to give a stable complex.

The conditions for cross-linking of DNA duplexes and oligonucleotides to DNA-binding proteins and a polypeptide are given in Table 3. Covalent binding of poly(L-Lys) with the 3′-end of an oligodeoxyribonucleotide with an attached oxidised cytidine was performed with a 2-fold excess of the polypeptide. An excess (~ 100-fold) of the reducing agent ($NaBH_3CN$) was introduced simultaneously with poly(L-Lys).[19] Affinity modification of the restriction-modification enzymes *Eco*RII and *Mva*I was carried out with an excess of the protein with respect to the DNA duplex. The endonuclease *Eco*RII and the methylases *Eco*RII and *Mva*I were incubated with the modified reagents for 5 min at room temperature and then for 15 min at 0 °C. This was followed by the reduction with a 1000-fold excess of $NaBH_4$ with respect to the protein (40 min, 0 °C).[25] The reduction of the covalent complex of T7 RNA polymerase with a DNA duplex containing an oxidised 2′-*O*-ribofuranosylcytidine residue was carried out with a 100-fold excess of $NaBH_4$ for 1 h at room temperature.[30]

What are the results of cross-linking of dialdehyde-containing oligonucleotides to poly(L-Lys) and dialdehyde-containing DNA duplexes to various enzymes? What is the practical significance of these results?

Cross-linking of 13–15-membered synthetic oligodeoxyribonucleotides, containing two 3′-terminal aldehyde groups, complementary to the site of initiation of translation of mRNA of the vesicular stomatitis virus protein to poly(L-Lys) has made it possible to increase considerably the efficiency of their incorporation into cells.[19] Such conjugates possess antiviral activity. No inhibitory effect on viral protein synthesis was found after incubation of cells containing the vesicular stomatitis virus with an oligodeoxyribonucleotide–poly(L-Lys) mixture, *i.e.*, the cross-linking of a modified oligonucleotide to the polypeptide is a prerequisite for the manifestation of antiviral activity.[19]

DNA duplexes containing an oxidised galactose residue in the recognition site of the *Eco*RII (or *Mva*I) restriction-modification enzymes (duplex **25***) or in the flanking nucleotide sequence (duplex **27***) were cross-linked to the *Eco*RII and *Mva*I methylases in high yields (see Table 3).[25] The efficiency of cross-linking depended on the location of the reactive group and the nature of the enzyme and correlated with the efficiency of binding of these enzymes with the corresponding reagents. The specificity of covalent binding of the DNA duplexes **25*** and **27*** with M.*Eco*RII was investigated by examining competitive inhibition of affinity modification of the enzymes with the DNA duplexes containing and devoid of a canonical recognition site. Thus the substrate containing a recognition site and used in a 20-fold excess caused practically complete inhibition of the cross-linking of DNA duplexes containing aldehyde groups to *Eco*RII methylase. The duplex devoid of the recognition site and used in a 10-fold excess did not influence the ability of the DNA duplexes **25*** and **27*** for cross-linking to M.*Eco*RII. The data obtained confirm the specificity of cross-linking of M.*Eco*RII to DNA duplexes containing aldehyde groups.

Partial cleavage of protein fragments of conjugates of the *Mva*I methylase and DNA duplexes **25*** and **27*** with chemical reagents specific for methionine, cysteine or tryptophan residues has been carried out.† Preliminary results of partial cleavage have made it possible to localise the site of cross-linking of the DNA duplexes **25*** and **27*** containing aldehyde groups to M.*Mva*I. It was found that this region is identical for both complexes and is localised in the C-terminus of the enzyme.

Affinity modification of the *Eco*RII restriction endonuclease was carried out only with a reagent containing aldehyde groups in the flanking nucleotide sequence (duplex **27***).[25] The yield of the

† O M Gritsenko, E S Gromova, unpublished results.

Table 3. Conditions for the preparation of covalent complexes of DNA-binding proteins with oligonucleotides containing dialdehyde fragments.

Protein or polypeptide (concentration)	Modified analogue of NA (concentration)	Reducing agent	Amount of the reducing agent	Duration of reduction / min	Yield of cross-linked products (%)	Ref.
Poly(L-Lys) (80 nmol)	oligodeoxyribonucleotides: 5′CATTTTGATTACTGTC*3′, 5′AACGAGGCACATTC*3′ (40 nmol), where C* is the oxidised cytidine residue	$NaBH_3CN$	10 μmol	120	25 – 30	19
*Eco*RII or *Mva*I methylases (M.*Eco*RII — 3.16 μmol litre^{-1}; M.*Mva*I — 3.3 μmol litre^{-1})	14-membered DNA duplex containing an oxidised galactose residue (0.23 μmol litre^{-1}) (duplexes **25*** and **27***)	$NaBH_4$	3.75 mmol litre^{-1}	40	9 – 20	25
Restriction endonuclease *Eco*RII (3.16 μmol litre^{-1})	14-membered DNA duplex containing an oxidised galactose residue (0.23 μmol litre^{-1}) (duplex **27***)	$NaBH_4$	3.75 mmol litre^{-1}	40	4	25
RNA polymerase of bacteriophage T7 (0.1 – 0.5 μmol litre^{-1})	22-membered DNA duplex containing an oxidised 2′-*O*-ribofuranosylcytidine residue (0.1 – 0.5 μmol litre^{-1})	$NaBH_4$	0.05 μmol litre^{-1}	60	–	30

cross-linked product did not depend on the presence of the cofactor (Mg^{2+} ions). This led the authors[25] to assume that cross-linking of DNA duplexes containing aldehyde groups to R.*Eco*RII occurs not at the active site.

None of the DNA duplexes containing an oxidised 2′-*O*-ribofuranosylcytidine unit was able to cause affinity modification of T7 RNA polymerase.[30] However, covalent cross-linking of the DNA duplex containing an oxidised 2′-*O*-ribofuranosylcytidine residue at position +2 of the consensus promoter to the mutant form of T7 RNA polymerase (Tyr639 → Lys) did take place. In wild type enzyme, Tyr639 is apparently involved in the NTP binding and should be located in close proximity to the initiation site, which suggests that Lys639 is the residue that was subjected to modification.[30]

The first results of affinity modification of DNA-binding proteins are important for determining specific DNA – proteins contacts and for studying the structure of active centres of enzymes as well as for elucidating the mechanisms of catalysis by restriction-modification enzymes and RNA polymerases.

IV. Conclusion

Introduction of reactive aldehyde groups into NA makes it possible to use them for attachment of fluorescent or any other label. In addition, oligonucleotides containing dialdehyde fragments are convenient for the synthesis of antisense oligonucleotides. Affinity modification of proteins with DNA duplexes containing regiospecifically introduced aldehyde groups proceeds with sufficiently high yields and results in stable covalent bonds. The results of cross-linking of this type of reagents to proteins allow one to recommend them as an efficient and flexible tool in the study of a broad range of DNA – protein interactions.

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