Russian Chemical Reviews **62** (4) 387–407 (1993)
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UDC 577.113.7;547.963.92

Imperfectly matched nucleic acid complexes and their biochemical manifestation

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Abstract. The review is devoted to the analysis of experimental data on the selectivity of the interaction of nucleic acids with antisense oligonucleotides and their derivatives, which lead to the prospect of achieving a highly selective influence on many biochemical and molecular-genetic processes in living organisms. Theoretical estimates of the level of specificity of the interaction of nucleic acids and the thermodynamic parameters of the formation of perfectly and imperfectly matched complementary complexes are examined in the review. Attention is concentrated on the complementation accuracy of the interaction of DNA and RNA with oligonucleotides and their derivatives in various model systems both *in vitro* and *in vivo*. Data on the specificity of the inhibition of translation with the aid of antisense oligonucleotides in model systems *in vitro* are analysed. The question of the influence of the steric structure of the nucleic acid molecule on the accuracy and efficiency of the interaction with antisense oligonucleotides and their derivatives is discussed. The bibliography includes 247 references.

I. Introduction

The selective interaction between nucleic acids is of cardinal importance in ensuring the occurrence of key life processes. It is sufficient to recall that the specific interaction of complementary nucleic acid sequences constitutes the basis of the double helix DNA structure established by Watson and Crick. The principle of complementarity in fact determines the selective interaction of nucleic acids with antisense RNA, oligonucleotides, and their derivatives. (Such compounds are referred to as antisense on the basis of their ability to form a complementary complex with the selected nucleic acid sequence — the NA target, which is a sense sequence.) All this leads to the prospect of being able to exert a site-specific ('addressed') influence on many key biochemical and molecular-genetic processes occurring with participation of nucleic acids both *in vitro* and in the living cell *in vivo*.[1,2] By virtue of the numerous interesting possibilities of this approach, including specific influence on intracellular processes, as well as the advances in the field of oligonucleotide synthesis, the method of site-specific action, based on the use of antisense oligonucleotides, has attracted increasing attention in recent years.[3–5]

One of the versions of this approach, first formulated at the end of the 1960s, has been called complementarily addressed (site-specific in modern parlance) modification.[6–8] It is based on two processes: in the first, a reactive derivative of the oligonucleotide, complementary to a specific section of the NA target, forms with this section a specific complementary complex and thus directs (addresses) the action of the reactive part of the molecule; in the second, there is a direct chemical reaction (alkylation, photomodification, cleavage) with a nucleic acid base near this section. This approach is regarded nowadays as the most universal method whereby nucleic acids can be highly selectively influenced in order to suppress the development of viral infections and the tumour process induced by mutations in protooncogenes, which are responsible for the malignant degeneracy of the cell.[5,9]

For the wide scale employment of this approach both in research and for therapeutic purposes, it is important that the interaction of the oligonucleotide or its reactive derivative with the NA target should occur with a high selectivity. According to statistics,[1] the sequence of a random 17-unit oligonucleotide is repeated in the human genome only once and one may therefore expect an extremely effective interaction of the NA target with oligonucleotides of this length. However, a fairly extensive set of experimental data, showing that the specificity of the interaction of nucleic acids (the formation of a perfectly matched complementary complex) is low has now accumulated. There is a high probability of the formation of imperfectly matched (containing noncanonical base pairs or loops) or partial (only part of the antisense oligonucleotide is in the complex) complexes with different sections of the NA target. Furthermore, the efficiency of complex formation with an antisense oligonucleotide is influenced enormously by the steric structure and by the accessibility of the selected section for the binding of the NA target. The same problem arises also when oligonucleotides or their derivatives are used for site-specific mutagenesis or as hybridisation probes.

The experimental data on the selectivity of the interaction of nucleic acids which have accumulated hitherto are analysed in the present review. They are examined in order of increase in the complexity of the model system investigated: from perfectly and

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Received 7th December 1992
Uspekhi Khimii **62** (4) 414–435 (1993); translated by A K Grzybowski

imperfectly matched complementary complexes formed by oligonucleotides to the interaction of antisense oligonucleotides with high-molecular-mass nucleic acids in systems *in vitro*. To facilitate the assimilation of such data, the first Section of the review quotes theoretical estimates of the level of selectivity of the interaction of nucleic acids and the second quotes the thermodynamic parameters of the formation of perfectly and imperfectly matched complementary complexes. The section devoted to the interaction of reactive derivatives of oligonucleotides with NA targets is based largely on the results of studies carried out at the Novosibirsk Institute of Bioorganic Chemistry of the Siberian Division of the Russian Academy of Sciences. It should be noted that the present review analyses only those studies concerning the interaction of high-molecular-mass NA targets with oligonucleotides in which the level of accuracy of the interaction of the nucleic acids was estimated or if sufficient experimental data were available to make such an estimate.

II. Theoretical estimates of the specificity of the interaction of nucleic acids

One of the sources of errors in the application of site-specific influences to nucleic acids is the possibility of the formation of imperfectly matched complexes. Theoretical estimates of the level of modification of nucleic acids in imperfectly matched complexes have been carried out.[10-12]

The influence of temperature and the concentration of the reagent (reactive oligonucleotide) on the specificity of the interaction of the oligonucleotide probe and the NA target has been analysed.[10] In order to estimate the contribution of the imperfectly matched complexes, it was suggested that the discrimination parameter (∇), characterising the ratio of the degrees of binding of the reagent in the regular and imperfectly matched complexes, be used to estimate the contribution of imperfectly matched complexes. The final expression for ∇ is as follows:

$$\nabla = \{\alpha - \lg[X]\}\frac{n-n'}{n'},$$

where [X] is the concentration of the reagent and n and n' are the lengths of the perfectly and imperfectly matched duplexes. Hence it follows that the value of ∇, i.e. the discrimination between perfectly and imperfectly matched duplexes, is determined by the length of these duplexes and depends logarithmically on the probe concentration, diminishing significantly with its increase. According to estimates made for oligonucleotide concentrations of 10^{-8} and 10^{-2} M, the parameter ∇ attains the maximum value at a reaction temperature equal to the melting point (T_m) of the perfectly matched duplex.

The ratio r' of the sum of the equilibrium nonspecific binding constants $\sum K_n^i = K_n$ for different types of imperfectly matched complexes [noncanonical pairs ('mismatch'), dangling ends, and 'extra' or projecting bases ('bulge') in the target and the oligonucleotide] to the formation constant of the perfectly matched duplex K_s ($r' = K_n/K_s$) has been estimated[11] with the aid of a statistical approach. The results enabled the authors to reach the following conclusions. The main contribution to K_n comes from complexes with dangling ends. In order to diminish the fraction of such complexes, it is necessary to employ reagents containing G · C pairs at the ends, because the difference between the stabilities of the perfectly and imperfectly matched duplexes, associated with the absence of the terminal G · C pair from the imperfectly matched complex, is 5–6 times greater than that due to the absence of the terminal A · T pair. The fraction of binding into imperfectly matched complexes can be significantly reduced if long oligonucleotides are employed or the condition $K_s x_0 \ll 1$, where x_0 is the oligonucleotide concentration, is maintained. It is noteworthy that all the calculations in the above study[11] were performed subject to certain assumptions. Thus the authors assumed that the terminal noncanonical pairs are fully fused and do not contribute to the stability of the duplex. The numerical changes in the free energy of the duplex associated with the formation of noncanonical pairs, calculated from the data of Aboul-ela et al.,[13] were averages and did not take into account the immediate environment. The degree of destabilisation of the duplex induced by the extra base was determined from experimental data of the same study for the extra cytosine and was assumed to be the same for all the bases. However, the assumptions made by the authors should not alter fundamentally the conclusions reached.

The kinetic aspects of the specificity of the interaction of the addressed reagent and the matrix have been examined[12] and it was shown that the nonspecificity of the interaction of the oligonucleotide reagent and the matrix may be of two types. Firstly, there is the nonspecificity associated with the reaction outside the complex, previously considered in a number of studies.[14-16] Secondly, the nonspecificity is induced by the formation of imperfectly matched complexes. The results of the analysis of the possible level of the nonspecific modification of both types for the 302-membered DNA fragment by reagents of different lengths (6–14 units) showed that the contribution of the nonspecific modification of the second type increases with decrease in the length of the nucleotide part of the reagent. It is noteworthy that the nonspecificity of the modifications of both types depends on the reagent concentration: the specificity of the modification of the NA target falls with increase in the reagent concentration.

III. Thermodynamic parameters of the formation of perfectly and imperfectly matched duplexes and the characteristic features of their steric structure

1. Perfectly matched duplexes

According to modern ideas, the characteristic features of the behaviour of double-helix nucleotide structures in solution are determined by the interactions between the heterocyclic bases, the influence of the solvent, the ionic strength, and the electrostatic interactions of the phosphate groups. In the complementary complex, the change in the state of each nucleotide unit cannot take place independently of the neighbouring units, which is responsible for the cooperative nature of the formation and dissociation of the double helix structures. Thermodynamic data for the DNA duplexes have been examined in detail in a number of reviews.[17-19] Natural RNA produce the right-handed *A* form of the double helix, which differs from the *B* form of DNA by the pitch of the helix in eleven bases (one more than in the *B* form of DNA). The most important differences between DNA and RNA involve the conformation of the nucleotide: in RNA the sugar residue has the C(3′)-*endo*-conformation, whilst in DNA it has the C(2′)-*endo*-conformation. The differences between the sugar conformations lead to the appearance of differences in the distances between the phosphates of the polynucleotide chain: 5.9 Å in the RNA duplexes and 7 Å in the DNA duplexes.[20] The relative stability of the *B* form of the DNA duplexes depends on the sequence of chains forming the duplex or, in other words, the stability of the base pair is determined by its immediate neighbours.[13,21-24] The overall stability and the conditions governing the fusion of any DNA duplex can be predicted on the basis of its primary structure, provided that the parameters ΔG_0, ΔH_0, and ΔS_0 are known for each pair of nearest neighbours[24] (Table 1). The free energy of formation of the perfectly matched duplex (ΔG_0) is determined by the sum of the free energies of formation of the pairs of nearest neighbours (ΔG_x) and can be readily estimated from the data in Table 1 by the formula

$$\Delta G_0 = -(\Delta G_{in} + \Delta G_{sym}) + \sum_x \Delta G_x,$$

Table 1. Thermodynamic parameters of the formation of pairs of nearest neighbours in DNA duplexes[24] (1 M NaCl, 25 °C, pH 7.0).

Pair	$-\Delta H_0$ / kcal mol^{-1}	$-\Delta S_0$ / cal mol^{-1} K^{-1}	$-\Delta G_0$ / kcal mol^{-1}
AA/TT	9.1	24.0	1.9
AT/TA	8.6	23.9	1.5
TA/AT	6.0	16.9	0.9
CA/GT	5.8	12.9	1.9
GT/CA	6.5	17.3	1.3
CT/GA	7.8	20.8	1.6
GA/CT	5.6	13.5	1.6
CG/GC	11.9	27.8	3.6
GC/CG	11.1	26.7	3.1

where ΔG_{in} is the change in the ΔG_0 of formation of the duplex as a result of initiation and ΔG_{sym} is a quantity which explains the difference between the entropies of the duplex formed by a self-complementary sequence and the duplex formed by two complementary sequences; ΔG_{in} = 5 kcal mol^{-1} for the complex originating from the G · C pair and 6 kcal mol^{-1} for the complex originating from the A · U pair; ΔG_{sym} = 0.4 kcal mol^{-1} for the duplex formed by a self-complementary sequence and is zero for the complex formed by two complementary sequences. ΔH_0 and ΔS_0 for the formation of the perfectly matched duplex are determined similarly. The thermodynamic parameters of the formation of a long DNA duplex can be readily determined from the three-dimensional diagram presented in a review.[19]

Table 2 lists the thermodynamic parameters of the formation of the G · C or A · U pairs in RNA duplexes obtained by the method of nearest neighbours.[25-28] The thermodynamic parameters of the formation of each duplex are determined in the same way as for the DNA duplexes.[24]

The most characteristic feature of the secondary structure of RNA is the formation of hairpins. The groups at the ends of the hairpin usually comprise 4–6 bases and stabilise the entire hairpin. The quantity $\Delta\Delta G_0$ depends to a large extent on the sequence of bases in the loop and on the base pair closing the loop, varying from −3 to 1.5 kcal mol^{-1}.[29-33] Under these conditions, all the bases even in the short groups (3–5 nucleotides) are stacked together with the ribose ring and each ribose residue has the N conformation, which enables the nucleotide to stretch to the maximum length.[20,30] In the general case, the loops formed by deoxyribooligonucleotides stabilise the entire hairpin to a somewhat lesser extent, but the dependence of the stabilising effects on the base sequence in the loop is retained.[32,33]

The only form in which the double helix RNA exists is the *A* conformation. Depending on the conditions in their formation and the sequence, DNA duplexes can exist in the form of the *A* and *B* conformations or the left-handed *Z* conformation. According to a number of data,[18,19,34-37] the *A* form of the helix is more characteristic of the RNA–DNA duplex, although under certain conditions and depending on the sequence, other conformations can also occur. A comparative analysis of the thermodynamics and kinetics of the formation of hybrid duplexes has been carried out.[18,35,37-41] On the basis of the analysis of the fusion curves and NMR spectroscopic data, the following stability series has been established for RNA–DNA duplexes: $dA_5 \cdot dT_5 > rA_5 \cdot dT_5 > rA_5 \cdot rU_5 > dA_5 \cdot rU_5$ (T_m = 27.5, 22.9, 19.1, and 0 °C respectively).[35] It is noteworthy that very few data are available on the stabilities of the RNA–DNA duplexes and they are contradictory. For the majority of the duplexes investigated, it has been found that the hybrid duplexes are less stable than the corresponding RNA or DNA duplexes.[42] Evidently the stability of the hybrid duplexes, like that of deoxyribo- and ribo-analogues, is determined by their primary structure.

Table 2. Thermodynamic parameters of the formation of pairs of nearest neighbours in RNA duplexes[28] (1 M NaCl, 25 °C, pH 7.0).

Pair	$-\Delta H_0$ / kcal mol^{-1}	$-\Delta S_0$ / cal mol^{-1} K^{-1}	$-\Delta G_0$ / kcal mol^{-1}
AA/UU	6.6	18.4	0.9
AU/UA	5.7	15.5	0.9
UA/AU	8.1	22.6	1.1
CA/GU	10.5	27.8	1.8
CU/GA	7.6	19.2	1.7
GA/CU	13.3	35.5	2.3
GU/CA	10.2	26.2	2.1
CG/GC	8.0	19.4	2.0
GC/CG	14.2	34.9	3.4
GG/CC	12.2	29.7	2.4
Initiation	0	10.8	−3.4
Symmetry of duplex	0	1.4	−0.4

2. Imperfectly matched nucleic acid duplexes

Interest in imperfectly matched nucleic acid duplexes arose initially apparently in the course of the study of the mechanisms of the transmission of kinetic information. In replication, transcription, or reverse transcription, the accuracy of the transmission of genetic information is ensured by the complementary interactions of nucleic acids, but this entails also the inclusion of error bases.[43,44] On the other hand, complexes of this type are formed in codon–anticodon interactions on formation of the steric structure of RNA (G · A and G · U pairs). The formation of imperfectly matched duplexes is widely used in the technique of site-specific mutagenesis.

a. Duplexes with dangling bases at the ends

Terminal unpaired bases, adjoining a long double helix (core), are a characteristic element of the secondary structure of RNA and are involved in many biological processes. The addition of an unpaired base at the 3′- and 5′-end of the RNA duplex stabilises the core duplex as a result of the stacking interaction of the dangling base with the last complementary pair of the duplex. The paired base at the 3′-end of the RNA duplex stabilises the duplex to a greater extent under these conditions than the 'dangling' 5′-end; the difference between the melting points of such duplexes is 8 °C at an oligonucleotide concentration of 10^{-4} M. The addition of a 3′-terminal unpaired base is comparable to the addition of the A · U pair. The stabilising effect of the noncomplementary bases at the ends of the RNA duplex diminishes in the sequence[45,46] 3′-A > 3′-U > 5′-A ≃ 5′-U. Similar results were obtained in the study of the influence of unpaired bases at the ends on the stability of the core duplexes[28,47] GGCC/GGCC, CCGG/CCGG, and CGCG/CGCG. The stabilising effect of unpaired terminal bases increased in the sequence 3′-C < 3′-U < 3′-G ≃ 3′-A and the increase in the free energy of the duplex as a result of the stabilising effect of the unpaired bases at the ends was then 10%–20%, 30%–70%, and 60%–95% of the increase in ΔG_0 due to the formation of the corresponding complementary pair. On average, the 5′- and 3′-noncomplementary bases contribute −0.2 and −(0.4–1.8) kcal mol^{-1} to the ΔG_0 of formation of an RNA duplex.[48,49]

The influence of the unpaired terminal base on the stability of the core DNA duplex is the opposite of the influence of the dangling base on the RNA duplex: the DNA duplexes are stabilised to a greater extent by the unpaired base at the 5′-end.[50,52] A study of the structure and thermodynamic features of the behaviour of the core duplexes containing unpaired thymidine residues at the 5′- and 3′-ends showed that the addition of a dangling 5′- or 3′-thymidine to the core duplex increases the T_m of the duplex. The 5′-terminal thymidine exerts a greater stabilising effect: T_m = 8.3–9.3 °C and 5.0–3.0 °C for the unpaired 5′- and 3′-thymidine residues respectively. Analysis of the circular dichroism (CD) spectra of the core duplexes and their derivatives with unpaired thymidine residues at the 3′- or 5′-ends

showed that the entire duplex, including the dangling end, is in the *B* conformation whilst the unpaired base is in a stack with the core duplex.[52]

b. Duplexes with extra (bulging) bases

The inner loop, consisting of a single base, or the extra (bulging) base are frequently encountered elements of the secondary structure of DNA and RNA. The special interest in projecting bases is associated with the fact that in DNA such a base is a 'hot' spot for mutations, whilst in RNA an extra adenosine frequently appears at the protein binding sites. The main question concerning this element of the secondary structure is whether the extra base belongs to the double helix or whether it forms an inner loop and is displaced from the stack.

The extra adenine in the duplex **1** is involved in a stacking interaction with neighbouring cytidine and guanine residues and does not form an inner loop. The conformation of the complementary pairs on either side of the projecting base is not altered, but the phosphodiester linkage opposite the extra adenine is somewhat lengthened, which leads to the loosening of the structure of the duplex and to a slight change in the torsional angles of the phosphodiester linkage. The melting point of the duplex with bulging adenine is lower by 19 °C than T_m for the perfectly matched duplex, but the fusion of such a duplex takes place cooperatively, including the bulging adenine.[53,54]

$^{5'}$CGC^{A}GAATTCGCG$^{3'}$ / $_{3'}$GCG–CTTAAGCGC$_{5'}$ **1**

$^{5'}$CAAA^{C}AAAG$^{3'}$ / $_{3'}$GTTT–TTTC$_{5'}$ **2**

The activation energy for the formation of the duplex **1** ($E_{act\text{-}A}$) is appreciably higher than the activation energy for the formation of the perfectly matched complex.[55] Whereas the rate-limiting stage in the formation of the perfectly matched duplexes is the formation of the nucleation core,[35] in the duplex with projecting adenine complex formation is inhibited at a defective section of the structure,[55] which leads to an appreciable increase in the activation energy and a broadening of the temperature range in which duplex **1** fuses.

The NMR spectra of nonexchanging protons and the nuclear Overhauser effect (NOE) spectra of the imino-protons of the bases, located on either side of the extra cytidine in duplex **2**, led to the conclusion that such cytidine is outside the helix and that the conformation of the A · T pair adjoining it is greatly modified. Duplex **2** is highly destabilised compared with the perfectly matched complex (T_m for duplex **2** is lower by 15 °C than T_m for the perfectly matched complex and $\Delta\Delta G$ = 2.9 kcal mol^{-1}). The decrease in the stability of the duplex has an enthalpy origin and is a consequence of the breakdown of the stacking interactions in this duplex.[56] At a low temperature (about 0 °C), the projecting cytidine is outside the duplex and, on raising the temperature (>40 °C), the fraction of conformations in which the extra cytidine forms part of the helix increases.[57]

The extra adenine and guanine adjoining the G · C pair (duplexes **A** and **G**) participate in the stacking interactions with the adjoining bases and are clearly part of the double helix (Scheme 1).

Scheme 1

$^{5'}$GATGGGCAG$^{3'}$ / $_{3'}$CTACCCGTC$_{5'}$ **N**

$^{5'}$GAT–GGGCAG$^{3'}$ / $_{3'}$CTA$_{C}$CCCGTC$_{5'}$ **C**

^{5}GAT–GGGCAG$^{3'}$ / $_{3'}$CTA$_{A}$CCCGTC$_{5'}$ **A**

$^{5'}$GAT–GGGCAG$^{3'}$ / $_{3'}$CTA$_{G}$CCCGTC$_{5'}$ **G**

The destabilising effect of these bases is strictly localised and does not extend over the entire duplex. The bulging cytidine is in the $C_{(4)}$ backbone of duplex **C** and is, as it were, delocalised over the $C_{(4)}$ sequence. This decreases the destabilising effect of the extra cytidine on the double helix; T_m for duplex **C** is higher by 5.7 °C than for duplex **A** or **G**.[58,59] Comparison of the structures of ribo- and deoxyribo-duplexes containing bulging uridine or thymidine (duplexes **3** and **4**) showed that the bulging thymidine in **4** is built into the double helix without causing any appreciable distortions in the *B* form of DNA, while the extra uridine in duplex **3**, having the same composition, is outside the helix. The regular structure of the *A* form of RNA is also unaffected under these conditions.[60,61]

$^{5'}$r(CUGG^{U}GCGC)$^{3'}$ / $_{3'}$(GACC–CGCG)r$_{5'}$ **3**

$^{5'}$d(CTGG^{T}GCGC)$^{3'}$ / $_{3'}$(GACC–CGCG)d$_{5'}$ **4**

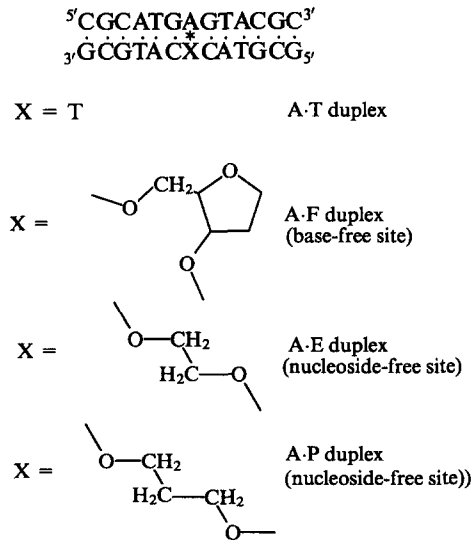
There are comparatively few experimental data concerning the influence of the projecting base on the structure and stability of RNA duplexes. The study of the thermodynamic behaviour of 14 synthetic ribooligonucleotides, assuming in solution the hairpin shape with four bases in the loop and one projecting base in the stem, showed that the introduction of the extra base into the double helix region destabilises the duplex. The magnitude of the effect is determined by the type of base: the greatest destabilising effect is shown by the extra adenine and guanine ($\Delta\Delta G_A^{37\,°C}$ = 3.3 kcal mol^{-1} and $\Delta\Delta G_G^{37\,°C}$ = 3.5 kcal mol^{-1}) and the smallest is exerted by the extra uridine ($\Delta\Delta G_U^{37\,°C}$ = 2.7 kcal mol^{-1}).[31]

c. Duplexes with point breaks

Point breaks as well as nonpurine and nonpyrimidine sites are frequently encountered in the DNA structure. However, comparatively few data have accumulated at the present time concerning the influence of such damage to DNA on the structure and stability of the DNA duplexes. Similar data on RNA duplexes are altogether lacking. Four 12-unit duplexes of identical composition with a point break have been investigated by NMR and NOE spectroscopy. It was found that they all have a helix in the *B* form and differ slightly in stability from the perfectly matched duplex. Fusion of the duplex with a point break takes place cooperatively. Apparently the point break does not alter appreciably either the stability or the structure of these DNA duplexes.[62] Somewhat different results for the influence of point breaks on the stability of a DNA duplex have been obtained in another study.[63] The introduction of a point break into the ring structure diminishes T_m for such a duplex by 27 °C (1 M NaCl).

The introduction of a base-free site into the structure of the duplex lowers the melting point of the duplex by 10–12 °C compared with the perfectly matched duplex. The destabilisation of the duplex by a 'hole' is comparable to the effect of the introduction into the duplex of a T · T pair (T_m = 66, 53–56, and 56 °C for the perfectly matched 13-unit duplex, the duplex with a nonpyrimidine site in the 7-position, and the duplex with a T · T pair in the 7-position respectively.[64] The influence of the base-free site and the nucleoside-free site on the structure of the DNA duplex has been investigated in greatest detail[65] in relation to the duplex having the composition shown in the next column.

The changes in all the thermodynamic parameters induced by the damage to the duplex resulting from the formation of a 'hole' are very great (ΔT_m = 13.9, 12.9, and 14.4 °C for the duplexes A · F, A · E, and A · P respectively) and greatly exceed the values predicted for such duplexes by the nearest neighbours method. The results show that these changes in the stability of the duplex are associated with the absence of a base and not the absence of a sugar residue. The cooperative nature of the helix–coil transition hardly changes on passing from the duplex A · T to the duplexes A · F and A · E. This suggests[57,65] that the sugar phosphate skeleton of the DNA molecule is in fact responsible for the cooperative nature of the helix–coil transition on fusion.

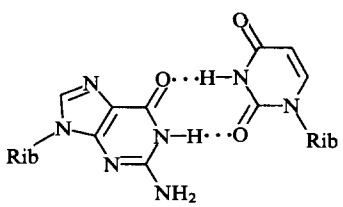


The data presented permit the conclusion that, although the introduction of a point break lowers the stability of the DNA duplex, it does not affect its structure, whereas nonpurine and nonpyrimidine sites lead to appreciable changes in the structure of the duplex as a result of the disturbance of the stacking interactions in the double helix.

d. Duplexes containing noncanonical base pairs

Noncanonical or irregular base pairs are very often encountered in the molecules of both RNA and DNA. All noncanonical pairs can either form an inner loop made up of two bases, or can participate in the stacking interactions of the double helix, or can form a noncanonical ('wobble') pair. In analysing any noncanonical pair, it is essential to answer the following questions: (1) what structural changes are introduced by the given irregular pair into the conformation of the duplex; (2) what thermodynamic and kinetic parameters of the formation of the duplex are altered under the influence of the given noncanonical pair; (3) in what way are hydrogen bonds formed in the given noncanonical pair. All these questions have been examined in detail in a review.[18] This section presents data concerning only the influence of noncanonical pairs on the stability of RNA and DNA duplexes.

The G · U or G · T pair. The G · U pair is one of the most thoroughly investigated, most frequently encountered, and most stable of the irregular pairs. The formation of hydrogen bonds in the G · U pair, put forward by Watson and Crick, is illustrated in the scheme below:



G · U

X-Ray diffraction analysis of an 8-unit duplex with two central G · T pairs as well as NMR spectroscopy of poly(dGT) [the appearance in the spectra of two signals in the region of 11 p.p.m. (resonance of the >N–H...O = C< hydrogen bond) and two signals in the region of 8 ± 0.5 p.p.m. (-resonance of the free NH_2 group)] yielded rigorous experimental evidence showing that the bases in the G · T and G · U pairs in long DNA or RNA duplexes are paired and built into the double helix.[22, 23, 54, 66–69]

The G · T pairs exert extremely little influence on the overall structure of the duplex and hardly disturb the stacking interactions in it. On formation of such a pair, small changes occur only in the torsional angle of the *N*-glycoside component of uridine, which is the main reason for the destabilisation of duplexes with G · U pairs compared with perfectly matched duplexes.[66, 70] Comparison of three 8-unit duplexes,[71] containing two G · T, two G · C, or two A · T pairs in the 3- and 4-positions, showed that both G · T pairs participate in the formation of the double helix, but the rotation of the helix at the G · T pair is by 44.3° (instead of 31.5° for the classical pair), while at the G · C pair, which follows G · T, the rotation is by only 22°.

The melting point of the 8-unit duplex with two G · T pairs in the centre is lower by 20 °C than T_m for the perfectly matched duplex (52 and 72 °C respectively), while $\Delta\Delta G_0 = 2-3$ kcal mol^{-1}.[66, 71] Similar results have been obtained in the X-ray diffraction analysis of an 8-unit duplex containing two I · T pairs in the centre.[72] It was found that a thermodynamically more favourable situation arises if the two G · T pairs follow one another and are not separated by classical pairs. The degree of destabilisation of the duplex ($\Delta\Delta G_0$) is in this case 1 kcal mol^{-1} per G · T pair.[71]

The rate of exchange of the protons of the G · T pairs[22, 23] in the 3- and 10-positions in a 12-unit duplex at 35 °C exceeds (approximately by an order of magnitude) the rate of exchange of the protons of the G · C pairs in the same positions in the duplex, which indicates a looser structure of the G · T pair, more accessible to the solvent, compared with the G · C pair. Data confirming the formation of the looser structure in duplexes with G · T pairs have also been obtained in other studies.[67, 68]

Comparison of the thermodynamic parameters of the formation of 16 duplexes containing an inner G · U or A · U pair revealed that the G · U pair stabilises the duplex (compared with the loop at this site) and that its contribution to ΔG_0 of formation of the duplex depends on the immediate environment.[73] If the G · U pair adjoins an A · U pair, then the increase in the free energy of the duplex is $-(0.5-0.7)$ kcal mol^{-1}, but, when it adjoins a G · C pair, then $\Delta G_0(G \cdot U) \simeq \Delta G_0(A \cdot U) = -(1.3-1.9)$ kcal mol^{-1}. On average, the inner G · U pair is less stable to the extent of 0.7 kcal mol^{-1} than the A · U pair.[48, 70, 73] The greater stability of the G · U pair with a G · C pair on its side, compared with the G · U pair adjoining an A · U pair, can be explained by more effective stacking interactions of the G · U and G · C pairs compared with the G · U and A · U pairs. Comparison of the ribo-duplexes containing a central G · U, G · C, or A · U pair showed that the T_m of the duplex with a G · U pair is approximately equal to the T_m of the duplex shortened by one unit. The G · U pair itself is a site of instability in the double helix.[74]

The G · A pair. This 'wobble' pair is of special interest, because it is the usual element forming the steric structure of RNA.[75] At neutral pH, one can postulate four different versions of the formation of hydrogen bonds in the G · A pair (Fig. 1).[75, 76] It is noteworthy that the conformation *a* has been predicted and detected in the RNA double helix,[75] while the conformation *b* has been confirmed experimentally in a study of model DNA duplexes.[77, 78] Until recently, there was no experimental confirmation of the existence of the conformations *c* and *d* for the G · A pair. In the conformations *a* and *b*, there is a characteristic propeller-like disposition of the bases relative to one another (the angle of rotation is 24.8°), which facilitates the formation of hydrogen bonds between guanine and adenine.[77, 79] The G · A pair fits satisfactorily into the double helix of the *B* form, causing virtually no disturbance to the stacking interactions in the duplex, while the changes in the torsional angles of the bases are within the limits characteristic of canonical base pairs.[77, 80, 81] The destabilising effect of the G · A pair is manifested by a decrease in the T_m of the duplex by 10 °C per G · A pair compared with the perfectly matched duplex. The perturbations introduced by the G · A pairs are local in character and are not propagated along the helix.[13, 77]

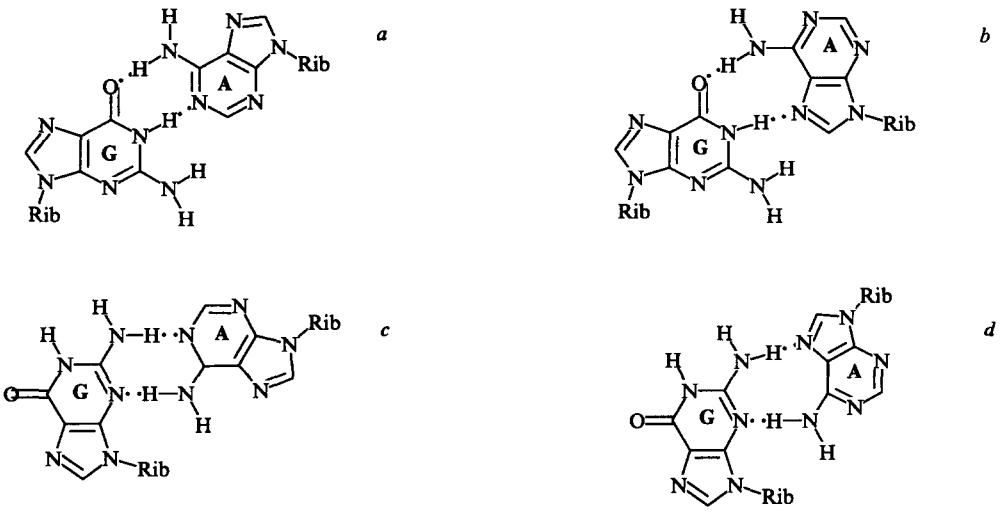


Figure 1. Four versions of the formation of hydrogen bonds in the G · A pair.

Comparative study of 13 dodecamers, differing in the number of G · A pairs, their positions, and their environments, revealed the influence of the nearest neighbours on the stability of the imperfectly matched duplex with such noncanonical pairs. It was found that the most stable sequences, containing two adjoining G · A pairs, have thermodynamic parameters of formation approximately equal to the parameters of formation of the perfectly matched duplex. However, the same sequences with a single G · A pair are appreciably destabilised compared with the perfectly matched duplex.[82,83]

In a comparatively recent study of a 10-unit duplex formed by the oligonucleotide d(ATGAGCGAATA), containing four G · A pairs and a noncomplementary adenine unit at the 3'-end, direct evidence was obtained for the existence of the conformation *d* of the G · A pair. This duplex produced the *B* form of the double helix and was distinguished by an anomalously high stability, approximately equal to the stability of the perfectly matched complex, which was explained by the very effective stacking interactions between the bases of the opposite chains (G_3 with G_7 and A_4 with A_8).[84] The study of the contribution of the hydrogen bonds formed within the G · A pairs to the stability of the RNA duplexes (not less than -1.4 kcal mol^{-1} per pair) makes it possible to explain this anomalously high stability of the imperfectly matched duplex with G · A pairs.[83]

The A · A pair. The A · A pair does not form stable hydrogen bonds. The incorporation of the d(A · A) pair in the double helix takes place as a result of the stacking of adenine units with neighbouring bases and is accompanied by the distortion of the planar structure of the heterocyclic base, in order to decrease the overlap of the exocyclic amino-groups of the adenine units, and by appreciable rearrangements of the sugar phosphate skeleton of the duplex.[85]

The G · G pair. Indirect evidence for the existence of this pair has been obtained in a number of studies,[86-88] but the existence of this wobble pair in a duplex was first demonstrated by Cognet et al.[89] According to two-dimensional NMR data, the bases of this pair participate effectively in the stacking interactions of the double helix and the double helix itself has the classical *B* form. The imino-protons of the bases participate in the formation of the hydrogen bond of the G · G pair; one of the guanine units assumes the normal *anti*-conformation, whilst the other assumes the *syn*-conformation or one close to it. Such a pair has a symmetrical structure and does not introduce perturbations into the regular structure of the *B* form of DNA. There are no data on the thermodynamic stability of duplexes with G · G pairs.

Purine–purine pairs cause a marked destabilisation of the double helix. Comparison of the thermal stabilities of oligomer-polymer duplexes having the structures $dG_ndG_{12}A_mC_x$ and $dG_ndG_{10}G_mC_x$ ($n = 100$, $m = 0-6$, $x = 5-41$) with different numbers of d(A · G) and d(G · G) pairs showed that the destabilisation of the duplex is proportional to the number of noncanonical pairs.[87] The presence of a cluster of noncanonical G · A or G · G pairs does not, however, affect the cooperative nature of the fusion of the duplex, which constitutes additional confirmation that the irregular purine–purine pairs are built into the regular double helix. Long DNA duplexes, containing one noncanonical pair, are resistant to the action of S1 nuclease. The increase in the length of the section with noncanonical pairs from 2 to 6 bases increases the sensitivity of the duplex to S1 nuclease: the duplex is cleaved by S1 nuclease under identical conditions to the extent of 4% and 30% for duplexes with one and six G · G pairs respectively.[87]

The influence of nearest neighbours on the destabilising effect of the purine–purine noncanonical pair has been investigated.[70,74,82] According to NMR spectroscopic data, 5-unit duplexes are not formed if the G · A or G · G pairs are flanked by A · U pairs, whereas duplexes in which the G · A or G · G pairs are flanked by G · C pairs are fairly stable (T_m = 26 °C). Data confirming the marked dependence of the stability of the duplex with purine–purine pairs on its base sequence have also been published.[82,84,87,90]

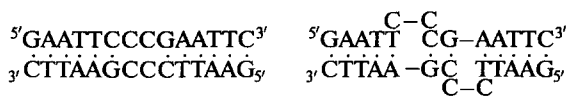
The A · C pair. According to literature data,[77,91] the A · C pair is stabilised by one hydrogen bond between the N(6) protons of the amino-group of adenine and the N(3) or O(2) protons of cytidine, but the possibility of the formation of an A · C pair, stabilised by two hydrogen bonds in the case of adenine in the tautomeric form, is not ruled out. The rate of exchange of the imino-protons in the G · C and A · T pairs adjoining a noncanonical A · C pair in a duplex with two A · C pairs increases compared with the perfectly matched duplex by factors of 3 and 2 respectively. Consequently the destabilising influence of the A · C pair is not localised and is propagated along the double helix.[91] The incorporation of an A · C pair in a DNA duplex is accompanied by small changes in the sugar phosphate skeleton of the duplex, ensuring the occurrence of stacking interactions between the A · C pair and the adjoining bases.[92] The introduction of an A · C pair into the centre of a 17-unit duplex lowers its melting point compared with the perfectly matched duplex by 10 °C. The stability of the duplexes containing noncanonical pairs diminishes in the sequence[93] G · C > A · T > G · U > A · C. Comparative analysis of data on the hybridisation of 11-, 14-, and 17-unit oligonucleotides with the DNA of the phage φX 174 and with the

DNA of a mutant phage in which a point exchange of G · A has taken place at the oligonucleotide binding site demonstrated that all three probes are bound much more weakly with the mutant DNA. The introduction of one A · C pair into the duplex lowers the dissociation temperature of these probes by 12 °C, which corresponds to the 'loss' of approximately three G · C pairs.[94]

The U · U pair. The existence of the U · U pair has not been demonstrated experimentally. The five-membered self-complementary oligonucleotide AGUCU exists preferentially in the single-stranded form. The U · U pair in the centre of the duplex leads to its complete destabilisation, while the duplex with the G · C pair at the same site has[74] a melting point of 25 °C.

The T · T pair. According to NMR data, the T · T pair is not stabilised by a hydrogen bond,[13] but it can be built into the double helix under these conditions with minimum disturbances to the structure of the duplex and leads to a decrease in its T_m compared with the perfectly matched 12-unit duplex by 11 °C. This pair forms a locally unstable section in which the dissociation of the duplex is initiated on raising the temperature simultaneously with a process occurring from the end of the duplex.[51,95] The T · T pair destabilises the duplex to the greatest extent[13,85] if it is incorporated in the ATA/TTT sequence, exerting the least effect in the CTG/GTC sequence.

The C · C pair. There is at present no experimental evidence in the literature for the formation of hydrogen bonds between the bases of the C · C pair. The absence of hydrogen bonds stabilising the C · C pair as well as the poor overlap (stacking) of the unpaired cytosine with the adjoining bases of the duplex lead to the bulging of the C · C pair with formation of an inner loop.[70,74] It has been shown[96] that duplex **5** exists not in the form of a hairpin with an inner loop comprising four cytidine units but has the structure **6** with bulging cytidine units. Such rearrangement of the duplex is accompanied by a change in the sugar phosphate skeleton only in the looped out nucleotide, whereas the entire remainder of the duplex has the *B* form of DNA.



5 **6**

The appearance of the C · C pair in the duplex causes an appreciable increase in the enthalpy of formation of the duplex: $\Delta H_0 = -77$ kcal mol^{-1} for $[(Ap)_3A(pU)_4]_2$ and $\Delta H_0 = -43$ kcal mol^{-1} for $[(Ap)_4C(pU)_4]_2$, and reduces its melting point by 10–17 °C, which corresponds to the loss of two A · U pairs.[97]

In the study of the crystal structure of the dodecameric RNA duplex containing three noncanonical pairs in the centre, it was observed that four noncomplementary bases do not form an inner loop, giving rise to a fairly regular helix incorporating the G · U and U · C pairs.[98] These results are the first experimental evidence for the existence of the U · C pair in the crystal. This pair forms one hydrogen bond and is stabilised by hydrogen bonds with water molecules, forming bridges between the nitrogen atom of the heterocyclic base and the oxygen atom attached to phosphorus. The authors mention particularly the influence of the hydrogen bonds between the OH groups of ribose, the oxygen atoms at the phosphorus, and the water molecules on the stabilisation of the duplex structure.[98]

3. Comparative analysis of the influence of noncanonical base pairs on the stability of DNA and RNA duplexes

It is quite evident that the less the stability of an imperfectly matched complex, formed fortuitously by an oligonucleotide and the NA target, differs from the stability of the perfectly matched duplex, the higher the level of their nonspecific interaction. Comparison of the destabilising influence of various structural anomalies (noncanonical pairs, projecting bases, dangling ends) in order to discover those which destabilise the duplex to the least extent is therefore extremely urgently required. An extensive set of experimental data, including also the thermodynamic parameters of the formation of imperfectly matched complexes with structural anomalies, has now accumulated, but these data are somewhat heterogeneous and do not permit unambiguous conclusions about the degree of the destabilising influence of each separate noncanonical base pair or some other structural anomaly in the duplex.[13,18] It is noteworthy that the destabilising influence of each structural anomaly depends also on the environment in which it is located i.e. on the nearest neighbours.[13,84,89] Thus the core component of the duplex usually remained unchanged in studies where the relative stabilities of different noncanonical pairs were estimated. This was the reason for the considerable discrepancies between the estimates of the stability of noncanonical base pairs obtained by different workers:[13,23,99]

T · G > G · G = G · A > G · T > A · G = C · T >
T · C = A · A = T · T > C · A > C · C > A · C;[13]
G · T = T · G > A · G > T · T = C · T > G · A = G · G >
T · C > A · A > A · C > C · A > C · C;[99]
G · T > G · A > A · A = T · T = C · A > C · T.[23]

Noncanonical base pairs are arranged in order of decreasing stability.

Considerable discrepancies are observed not only in the estimates of the relative stabilities of various noncanonical base pairs but also in the absolute differences between the thermodynamic parameters for perfectly and imperfectly matched complexes incorporating the same noncanonical pair. Thus, the destabilising influence of the A · C pair with a constant environment can be determined from the results of different studies:[13,100] $\Delta\Delta G_0$(A · C) = 2.7 kcal mol^{-1} (Ref. 100) and 3.5 kcal mol^{-1} (Ref. 13). However, such a difference between the destabilising effect of the given noncanonical base pair cannot be accounted for by the differences either between the experimental conditions or between the lengths of the duplexes investigated.

The destabilising effect of different noncanonical bases on the stability of core duplexes has been studied in greatest detail in several investigations.[13,56,99,100] Corresponding parameters for the destabilisation of the duplex owing to the formation of noncanonical pairs as a function of nearest neighbours have been determined experimentally.[13] These data are presented in Table 3.

The influence of all possible noncanonical pairs on the thermodynamics of the formation of a 17-unit DNA duplex (with mismatches located within the centre of the duplex) has been investigated.[99] On the basis of the analysis of the ΔH_0 and ΔS_0 for a noncanonical base pair, i.e. the parameters which describe the stacking interactions of the given pair with the adjoining bases, the authors classified different mismatches in terms of their involvement in the formation of the duplex: (1) Wobble pairs or irregular pairs stabilised by hydrogen bonds. These are characterised by a strong stacking interaction with the adjoining bases. Such pairs do not disturb either the overall stacking of the duplex or the conformations of the sugar phosphate residue; the entropy of formation of the wobble pair is approximately equal to the entropy of the Watson–Crick pair, while the enthalpy of formation is somewhat lower. The T · G, G · G, C · A, A · A, and A · G pairs belong to the class of wobble pairs. (2) Open pairs, i.e. pairs which do not form hydrogen bonds but are involved in the stacking interaction with

Table 3. Destabilisation of the double helix by the formation of noncanonical base pairs.[13]

Base pairs	ΔG_0 / kcal mol^{-1}	ΔH_0 / kcal mol^{-1}	ΔS_0 / cal mol^{-1} K^{-1}
A–T–A / T–G–T	0	−8.1	−27
A–G–A / T–G–T	0.2	−6.0	−20
A–G–A / T–A–T	0.3	−5.1	−18
A–G–A / T–T–T	0.7	0.8	1
A–A–A / T–G–T	1.2	7.6	22
A–C–A / T–T–T	1.2	−5.7	−23
A–T–A / T–C–T	1.5	−2.5	−13
A–A–A / T–A–T	1.5	10.6	31
A–T–A / T–T–T	1.5	−7.1	29
A–C–A / T–A–T	1.9	7.2	38
A–C–A / T–C–T	2.0	−7.8	−33

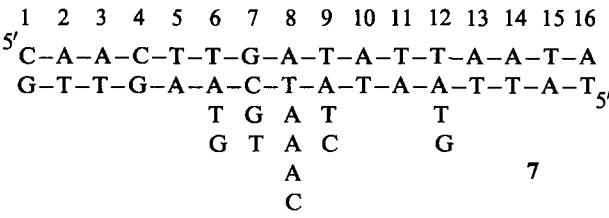
the bases of the chain. Such pairs introduce the greatest perturbation and destabilise the duplex. (3) Weak pairs linked by only one hydrogen bond; the stacking interaction is unlikely in this instance. The enthalpy of formation of a weak pair is approximately equal to half the enthalpy of formation of a wobble pair.

It is noteworthy that the melting points of the duplexes differing only in the orientation of the irregular pair are not the same although the environment of these pairs and hence the interplanar contacts do not change. Data obtained recently indicate the extremely anomalous influence of adjacent inverted G · A pairs on the stability of DNA duplexes.[82,84] The replacement of two canonical pairs in the decameric duplex by two isolated G · A pairs lowers the association constant by a factor of $10^3-4\times10^5$. Four inverted G · A pairs arranged in pairs destabilise the duplex by a factor of 4.5, but, in the presence of the same number of G · C pairs in this duplex as in the perfectly matched complex, an anomalously stable duplex is produced (the association constant increases by a factor of 5–40). Similar data have been obtained[90] also for ribo-duplexes containing a G · A pair.

The influence of the location of the noncanonical pairs on the stability of duplex **7**, which is a fragment of the DNA of the bacteriophage φX 174 and is the binding site of the gene A protein, has been investigated.[100] Duplexes containing noncanonical pairs fuse at a lower temperature than the perfectly matched duplex **7**. The destabilisation of the duplex is greater when the irregular pair substitutes a G · C not a A · T pair and also if the noncanonical pair is located closer to the centre of the duplex (cf. the duplexes 8-AA and 12TG in Scheme 2).

Three probable structures were analysed for all the duplexes in Scheme 2: (1) the formation of inner loops. This process should be accompanied by the loss of the stacking interaction of two base pairs, an appreciable increase in ΔH_0, and a change in the free energy of the duplex ($\Delta\Delta G_0$) in the range 5.5–7.0 kcal mol^{-1}. (2) The formation of a structure with bulging bases. This should be accompanied by the elimination from the stacking interaction of one base pair and should involve $\Delta\Delta G_0$ = 7.0 kcal mol^{-1}. (3) The formation of a noncanonical base pair. This process is characterised by the retention or slight alteration of the values of ΔH_0 and low values of $\Delta\Delta G_0$. According to the thermodynamic parameters presented above, these duplexes, illustrated in the scheme, form noncanonical pairs and not any other structures.

Scheme 2



Duplex	T_m / °C	$-\Delta G_0$ / kcal mol^{-1}	$\Delta\Delta G_0$ / kcal mol^{-1}
7	52.0	15.8	–
6-TG	45.1	13.2	2.6
7-GT	42.9	12.7	3.1
8-AA	42.1	12.6	3.2
8-AC	40.6	12.1	3.7
9-TC	43.0	13.1	2.7
12-TG	46.9	14.0	1.8

The influence of the terminal noncanonical pair on the stability of the duplex has been studied only for oligoribo-nucleotide duplexes.[49,101] The free energy increment following the addition of the terminal noncanonical pair to the core duplex was on average −0.5 to −2.3 kcal mol^{-1} depending on the form of the noncanonical pair and the last base of the core duplex: $\Delta\Delta G_0$(G · U) ≃ $\Delta\Delta G_0$(A · U) ≃ −1.3 kcal mol^{-1}; the addition of a pyrimidine–pyrimidine or purine–pyrimidine pair (but not G · U) increased the stability of the duplex by −0.5 kcal mol^{-1}, while the addition of a purine–purine pair had an effect comparable to that of the addition of a dangling end (the contribution to ΔG_0 by this pair is −0.2 kcal mol^{-1}). It has been shown[102] that the stabilising influence of the terminal mismatch increases in the sequence A · C ≃ A · A < A · G ≃ A · U (data obtained for the CCGG/GGCC core duplex).

Comparative analysis of the data presented in a number of studies[13,49,56,99,101] permits the claim that the addition of the weakest noncanonical pair U · C at the end of the duplex does not alter its stability, while the appearance of an isolated G · U pair in the central part of the duplex causes its appreciable destabilisation.

The study of Gillam et al.[42] is the only one in which the stabilities of imperfectly matched heteroduplexes were compared. The melting points of the mismatched duplexes were determined from the temperature dependence of the degree of binding of the oligonucleotides to d(pT)$_9$ immobilised on a resin. The following noncanonical pairs were analysed: T · dC; T · rC; T · dG; T · rG; T · T; T · U. Ribooligonucleotides form less stable duplexes with immobilised d(pT)$_9$ than do deoxyribooligonucleotides. Only duplexes containing the T · rG pair are an exception. The destabilising effect of this pair is much smaller than that of the dT · dG pair. The stability of the duplex with the T · rG pair corresponds to that of the duplex shortened by one A · U pair. Furthermore, the influence of the position of the irregular pair in the duplex on its stability was demonstrated in the same study. The terminal irregular pair induces a minimal destabilisation of the double helix (ΔT_m = 4 °C for dT$_9$ · dA$_8$ and dT$_9$ · dA$_8$G) compared with the irregular pair at the centre of the duplex (ΔT_m = 15 °C for dT$_9$ · dA$_8$ and dT$_9$ · dA$_5$GA$_3$).

In considering the problem of the reparation of the noncanonical pairs,[88,90] the authors conclude that the stable pairs (G · T and T · G), introducing minimal perturbations into the DNA structure, are repaired most effectively. The degree of reparation is 93% for the G · T pair and 55% for the T · G pair, whereas other mismatches are recognised by the cell repair system to a much lesser extent. The degree of reparation is less than 5% for the A · G and C · T pairs and 42% for the G · A pair.

The experimental data considered in this Section indicate unambiguously that the greatest contribution to the decrease in the complementary accuracy of the interaction of nucleic acids should come from imperfectly matched complexes with structural

anomalies at the ends of the duplex, which agrees with theoretical estimates of Perelroyzen and Vologodskii.[11] However, one should not forget at the same time the possibility of the formation of imperfectly matched complexes with an increased stability, e.g. such as the duplexes with inverted noncanonical G · A pairs.

IV. Accuracy of the interaction of RNA and DNA molecules with oligonucleotides and their derivatives

A number of approaches have been developed in recent years for the investigation of the structure and functions of nucleic acids based on the use of antisense oligonucleotides and their derivatives.[1] The antisense oligonucleotides, which are complementary to different sequences in the composition of single-stranded and double-stranded nucleic acids, have the ability to form specific complexes with these nucleic acids (duplexes and triplexes), which prevents the interaction of the nucleic acids with ribosomes and DNA or RNA polymerases both *in vitro* and *in vivo*.[2,9]

There exist several main requirements on which these approaches are based. Firstly, oligonucleotide derivatives must permeate the cell and must be sufficiently stable under *in vivo* conditions. Secondly, oligonucleotides or their derivatives must form a complex with the selected nucleic acid sequence. Thirdly, the formation of the complex must inhibit the functions of the given nucleic acid. Fourthly, the antisense oligonucleotides must not interact with other sequences in the cell apart from the one chosen.[9] It is quite evident that the last two principles are closely interrelated — a specific interaction with the selected sequence is impossible without the formation of a highly stable complementary complex.

The thermodynamic parameters of the formation of perfectly matched DNA and RNA duplexes as well as the defects in the double helix having a destabilising effect on the duplex were examined in the previous Sections. All these data were obtained in the study of model oligomeric duplexes. The next section will deal with studies on the interaction of oligonucleotides or their derivatives with the NA target, in which the accuracy of the interaction of the oligonucleotide with the selected nucleic acid was estimated.

1. Model systems

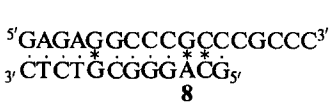
a. Oligonucleotides with the usual phosphodiester linkage

The specificity and effectiveness of complex formation by a series of synthetic oligonucleotides with a length corresponding to 8–14 units (ON) with the M13mp3 phage DNA have been investigated.[103] Two oligonucleotides out of the eleven investigated [one a 12-unit oligonucleotide (ON-12) and the other a 14-unit oligonucleotide (ON-14)], complementary to the 3366-3377 and 2216-2230 sections of the phage DNA respectively, were bound at 4 °C to the DNA target at more than one site (the ON-12 and ON-14 concentrations were 2 μM). The binding of these oligonucleotides to the DNA target with formation of imperfectly matched complexes was confirmed by the elongation of ON-12 and ON-14 as primers under the influence of DNA polymerase at 20 °C. It is noteworthy that under the same conditions two 10-unit oligonucleotides, forming with the M13mp3 DNA duplexes with a length equivalent to 10 base pairs and containing a central G · T pair, were not built up by DNA polymerase, while a 13-unit oligonucleotide containing an analogous noncanonical pair was built up by the enzyme until the phage DNA was replicated.

The influence of noncomplementary pairs in the matrix · primer duplex on the effectiveness of the chain elongation reaction with the aid of the Taq DNA polymerase and the AMV reverse transcriptase has been investigated in a series of studies.[104–108] The presence of a single mismatch within the matrix · primer duplex did not produce an appreciable effect on the yield of the product of the amplification of the HIV-1 gag-region, whereas the presence of a noncanonical base pair at the 3′-end affected the yield of the PCR-product. The 3′-terminal T · C, T · G, T · T, G · G, and A · C pairs were amplified with the same effectiveness as the perfectly matched duplex, whereas the A · G, G · A, and C · C pairs at the 3′-end of the duplex decreased the overall yield of the PCR product by a factor of 100 and the A · A pair decreased it by a factor of 20. The influence of the G · A and A · G mismatches on the yield of the product was the same. The minimum effect on the yield of the PCR-product was exerted by mismatches generated by T with G and by C with T. The presence of two irregular pairs in the vicinity of the 3′-end of the primer · matrix duplex (the last four bases of the duplex), one of the mismatches being 3′-terminal (apart from the irregularly paired thymidine), leads to the virtually complete cessation of the elongation reaction.[104] It is noteworthy that the data on the thermodynamic stability of such imperfectly matched duplexes do not explain the results obtained by the authors. Similar data have been obtained[105] for the elongation of an imperfectly matched primer · matrix duplex with the aid of the AMV reverse transcriptase. The decrease in the overall concentration of dNTP from 800 to 50 μM[104] or from 200 to 2 μM[106] led to the elongation mainly of only the perfectly matched duplex (the G · G and C · T mismatches respectively). With increase in the temperature of the annealing of the primer · matrix duplex from 44 to 55 °C, only the perfectly matched duplex was elongated, while no elongation was observed in the duplexes with the 3′-terminal A · A and T · T mismatches.[107] The elongation of only the perfectly matched duplex could be achieved also using shorter primers: 10 out of the 12 possible noncanonical pairs at the 3′-end of the duplex completely stopped the elongation if short (10-unit) primers were employed.[108]

Analysis of a series of oligonucleotides, complementary to the Shine–Dalgarno sequence in the *E. coli* 16S rRNA, forming with the latter perfectly (AGAGGAGGU, ON/r) and imperfectly matched complexes (GGAAGGAGC, ON/i-1; GGAGAAGC, ON/i-2; AUUUCUUCUAUG, ON/i-3) showed that the effectiveness of the inhibition of the binding of Qβ RNA to the 70S ribosomes of *E. coli* depends on the stability of the 16S rRNA · oligonucleotide duplex: ON/r: ΔG = 16.9 kcal mol^{-1}, binding inhibited by 87.2%: ON/i-1: ΔG = −11.1 kcal mol^{-1}, binding inhibited by 56.6%; ON/i-2: ΔG = −7.9 kcal mol^{-1}, binding inhibited by 42.6%; ON/i-3: ΔG = −1.1 kcal mol^{-1}, no inhibition observed.[109] The oligonucleotide 'probing' of the 16S rRNA molecule forming part of the composition of the 30S ribosomal subparticles in *E. coli* by 8–15 unit oligonucleotides demonstrated that, at an oligonucleotide concentration of 1 μM, there is a possibility of the formation of an imperfectly matched complex with exposed RNA sections, containing five classical Watson–Crick pairs at the 3′-end of the duplex.[110]

The site-specific cleavage of mRNA of prothymosin-α, isolated from the cytoplasm of lymphocytes stimulated by the staphylococcal enterotoxin, in the presence of a 12-unit oligonucleotide (GCAGGGCGTCTC) and RNase H takes place within both perfectly and imperfectly matched duplexes. The imperfectly matched ON-12 binding site was identified from the size of the hydrolysis product and was found to contain three irregular pairs (structure **8**).[111]



8

The mRNA of γ-interferon was cleaved in the same investigation in the presence of a 24-unit oligonucleotide ON-24 (at a concentration of 1 μM). Although, apart from forming a complex with the 603–625 sequence of the mRNA of γ-interferon, ON-24 can form an imperfectly matched duplex with a length equivalent to seven base pairs with the TGCCTGC sequence of mRNA, the products of the cleavage of RNA at this site were not detected. Evidently the difference between the

stabilities of the perfect and imperfect ON-24 · mRNA of γ-interferon duplexes proved insufficient for discrimination between these sites by RNase H.

The accuracy of the formation of the duplex is particularly important in site-specific mutagenesis. 8–12-Unit oligonucleotides containing the base to be replaced in the centre proved to be most convenient for the introduction of point mutations into the DNA molecule.[112] The stability of the imperfectly matched duplex containing one noncanonical pair proved to be the decisive parameter on which depended the yields of the mutant DNA. The yield of the mutant DNA increased from 0.05 to 20% on reducing the hybridisation temperature from 33 to 0 °C for the 8-unit oligonucleotide with the G · T pair in the centre. The use of a 10-unit oligonucleotide with a similar irregular pair[113] increased the yield of the mutant DNA to 38% at 10 °C. When the base to be replaced was shifted from the 3'-end to the centre of the oligonucleotide, the yield of the mutant DNA rose from 8 to 20% respectively. Although duplexes with an irregular pair at the end are in fact more stable, the oligonucleotides forming such duplexes are probably poor primers for DNA polymerase.[113]

When oligonucleotides complementary to the mutation site were used, it was shown that the effectiveness of the correction of the DNA sequence with the aid of DNA polymerase is linked directly to the stability of the oligonucleotide (primer) · DNA duplex. With increase in the length of the primer from 11 to 17 bases, the yield of the 'corrected' phage φX 174 DNA (the mutant strain am3 was corrected) increased from 0.02 to 13.9%. Furthermore, the two oligonucleotides placed one immediately behind the other (in a tandem fashion) (simulation of point dissociation) were more effective in the synthesis of the 'corrected' DNA than short oligonucleotides [yield of φX 174 phage DNA (%): 1.2 for ON-11 + ON-6 and 0.02 and 0 for ON-11 and ON-6 respectively; 14.0 for ON-14 + ON-6 and 1.9 for ON-14; 32.5 for ON-17 + ON-6 and 13.9 for ON-17].[114]

The pentadecadeoxyribonucleotide, fully complementary to the RNA segment (synthesised on the *E. coli* tryptophan operon) responsible for the regulation of attenuation, was used[115] to investigate the role of the secondary structure of RNA in the termination of transcription. The essential feature of the approach employed in the above study[115] is that the oligomer is bound in a complex with a selected section of the synthesised transcript, preventing thereby the formation of the corresponding 'terminator' secondary structure in this segment of RNA and behaving therefore as an antiterminator. It was shown that, in the presence of a 500-fold excess of the oligomer (2 μM, 37 °C) relative to the matrix, the yield of the transcript exceeded by a factor of approximately four the yield of the product in the control experiment (without the oligonucleotide). On the other hand, the yield of the transcript synthesised in the presence of the same oligomer (and under the same conditions) on the *Ser. marcescens* tryptophan operon exceeded the yield of the product in the control experiment by a factor of only 1.2. In the latter case, the oligomer might have formed a duplex containing two noncanonical pairs with the corresponding section of the transcript, in contrast to the perfectly matched complex formed by the oligomer with the selected section of RNA synthesised on the *E. coli* tryptophan operon.

A general approach to the formation of oligonucleotide probes for mRNA with an unknown primary structure has been proposed[116] (if it is assumed that the aminoacid sequence of the corresponding protein is at least partly known). This approach is based on the use as the primer or the probe of an oligonucleotide complementary to the section of mRNA coding for tetrapeptides consisting of the aminoacids Phe, Tyr, His, Asn, Asp, Cys, Gln, Lys, Glu, Trp, and Met. Taking into account the degeneracy of the code, such an oligonucleotide will form with mRNA a duplex containing either G · C or G · U pairs. Furthermore, T · G or T · A pairs may be encountered. The probe, constructed in accordance with this principle for gastrin mRNA (containing two G · U pairs) was hybridised with the mRNA, but its efficiency as a primer was lower by a factor of 2 than the efficiency of the oligonucleotide fully complementary to the given section. Furthermore, in the case of oligonucleotides with two G · U pairs, the primer underwent both hybridisation and elongation only on denaturation of the mRNA.

The exact selection of hybridisation conditions makes it possible to distinguish the RNA or DNA sequences containing at least one replacement at the probe binding site.[117,118] Thus the presence of one G · T pair (instead of G · C) at the centre of the binding site for a 14-unit oligonucleotide (ON-14-B) decreased the degree of its hybridisation with the rabbit β-globin mRNA to 8% compared with the degree of hybridisation of the oligonucleotide fully complementary to the same section (ON-14-A) {1 M NaCl, 37 °C, [ON-14-A] = [ON-14-B] = 0.15×10^{-9} M}. The dissociation temperatures of the complexes[117] mRNA · ON-14-A and mRNA · ON-14-B differed by 5 °C.

The hybridisation of 23-unit oligonucleotides with the initial and mutant DNA depended on the type of irregular pair at the oligonucleotide binding site. The oligonucleotide ON-23, complementary to the adenovirus Ad-2 DNA, was hybridised also with the DNA of the Cyt-15 mutant strain. The duplex ON-23 · Cyt-15 DNA contained the T · T pair. On the other hand, the oligonucleotide ON-23M, complementary to the Cyt-15 DNA, gave rise to a very weak signal due to hybridisation with the Ad-2 DNA. In the latter case the duplex contained the A · A pair, which can be explained by the greater destabilising effect of this pair on the double helix than the effect of the T · T pair.[118]

The differences between the stabilities of the perfectly matched duplex and the duplex containing one irregular pair were used to diagnose sickle cell anaemia. The 19-unit oligonucleotides ON-19-A, complementary to the normal globin mRNA (mRNA-A), and ON-19-S, complementary to a mutant mRNA (mRNA-S), gave rise to cross-hybridisation at 45 °C (level of hybridisation 10%–15%); the complex mRNA-A · ON-19-S contained one A · A pair, while the complex mRNA-S · ON-19-A contained one T · T pair. An increase in the hybridisation temperature to 55 °C led[119] to a rigorously specific interaction of the above oligonucleotides with the corresponding mRNA. The accuracy of the interaction of short probes with lengths equivalent to 7, 9, or 12 oligonucleotide units proved low and led to intense cross-hybridisation. Thus the 9-unit and 12-unit probes, complementary to the gene for the phage T4 lysozyme, were hybridised to the extent of 15%–18% with the DNA of the lysozyme-free G223 phage and 10% with the total RNA of *E. coli*.[120] At a hybridisation temperature lower by 15–20 °C than the melting point of the perfectly matched complex, the 12–15-unit oligonucleotides were effectively hybridised with the DNA of the *lac* operon (of not only the wild strain but also of a series of mutant strains) with formation of duplexes containing noncanonical pairs of the type C · A, G · A, T · T, etc. The degree of cross-hybridisation depended under these conditions on the type of the irregular pair: the degree of hybridisation for oligonucleotides forming a complex with the G · A pair was lower than for those forming a complex with the C · A pair. An increase in the hybridisation temperature from 30 to 37 °C made it possible to avoid the cross-hybridisation of these probes.[121]

b. Nonionic analogues of oligonucleotides

Nonionic analogues of oligonucleotides (phosphotriesters, methylphosphonates) have been proposed as antisense oligonucleotides for the inhibition of the expression of genes *in vivo*. Unchanged oligonucleotides readily permeate into the cell via passive diffusion. The use of nonionic analogues of oligonucleotides as inhibitors of the translation of mRNA or the expression of genes has been examined in detail in a number of reviews.[1–3,122]

In the study of the binding of ethyl phosphotriesters of oligonucleotides complementary to the anticodon loop (dTpTpCpA, ON-I) and the CCA-end (dGpGpT, ON-II) of yeast and *E. coli* $tRNA^{Phe}$, it was observed that the constants for

the binding of these derivatives to $tRNA^{Phe}$ are higher than for the unmodified oligonucleotide. At 0 °C (1 M NaCl, 10 mM, Mg^{2+}), the binding constant (K_x) was 3600 litre mol^{-1} for ON-I and 2400 litre mol^{-1} for the diester analogue.[123] As expected, at 0 °C ON-II at a concentration of 270 μM inhibited the aminoacylation of all five tRNA investigated (specific to Phe, Lys, Tyr, Pro, and Leu) to approximately the same extent (60% – 80%). At a concentration of 364 μM, ON-I inhibited the aminoacylation of $tRNA^{Phe}$ as a result of the formation of a perfectly matched duplex with the tetranucleotide sequence in the anticodon loop of $tRNA^{Phe}$. Together with this finding, the inhibition of the aminoacylation reactions of other tRNA was also observed[124] in the presence of ON-I: by 35% for $tRNA^{Leu}$, by 12% for $tRNA^{Lys}$, and by 0.7% for $tRNA^{Pro}$. When the methyl phosphate derivatives (the modified phosphate is designated by an asterisk) of the oligonucleotides dAp*Ap*Ap*A (M-ON-1) and dGp*Gp*U (M-ON-2), complementary to the sequence in the anticodon loop of $tRNA^{Lys}$ and the CCA-end of tRNA respectively, were used as the aminoacylation reaction inhibitors, the nonspecific inhibition of the aminoacylation of $tRNA^{Phe}$ by 9% and of $tRNA^{Leu}$ by 12% was observed in the presence of M-ON-1 (0 °C). In the presence of a similar oligonucleotide derivative dAp*Ap*Gp*A (M-ON-3), forming with the anticodon loop of $tRNA^{Lys}$ a duplex with one G · U pair, the level of inhibition of the aminoacylation reaction of $tRNA^{Lys}$ was 35% (whereas for M-ON-1 it was 88%), while the degrees of aminoacylation of $tRNA^{Phe}$ and $tRNA^{Lu}$ did not change.[125]

The experimental data on the thermodynamics of the duplexes formed by phosphodiester and methylphosphonate oligonucleotides are represented in the most complete form in the communications of Quartin and Wetmur[126] and Miller et al.[127] The change in the free energy of the duplex as a consequence of the introduction of one methylphosphonate linkage instead of the diester linkage is $\Delta\Delta G_0 = -0.75$ kcal mol^{-1} (1 M NaCl, 25 °C) regardless of the type of base in the oligonucleotide. These duplexes are additionally stabilised as a result of the decrease in the overall charge per pair of nearest neighbours containing the methylphosphonate linkage.[126]

The increase in the stability of the duplexes formed by methyl phosphate derivatives of oligonucleotides influences the complementation accuracy of the interaction of such derivatives with nucleic acids and their fragments. The photomodification of a 35-membered oligonucleotide by dodecameric methylphosphonates carrying 4'-aminoalkyl-4,5',8-trimethylpsoralen at the 5'-end has been investigated.[128] It was shown that the degree of photomodification of the ON target depends greatly on the accuracy of the formation of the duplex with the reagent (Table 4). Noncanonical pairs adjoining the reactive component of the derivative reduced sharply the degree of photomodification of the target. These results agree well with data obtained in a study[129] where it was shown that the biological and biochemical activity of oligonucleoside methylphosphonates falls sharply when defects appear in the duplex.

Table 4. The influence of the accuracy of the formation of the duplex on the degree of photomodification of a 35-unit oligonucleotide by the psoralen derivative of a methylphosphonate dodecamer.[128]

Duplex	Degree of photomodification (%)
Perfectly matched	85
Central T·pair	53
3'-Terminal T · C pair	58
5 G · T pairs at one pair intervals	33
3 T · C pairs at one pair intervals	19
4 T · C pairs at one pair intervals	0

c. Phosphorothioate analogues of oligonucleotides

The phosphorothioate analogues of oligonucleotides, containing a sulfur instead of an oxygen atom at the phosphorus atom, also belong to oligonucleotide derivatives with a modified phosphodiester linkage. The replacement of an oxygen atom by a sulfur atom increases the resistance of such derivatives to the action of intracellular nucleases.[130] The thermodynamic parameters of the formation of duplexes between the usual DNA or RNA and the phosphorothioate derivatives of oligonucleotides have been published.[1,131–134] Unfortunately, there are no data concerning the accuracy of the interaction of the phosphorothioate derivatives of oligonucleotides with model NA targets.

d. Derivatives of oligonucleotides containing α-anomeric units

During the last few years, methods have been developed for the synthesis of oligonucleotides resistant to the action of nucleases and consisting of the α-anomers of nucleotides (see the relevant reviews[135–137]). α-Oligonucleotides form a double helix with a complementary chain consisting of natural β-anomers of α-anomers of nucleotides. Both chains of the α · β duplex have a parallel orientation and the duplex itself belongs, according to NMR data, to the *B* conformation of DNA and has a right-handed twist to the extent of 70 ± 10%.[138] The melting point of duplexes[140] of the type α · β{α[(Tp)$_7$T] · β[d(pA)$_8$]} (30 °C) is higher than the T_m of the corresponding β · β duplex (24 °C),[139] which indicates the greater stability of the α · β duplexes compared with the β · β duplexes. On the other hand, the melting point of the heterooligomeric duplexes of the type α[d(TCTAAAC)] · · β[d(AGATTTG)] hardly differs from that of the corresponding β · β duplex (33 and 36 °C respectively).[141] Detailed thermodynamic studies of the duplexes β[d(CCTTCC)] · β[d(GGAAGG)], α[d(CCTTCC)] · β[d(GGAAGG)], and β[d(CCTTCC) · ·α[d(GGAAGG)] permitted the conclusion that the stability of the α · β duplexes depends on the nature of the bases in the α-chain (purine or pyrimidine) at least for homopurine or homopyrimidine sequences.[142] Comparison of the β · β duplexes mentioned above (T_m = 19 °C) with the α · β duplexes (T_m = 28 °C) showed that the α · β duplexes are more stable in 1 M NaCl than the β · β duplexes, provided that the α-chain consists of pyrimidine bases.[136] The duplexes of the α-oligonucleotides [α(T$_p$)$_7$T] with natural RNA [poly(rA)] are more stable than the analogous duplexes with poly(dA) (T_m = 29 and 17 °C respectively), while the β-dT$_8$ · poly(rA) duplex is less stable than dT$_8$ · poly(dA) (T_m = 14 and 17 °C respectively).[143] Data on the accuracy of the interaction of α-oligonucleotides with natural DNA or RNA targets are lacking in the literature.

e. Derivatives of oligonucleotides bound covalently to duplex-stabilising groups

The oligonucleotides bound covalently via an oligomethylene bridge to a dye molecule form duplexes with an increased stability (ΔT_m = 20 – 30 °C) as a result of the intercalation of the dye in the mRNA · antisense oligonucleotide double helix.[131,144,145] The synthesis and the mechanism of the action of such oligonucleotide derivatives have been considered in detail in a number of reviews.[5,146,147] The stability of the duplex poly(rA) · dT$_{6-8}$ increases appreciably (ΔT_m = 20 – 30 °C) if the oligothymidylate is bound covalently via one of the ends to 9-aminoacridine.[139,144,148] Another dye — *N*-(2-hydroxyethyl)-phenazinium — has been used[149,150] to increase the stability of the DNA duplexes. The oligonucleotide carrying a phenazinium residue at the 5'-end activated the replication of the M13mp7 bacteriophage DNA to the extent of 65%, while the free oligonucleotide showed no inactivation activity.[150] Unfortunately, the accuracy of the interaction with the NA target was not specially estimated in the studies devoted to such oligonucleotide derivatives. All the investigations were performed either on homopolymer matrices or on short DNA fragments.

2. Inhibition of the translation of mRNA

In recent years, antisense oligonucleotides and their derivatives have been used rigorously as specific inhibitors of the expression of genes.[1,5,151,152] The approach is based on the hypothesis[158-161] that, after the formation of a duplex with an antisense oligonucleotide, mRNA either loses the ability to be translated by the ribosome[153-157] or is cleaved by RNase H.

Standard cell-free protein-synthesising systems [the lysate of rabbit reticulocytes, the extract from wheat germ (WGE), and the oocytes of *Xenopus laevis*] are used to study the inhibiting activity of oligonucleotides or their derivatives on the translation of mRNA. The activity of RNase H is particularly high in the wheat germ extract.[159,162,163] Tests showed that the low activity of RNase H in a freshly prepared lysate of rabbit reticuloycytes is sufficient to hydrolyse exogenic mRNA with an antisense nucleotide.[160] In *Xenopus laevis* oocytes, RNase H activity is observed in the nucleus and in the cytoplasm.[159,164]

The most readily available mRNA of rabbit[157,159,165-169] or mouse[160] globins were used as the matrix. In addition, viral RNA sometimes served as a matrix: from the Rous sarcoma (RSV),[170,171] the Sendai virus,[172] the encephalomyocarditis virus,[173] and the tobacco mosaic virus.[174] Since the level of specificity of the inhibition of the translation was estimated in some investigations, the results of the latter will be considered in greater detail.

It has been shown[170] that the translation of 35S RNA from the Rous sarcoma virus (RSV) was effectively translated in WGE in the presence of the 13-unit oligonucleotide d(AATGGTAAATGG), complementary to the 3'- and 5'-terminal sequences of this RNA. Simultaneously with the suppression of the translation of the RSV RNA (approximately to the extent of 88%), the inhibition of the translation of AMV (avian myeloblastosis), the rabbit globin mRNA, and the BMV (broom mosaic virus) RNA to the extent of 57, 25, and 30% respectively has been observed (the data are quoted for a RNA : ON-13 ratio of 1 : 20 at 25 °C). The degree of inhibition of the translation of all the types of RNA increased with increase in the ON-13 concentration, but at low ON-13 concentrations (RNA : ON-13 = 1 : 2) mainly the translation of the oncovirus RNA (RSV and AMV RNA) was inhibited — to the extent of 27% and 10% respectively. Analysis of the sequence showed that ON-13 could form an imperfectly matched complex with AMV RNA with a length equivalent to 13 base pairs and containing three noncanonical pairs — two in the centre and one at the 3'-end. The degree of homology of the above oligomer with BMV RNA and globin RNA was not determined.

The inhibition of the translation of the mRNA in WGE as a result of the formation of an imperfectly matched complex with an antisense oligonucleotide has been observed.[164] All three oligonucleotides investigated, specific to β-globin mRNA, had incomplete complementation sites on the α-globin mRNA. Thus ON-17 could form with α-globin mRNA an imperfectly matched duplex comprising ten base pairs, three of which were noncanonical, ON-12 could form an imperfectly matched duplex consisting of seven base pairs with a central A · C pair, and finally ON-8 could form a duplex containing seven base pairs with a terminal irregular pair. The inhibition of the translation of the α-globin mRNA was observed only in the presence of ON-8 (the degrees of inhibition of β- and α-globin mRNA were 65% and 15% respectively). Apparently, in the case of ON-17 and ON-12 the difference between the stabilities of the perfectly and imperfectly matched duplexes, formed by these oligonucleotides with the β- and α-globin mRNA, was sufficiently high to prevent altogether the formation of an imperfectly matched complex at 25 °C and a concentration of the oligonucleotides of 10 μM or the complex was not recognised by RNase H.

The formation of an imperfectly matched complex between the α-globin mRNA and a 30-unit oligonucleotide complementary to the 270-300 fragment of the β-globin mRNA was detected from the cleavage of α-globin mRNA in the presence of saturation concentrations of the 30-unit oligomer in the *Xenopus laevis* oocytes.[159] The degree of homology between the α-globin mRNA and ON-30 was 60%. The non-specific inhibition of the translation of globin mRNA by oligonucleotides complementary to the 5'-nontranslatable sequence has been observed.[160] This finding was accounted for by the high level of homology between the nontranslatable sequences in the α- and β-globin mRNA. As a result of the high degree of homology (about 50%) between the 5'-terminal sequences of all the mRNA of the Sendai virus, the 20-unit oligonucleotide, complementary to the 1-20 fragment of the nucleocapsidyprotein (NP) mRNA reduced the yields of the products of the translation of all the mRNA of this virus due to the formation of a partial complex with a length equivalent to ten base pairs and a 10-unit sequence, which is virtually common to all the mRNA of the Sendai virus.[172]

The nonspecific inhibition of the translation of the BMV RNA in the lysate of rabbit reticulocytes (RRL) by a 15-unit oligonucleotide complementary to the vesicular stomatitis virus (VSV) was explained by the inhibition of the translating capacity of ribosomes as a result of the binding of the above ON-15 to 18S rRNA. The level of the nonspecific inhibition of the translation of the BMV RNA depended on the ON-15 concentration and showed a distinct tendency towards saturation. The maximum attained degree of inhibition of the translation was 88% and 43% in the presence of 40 μM ON-15 and 41% and 2% in the presence of 10 μM ON-15 for VSV and BMV RNA respectively.[175] Although, according to the authors claim there is no homology between the BMV RNA, 18S rRNA, and the above ON-15, the inhibition of the translation of the BMV RNA as a result of the formation of an imperfectly matched complex with the oligonucleotide appears more likely than nonspecific inhibition of the translating activity of ribosomes.

It has been shown[173] that the translation of the encephalomyocarditis viral RNA in the RRL is inhibited approximately to the extent of 60% with the aid of 25 μM 13-unit oligonucleotide, which forms a perfectly matched complex with the 751-763 section. The replacement of the central G · C pair of this duplex by the G · A pair (one base of ON-13 is altered) leads to a fourfold decrease in the degree of inhibition of the translation (down to 14%) at the same ON-13 concentration.

Interesting data on the complementation accuracy of the interaction of antisense oligonucleotides or their methylphosphonate analogues with a NA target have been obtained in a series of studies by Miller and coworkers[165-167,176,177] of the inhibition of the translation of rabbit globin mRNA *in vitro*. Phosphodiester oligonucleotides with a length equivalent to 8-10 units inhibited the translation of the globin mRNA in the RRL and WGE (0-200 μM solution of the (oligonucleotide, 0.3 μg of mRNA). The above oligomers were fully complementary to the sequences located in the 5'-nontranslatable region and the region of the AUG codon in either α- or β-globin mRNA. All the oligonucleotides synthesised formed a duplex with the selected mRNA sequences, which was demonstrated by building up these oligonucleotides (as primers) using a reverse transcriptase. However, despite the fact that these oligonucleotides were effective translation inhibitors, the complementation accuracy of their interaction with mRNA was low. For example, the ON-12 oligonucleotide inhibited the translation of the α-globin mRNA (it was complementary to its 28-39 section) by 97% in the RRL and by 100% in the WGE at a concentration of 100 μM. However, the ON-12 inhibited simultaneously the translation of the β-globin mRNA by 96 and 100% in the RRL and WGE respectively. At a ON-12 concentration of 5 μM, the degree of inhibition of the translation of α- and β-globin mRNA was 15 and 14% in the RRL and 75 and 14% in the WGE respectively. Similar results were obtained for all seven nucleotides investigated.[165]

Short 4-12 unit oligonucleoside methylphosphonates (M-ON), exactly complementary to the same sections of the globin mRNA as the phosphodiester oligonucleotides used by

Blake et al.,[165] have been investigated.[166,176] The prehybridisation of M-ON with mRNA increased the degree of inhibition of the translation of the mRNA in the RRL by 20%–30%. It is surprising that at 37 °C the M-ON-4 ($Cp_*Cp_*Ap_*T$) (complementary to the 37–40 and 54–57 sections in α- and β-globin mRNA respectively) at a concentration of 100 μM did not inhibit the translation of mRNA, while at a concentration of 200 μM the degree of inhibition of the translation of α- and β-globin mRNA was 28% and 42% respectively. As in the study of Murakami et al.,[167] the M-ON complementary to the α-globin mRNA inhibit the translation of both mRNA to approximately the same extent, particularly at high M-ON concentrations (200 μM) and when the stage involving the transhybridisation of the mRNA with the M-ON is carried out.

Direct comparison of the inhibiting activities of the ON and M-ON permits the conclusion that the M-ON are not more effective and specific translation inhibitors than the phosphodiester oligonucleotides. The low specificity of the ON and M-ON with lengths equivalent to 4–12 units as well as their low effectiveness in inhibiting translation can be explained by several causes. Firstly, the 5'-nontranslatable region in the rabbit α- and β-globin mRNA exhibits a high degree of homology. Therefore, apart from the binding site selected on one mRNA, each nucleotide can form also imperfectly matched complexes with the other mRNA. However, the authors, in discussing the possibility of the formation of such complexes, seek only those sections of the mRNA which can form with the M-ON or ON a continuous duplex with a length equivalent to not less than four base pairs (the so called partial duplex[47]) and fully reject the possibility of the formation of longer duplexes containing noncanonical pairs or, in other words, imperfectly matched complexes.[165,166] Secondly, the influence of the secondary structure of mRNA on complex formation is not considered.[175]

A low specificity of the action of short M-ON (9-unit oligonucleotides) has also been observed in the inhibition of the translation of the mRNA of the N, NS, and GVSV proteins in the RRL at 30 °C. On concentrating M-ON-9 (complementary to the N protein mRNA, section 14–22), the inhibition of the synthesis of the N protein by 77% was accompanied by the inhibition of the syntheses of the NS and M proteins by 38% and 43% respectively.[178] The low specificity of the inhibition of the translation observed at high concentrations (100–150 μM) of M-ON-9, was explained by the formation of partial complexes between M-ON-9 and other VSV mRNA. Furthermore, despite the absence of experimental evidence, the authors suggest[178] that the secondary structure of the mRNA exerts a strong influence on the specificity and effectiveness of the inhibition of the translation.

The site-specific inhibition of the translation takes place also when the M-ON forms with the mRNA a duplex containing noncanonical pairs. Three octamers (M-ON-8_{1-3}), complementary to the AUG region of the codon for the ras p21 BALB protein mRNA, inhibited translation of the mRNA in conformity with the stability of the duplexes formed. Thus M-ON-8_1[177] inhibited the synthesis of the protein by 97%; M-ON-8_2, forming with the mRNA a duplex containing one irregular pair in the centre, caused 31% inhibition; whereas M-ON-8_3, forming with the mRNA a duplex with two noncanonical pairs in the centre, inhibited the translation to the extent of 9%.

At a certain temperature and certain concentrations of the antisense oligonucleotides, the degree of inhibition of the translation of mRNA depends on the length of the oligonucleotide employed, i.e. on the stability of the duplex. The 12-unit oligonucleotide complementary to the initiator region of trypanosomal mRNA is less effective in inhibiting translation[179] (degree of inhibition 20%–25%) than ON-22 and ON-34 (translation inhibited to the extent of 65%–70% and 95%–100% respectively) at 25 °C and a concentration of 15 μM. The decisive importance of the stability of the mRNA · ON duplex in inhibiting the translation of the given mRNA in the RRL and WGE can be additionally illustrated by data from other studies.[180,181] Thus the 12-unit oligonucleotide complementary to the 2-13 sequence of the *T. brucei* mRNA inhibits the translation of the mRNA but not that of *T. vivax* (another species of African trypanosomes) at 22 °C. It was found that ON-12 forms with the *T. vivax* mRNA a duplex (its dissociation temperature is 22 °C) containing two irregular pairs — G · A and T · T.

It has been demonstrated[145,182] that the 18-unit oligonucleotide carrying at the 3'-end a 9-aminoacridine residue inhibits the expression of pKH113 by 55% (at an ON-18 concentration of 30 μM). A slight inhibition of the expression of pBR322 (by 10%) is observed simultaneously, evidently owing to the formation of an imperfectly matched complex with a length equivalent to 11 units and containing two noncanonical base pairs at the 5'-end of the duplex (G · T and C · T) in the 3234 region of the pBR322 nucleotide residue.

In all the studies examined in this section, short (4-13)-unit oligonucleotides at high concentrations (40–200 μM) were used to inhibit the translation of mRNA *in vitro*. The low specificity of the inhibition of the translation was apparently associated with the fact that the difference between the stabilities of the imperfectly and perfectly matched complexes at high concentrations of the oligonucleotides virtually disappeared and both types of duplexes functioned as inhibitors of the initiation of translation (binding to ribosomes) or served as substrates of the RNase H.

The specificity of the inhibition of the translation of mRNA *in vitro* increases sharply when long antisense oligonucleotides are employed. Thus the translation of the mRNA of trypanosomes was inhibited specifically (degree of inhibition 95%–100%) by 22- and 34-unit oligonucleotides at a concentration of 25 μM in the WGE. The BMV RNA, the degree of translation of which in the presence of the same oligonucleotides remained unchanged, was employed as a control.[179] The translation of the tobacco mosaic virus (TMV) RNA was inhibited specifically (to the extent of 90%–95%) by two 26-unit oligonucleotides (at a concentration of 10 μM) in the RRL at 30 °C. Under the same conditions, the level of translation of the BMV RNA remained unaltered.[174] Specific inhibition of the translation of the rabbit β-globin mRNA in the presence of the α-globin RNA was achieved using 20–25- and 48-unit oligonucleotides at a concentration of 5.3 μM (2.6 μM for ON-48) at 37 °C in the RRL. The level of inhibition was[169] 55%–88% for ON-20(25) and 80%–98% for ON-48.

The use of antisense oligonucleotides with lengths equivalent to 21–49 units, complementary either to the translation initiation region or the part of the mRNA molecule coding for the enzyme dehydrofolate reductase–thymidylate synthetase of *Plasmodium falciparum*, led to the inhibition of the translation of mRNA in the RRL system by not more than 60% at a concentration of the 49-unit oligonucleotide of 190 μM. The main reason for the low inhibiting capacity of such long oligonucleotides relative to their length and concentration employed is apparently the inaccessibility of sections of the mRNA for the formation of such a long duplex.[156] Unfortunately, there are at present insufficient data to discuss the specificity of the inhibition of translation of the mRNA of the rabbit globins with the aid of 17-unit phosphorothioate oligonucleotide derivatives (S-ON) or α-oligomers having the same length in the RRL, WGE, and *Xenopus laevis* oocytes.[157,168]

3. Accuracy of the interaction of the oligonucleotides and their derivatives with the NA target *in vivo*

Antisense oligonucleotides and their derivatives can be regarded as possible future therapeutic antitumour, antiviral, and antibacterial agents. This explains the enormous interest in the study of the action of antisense oligonucleotides *in vivo*. The mechanism of the permeation of oligonucleotide derivatives into the cell and their utilisation have been examined in detail in a number of studies.[159,183–187]

a. *Xenopus laevis* oocytes

The *Xenopus laevis* oocytes are some of the simplest *in vivo* systems.[188] Exogenic mRNA and DNA, introduced into the *Xenopus* oocytes by microinjections, are expressed in the given system; under these conditions, the nuclear cytoplasmic transcripts are equally accessible for the interaction with (19–20)-unit oligonucleotides and antisense RNA.[189,190] As in the *in vitro* translation system, one of the mechanisms of the action of the oligonucleotides *in vivo* involves[189] oligonucleotide-specific cleavage of the exogenic mRNA by the endogenic RNase H. Unfortunately, there are no literature data concerning the influence of the accuracy of the interaction of antisense oligonucleotides on the degree of inhibition of the translation or transcription of exogenic mRNA or DNA in the *Xenopus laevis* oocytes. Oligonucleotides with a length corresponding to 18–23 nucleotide residues[191] inhibit by 85%–98% the translation of the mRNA of interleukins IL-2 and IL-3 in the *Xenopus laevis* oocytes at a concentration less than 1 μM; on the other hand, the inhibition of the synthesis of β-tubulin in the same system requires a concentration of the 27-unit oligonucleotide of 20 μM.[192]

b. The accuracy of the antiviral activity of oligonucleotides

The majority of studies of the functioning of antisense oligonucleotides *in vivo* have been carried out on cultivated cell strains infected with viruses. This is evidently associated with the fact that sequences of viral RNA or DNA and the characteristic features of the development of the viral infection of the cell are known for many viruses and also with the relative simplicity of the determination of the effect of an antisense oligonucleotide on the viral gene. Studies on the antiviral effect of antisense oligonucleotides have been carried out on the viruses of the Rous sarcoma (RSV),[193] vesicular stomatitis (VSV),[178,194] herpes (HSV),[129] human immunodeficiency (HIV),[195–200] and influenza.[201–203] However, the mechanism of the antiviral action of antisense oligonucleotides has not been demonstrated in any of the above studies. Furthermore, direct evidence has not been obtained for the formation of a duplex between the oligonucleotide and the viral RNA or DNA *in vivo*. Nevertheless, numerous experimental data have accumulated which it would be of interest to examine from the standpoint of the specificity of the interaction of oligonucleotides or their derivatives with the NA target.

Thus the virtually quantitative inhibition of the multiplication of the Rous sarcoma virus in a culture of fibroblasts from chick embryos took place[193] on treatment with a 13-unit protected oligonucleotide complementary to the 3′- and 5′-terminal sequences of the RVS RNA at an ON-13 concentration in the medium of 2 μM. Some data on the dependence of the degree of inhibition of the viral infection on the accuracy of the interaction of the oligonucleotide with the NA target have been obtained[129] in a study of the inhibition of the replication of the HVS DNA by the octanucleoside methylphosphonate d(TpCp*Cp*Tp*Cp*Cp*Tp*G) (M-ON-8). This octanucleoside methylphosphonate is complementary to the acceptor site for the splicing of the HSV-1 mRNA 4 and 5. A virtually quantitative inhibition of the multiplication of the virus (the extent of 90%–95%) occurred on treatment with 75 μM M-ON-8, added to the medium simultaneously with the virus or before the latter. When M-ON-8 was added to the medium (at a concentration of 150 μM) 1 h after infection, the degree of inhibition of the development of the virus did not exceed 40%. Simultaneously with the inhibition of the HSV, the synthesis of a number of low-molecular-mass proteins of the host cell by M-ON-8 to the extent of 10%–30% occurred. Furthermore,[139] M-ON-8 was able to form a complex with a length equivalent to 6 base pairs in the acceptor site for the splicing of the IE mRNA 4 and 5 of the HSV-2 genome and hence to inhibit the multiplication of the HSV-2 virus (by 70% at an M-ON-8 concentration of 5 μM) just as effectively as that of the HSV-1 virus. Moreover, M-ON-8 could form a partial duplex with a length equivalent to 7 base pairs with the mRNA of the L protein of the VSV and with the 72–80 sequence of the rabbit β-globin mRNA. In conformity with the above findings, M-ON-8 (150 μM) inhibited the synthesis of the VSV L protein by 85% during multiplication in the mouse L cells and the translation of the β-globin mRNA by 75%–78% in the RRL *in vitro*.[166]

The increase in the length of the antisense oligonucleotide with increase in concentration made it possible to achieve rigorously selective inhibition of the multiplication of the virus. It has been shown[194,204] that a 15-unit oligonucleotide (at a concentration in the medium of 200–400 mM), bound covalently by the 3′-end to poly-L-lysine, inhibits specifically (by 85%–98%) the multiplication of the VSV, without inhibiting at the same time either the synthesis of the host protein or the encephalomyocarditis virus. The function of poly-L-lysine (molecular mass 14 000) is to stimulate the permeation of the antisense oligonucleotide into the cell.

In contrast to model systems, antisense oligonucleotides can inhibit *in vivo* the development of the virus at any ripening stage. For example, for the human immunodeficiency virus (HIV), the target acted upon by antisense oligonucleotides may be the 5′-end of the viral RNA and the regions of all the initiator codons, the sections of the viral RNA involved in the splicing process (the donor and acceptor splicing sites), and the polyA backbone. Furthermore, with the aid of oligonucleotides it is possible to affect the activity of the viral reverse transcriptase, the region of the viral genome responsible for transactivation (the TAR region), and many other stages in the ripening of the virus. It has been shown[196,205] that the greatest inhibition of the multiplication of the HIV virus in the Molt-3 cells is observed in the interaction of ON-20 with the splice acceptor site (5349-5368) of the tat III gene. The repeating sequences, the nontranslatable 5′-regions of the mRNA of viral proteins, and the polyA backbone at the 3′-end of viral RNA are next as regards sensitivity to antisense nucleotides. It has been shown that the effectiveness of the inhibition of the virus depends on the length of the antisense oligonucleotide. Thus ON-12, ON-15, and ON-20, complementary to the 5349-5368 sequence, inhibit the development of synsytium by 64%, 64%, and 73% at concentrations in the medium of 26.0, 19.7, and 15.4 μM respectively.

Oligonucleotides forming complexes with viral RNA containing noncomplementary bases at the 3′-end of the oligonucleotide are effective inhibitors of viral revertase [reverse transcriptase]. Thus ON-26 and ON-14, which form with viral RNA duplexes having three and two noncomplementary bases at the 3′-end of the oligonucleotide, inhibit the enzyme activity of the HIV revertase by 80% and 75% respectively. The inhibition[205] of the synthesis of the viral proteins p15 and p24 in the presence of the same nucleotide takes place to the extent of 4%–8% only.

Tests of the antiviral activity of 15- and 20-unit oligonucleotides containing a modified linkage (phosphorothioates, butylphosphoramidates, phosphoromorpholidates, phosphoropiperazidates) led to the observation that not only antisense oligonucleotides but also ON-20 with a random sequence and homooligomers with the same length exhibit an anti-HIV activity.[196,199,200,206,207] According to the authors, there is a possibility here of several mechanisms of action of such oligonucleotides. Firstly, since the homooligomers are better substrates for the HIV revertase than the heterooligomers, they can function as competing inhibitors of the viral revertase. Secondly, the homooligonucleotides can form imperfectly matched long duplexes, which are sufficiently stable to be cleaved by RNase H. Thirdly, one cannot fully rule out a completely different inhibition mechanism. It is noteworthy that, whereas the homooligomers and oligonucleotides with a random sequence inhibit effectively the development of HIV when added to the medium simultaneously with the virus or even before the latter, the antisense oligonucleotides inhibit HIV satisfactorily also in chronically infected cells.[197,199,200] This difference can apparently be explained by the fact that any oligonucleotide and virus added to the medium simultaneously or with a small time interval (early

infection) have more possibilities for interaction (the oligonucleotide may prevent the permeation of the virus into the cell, may intensify the action of RNase H). It has been shown[197] that, in the early stages of the infection with the HIV virus, the concentrations of phosphorothioate derivatives of oligonucleotides [an antisense oligonucleotide (S-ON-I), an oligonucleotide forming an imperfectly matched duplex with eight mismatches (S-ON-II), and S-dA_{20}] necessary to inhibit the development of syncytia are approximately the same. In a chronic infection, the attainment of the same level of inhibition requires an increase in the S-ON-I, S-ON-II, and S-dA_{20} concentrations by factors of 3, 8, and 11 respectively. Similar results were obtained in other investigations.[196,206] In order to achieve 50% inhibition of the HIV virus in chronically infected cells, it was necessary to use concentrations of the antisense ON-20, the ON-20 with a random sequence, and $(dA)_{20}$ of 12, 46, and 60 μg ml^{-1}. Two random ON-20 oligonucleotides (manifesting an anti-HIV activity and serving as a control for antisense ON-20), complementary to the 5349-5368 and 280-299 HIV RNA sequences, were subjected to computer analysis.[206] It was found that the ON-20 with a random sequence, complementary to the 5349-5368 fragment, can form a perfectly matched duplex, having a length equivalent to six base pairs, with the 917–928 section or a duplex, having a length equivalent to 12 base pairs and containing two noncanonical pairs, with the same sequence. The ON-20 oligonucleotide, which is random as regards the 280–299 sequence, can form in the 8421-8437 region either a six-unit duplex or a duplex having a length equivalent to 17 base pairs with four irregular pairs. Furthermore, sequences such as T_4, T_5, A_4, and A_5 are encountered in the HIV genome 49, 13, 149, and 50 times respectively. Thus the antiviral activity of the "random" oligonucleotides and homooligomers may be associated with their ability to form imperfectly matched complexes with HIV RNA having a length equivalent to four base pairs and more and a stability which is sufficient for the manifestation of the activity of RNase H.

Oligonucleotides with a modified phosphodiester linkage exhibit a stronger antiviral activity than those with the usual phosphodiester linkage despite the virtually identical stabilities of the duplexes; O-ON-20 (T_m of the duplex 55 °C, determined at an oligonucleotide concentration of 5 μM) inhibits syncytia by 68% at a concentration in the medium of 100 μg ml^{-1}. The inhibition effected by MO-ON-20 (T_m of the duplex 51.5 °C, 5 μM), S-ON-20 (T_m of the duplex 49 °C, 5 μM), and *N*-butylphosphoramidate-ON-20 (T_m of the duplex 45 °C, 5 μM) is to the extent of 90%, 90%, and 100% respectively at concentrations in the medium of 25 μg ml^{-1} in each case.[197,199,206,207]

The study of the anti-HIV activity of antisense oligonucleotides with a modified phosphodiester linkage (phosphorothioates and nucleoside methylphosphonates) yielded a considerable amount of experimental data indicating a different inhibition mechanism, which rules out the formation of a complementary complex or, in other words, a sequence-independent inhibition of the development of the virus. Thus the methylphosphonate derivative of the 8-unit oligonucleotide d(CpCAATTCT) (the underlined nucleotides have been modified), complementary to the splice acceptor site of the HIV-1 tat-3 gene, inhibited specifically the development of synsytium and the activity of the viral revertase at a concentration of 100 μM. The synthesis of the viral RNA was inhibited both by the 8-unit nucleotide and by the oligonucleotide complementary to it (sense sequence).[198]

In the study of the antiviral activity of phosphorothioate derivatives of hetero- and homo-oligomers, it was observed that the 14-unit phosphorothioate analogues (25 μM) have the same inhibiting activity (93%–95%) as the analogous derivatives of oligonucleotides having the same sequence as the HIV RNA (78%–100%). In addition it has been observed that the decisive factor is the length of the phosphorothioate derivative:[195,199,208,209] the inhibiting effect of oligocytidine phosphorothioates increases in the sequence $d(p_SC) < d(p_SC)_{14} < d(p_SC)_{28}$. It has been suggested that such derivatives are inhibitors of the DNA polymerase activity of the reverse HIV transcriptase. It is also possible that the production of interferon is stimulated under the influence of phosphorothioate analogues of oligonucleotides.[195] The complete inhibition of the reproduction of HIV-1 in H9 cells has been achieved[209] with the aid of the phosphorothioate derivative of a 27-unit oligonucleotide (25 μM) complementary to the regular HIV-1 gene rev. The mechanism of such effective action of the above derivatives has not been ultimately elucidated. Phosphorothioate analogues of 2′-*O*-methylribooligonucleotides with a length equivalent to 10–30 units also exhibited an antiviral activity, while the 2′-*O*-methylated oligoribonucleotides and their deoxy-analogues did not inhibit HIV in MT-4 cells.[210]

c. Inhibition of the expression of genes *in vivo*

The expression of the c-myc gene constitutes a good model for the study of the inhibition of gene expression under the influence of antisense oligonucleotides,[211–214] since the lifetime of the c-myc mRNA and proteins is 20–30 min. In the study of the expression of the c-myc gene, T-lymphocytes of human peripheral blood, stimulated by phytohaemagglutinin (PTH), are usually employed. Antisense oligonucleotides are added to the medium up to a concentration of 30 μM 4 h before the stimulation of the T-lymphocytes by PTH. It has been shown[211] that a 15-unit oligonucleotide complementary to the first five codons for c-myc mRNA inhibits the mitogen-stimulated proliferation of cells to the extent of 80%, while an oligonucleotide with a random or coding sequence does not have an inhibiting effect on the cell proliferation. A similar pentadecamer induced in another study[212] a decrease in the level of synthesis of c-myc protein by 50%–80%. The inhibition of the expression of the c-myc gene is associated with the decrease in cell growth and the induction of myeloid differentiation. Treatment of HL cells with antisense 15-unit oligonucleotides (4 μM, 37 °C), which form complexes containing from 2 to 12 irregular base pairs (T_m = 56 °C and 41 °C for the perfectly matched duplex and the duplex with two G · T pairs respectively) did not alter the time required for the doubling of the number of cells. In the presence of the fully complementary nucleotide, the cell doubling time increased by a factor greater than 2.

The rigorously specific inhibition of the expression of the c-myc gene by the antisense 15-unit oligonucleotide complementary to the first five codons of the c-myc RNA apparently occurs[213] because, according to the calculated secondary structure of the c-myc mRNA, the ON-15 binding site is in the region of the loop, i.e. is open to interaction with ON-15. The specific inhibition of the expression of the c-myc gene, associated with the decrease in the level of both the c-myc protein and the c-myc mRNA, was achieved with the aid of a 17-unit antisense nucleotide bound covalently to poly-L-lysine.[214] At the concentrations employed (less than 1 μM), neither the random oligonucleotide nor one forming an imperfectly matched complex with six mismatches exerted an appreciably inhibiting effect on the expression of the c-myc gene.

Apart from the studies listed above, there is an enormous number of others devoted to the action of antisense oligonucleotides on different mRNA and genes in which the level of specificity of the interaction could not be determined simply. The inhibiting effect of the oligonucleotide was followed either from the enzyme activity of, for example, chloramphenicol : acetyl transferase[215] or from the inclusion of ^3H TTP in Balb/c3T3 cells after incubation with an oligonucleotide complementary to PCNA mRNA.[216]

4. Interaction of reactive derivatives of oligonucleotides with NA targets

The mechanism of the inhibiting activity of antisense oligonucleotides on different biochemical and molecular-genetic processes *in vitro* and *in vivo* includes as one of the main stages the formation of a complementary complex between the oligonucleotide and the NA-target. As shown in previous Sections, the stability of the ON-NA target duplex is one of the decisive parameters for the effective inhibition of both the translation of mRNA and the expression of genes.

In order to create more effective inhibitors, derivatives of oligonucleotides capable of forming duplexes with an enhanced stability have been devised. Thus the introduction into the oligonucleotide of intercalators such as acridine, *N*-(2-hydroxyethyl)-phenazinium, and ethidium bromide leads to an appreciable stabilisation of the duplex, but the complex formation itself remains an equilibrium process. Furthermore, it is well known that the translating ribosome possesses unwinding activity, i.e. the ability to melt fairly stable duplexes.[153,154] On the other hand, it has been shown in a number of studies that, in order to inhibit the translation of mRNA, RNase H is necessary apart from the complex formation with the antisense oligonucleotide. However, not all the heteroduplexes are cleaved by the RNase. For example, the derivatives of oligodeoxyribooligonucleotides with a modified phosphodiester linkage (phosphorothioates) are very stable in the cell and form stable complexes with RNA, which are not substrates of RNase H.

Derivatives of antisense oligonucleotides which would not only form a stable duplex with the NA target but would also damage the nucleic acid molecule as a result of a site-specific interaction, or, in other words, contain the reactive unit, are therefore very promising. There is then a possibility of several types of reactive groups bound covalently to the antisense oligonucleotide:

(1) alkylating groups which lead to the formation of a covalent bond between the oligonucleotide and the NA target;

(2) groups capable of being photoactivated, the activity of which is induced by UV radiation, resulting also in the formation of a covalent bond between the 'addressed' reagent and the nucleic acid;

(3) cleaving groups, that is groups such as Fe · EDTA, Cu · 1, 10-phenanthroline, and Fe(Co, Mn) · porphyrins, as a result of the activity of which the sugar phosphate skeleton of the nucleic acid is cleaved.

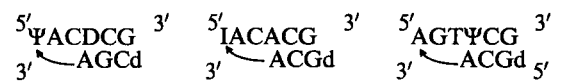
The methods of synthesis of oligonucleotides, the properties important for their use, and the results of the specific action of these derivatives on NA targets have been examined in a number of reviews.[5,9,217–222]

The majority of studies on the site-specific modification or site-specific damage of the NA target have been carried out on model matrices: synthetic oligonucleotides, homopolymers, short DNA fragments (up to 400 bases), tRNA, and 16S rRNA, oligonucleotides of different lengths being used. The advantages of the reactive groups used were demonstrated, the effectiveness of the reaction was estimated, and modification conditions were selected in these studies. In some investigations, the positional specificity of the modification was determined.[216–220] The specificity of the modification (complementation accuracy) was studied only in a few investigations, which will be examined in the present section.

Certain data on the accuracy of the interaction of the reagent and the NA target may be found in a series of communications (see the relevant reviews[2,5,9,219,221,229]) on the site-specific modification of nucleic acids by oligonucleotide derivatives containing the *N*-2-chloroethyl-*N*-methylaminophenyl group bound either to the 3′-end of the oligonucleotide via a benzylidene linkage or to the 5′-end via phosphoramide linkage.

The first experiments on the site-specific modification of natural nucleic acids (23S + 16S rRNA of *E. coli*) by derivatives of tetra-, penta-, and hexaadenylates carrying the 2′,3′-*O*-[4-*N*-(2-chloroethyl)-*N*-methylamino]benzylidene group (=CHRCl) at the 3′-end of $(Ap)_{n-1}A=CHRCl$ ($n = 4-6$)[229–231] demonstrated the highly effective modification of 16S + 23S rRNA. At a concentration of the reagents less than or equal to the concentration of the NA target, between 70 and 98% of the reagent present in the reaction medium was consumed on the alkylation of the RNA. This process was not distinguished by chemical selectivity—all the bases, except uridine, were modified.[230] The degree of modification of 16S + 23S rRNA was[230] 5 mol of $(Ap)_4A=CHRCl$ per mole of RNA at 20 °C and about 30 mol at 5 °C. According to the analysis of the sequence, in 16S and 23S rRNA there are no U_5 and longer fragments capable of ensuring the formation of a sufficiently long duplex with the reagent and only three U_4 and 24 U_3 sequences. Reagents of the type $(Ap)_{n-1}A=CHRCl$ ($n = 4-6$) apparently formed either partial duplexes with the U_3 and U_4 sequences or imperfectly matched complexes incorporating the U_3 and U_4 backbones. The values of $R_{x(app)}$ for the binding of the reagents $(Ap)_{n-1}A=CHRCl$ ($n = 4-6$) to RNA, found by Karpova,[229] proved to be similar and corresponded to $(3-7)\times10^5$, 7×10^5, and $(3-7)\times10^5$ litre mol^{-1} for reagents with $n = 6$, 5, and 4 respectively. Comparison of the values of $K_{x(app)}$ with those of K_x estimated by the method of Uhlenbeck et al.[97] showed that $K_{x(app)}$ is approximately equal to K_x for the duplex $A_4 \cdot U_4$.

The selectivity of the site-specific modification of nucleic acids was first estimated in the determination of the points where yeast $tRNA_I^{Val}$ was modified by the reagent d(pCpG)rA=CHRCl.[232,233] It was shown that three bases in $tRNA_I^{Val}$ with the same disposition relative to its CpG sequence, are alkylated. The modification occurred in the following duplexes:



where the arrow indicates the tRNA sequence. The adenine residue in these duplexes either forms an irregular pair or does not participate in complex formation.[234]

Subsequently, a similar analysis of the accuracy of the interaction of the addressed reagent and the NA target was carried out for the $ClRCH_2NH$ reagents. It was shown[235] that the reagent $ClRCH_2NH$-p(dAACCA) alkylates selectively the G_{24} $tRNA^{Phe}$ base of *E. coli*. Judging from the position of the modification point, G_{24} is alkylated in the imperfectly matched complex



where the arrow indicates the *p*-NA-CH_2-RCl group, whereas the section of the $tRNA^{Phe}$ molecule to which the reagent was addressed is involved in the formation of the secondary structure.

The reagent $ClRCH_2NH$-p(dATTTTCA), fully complementary to the sequence in the anticodon loop of $tRNA^{Phe}$, modifies three guanine residues:[236] G_{10}, G_{24}, and $G_{28/29}$. Whereas the alkylation of $G_{28/29}$ most probably takes place when it is part of the composition of a perfectly matched duplex with the selected sequence, G_{10} and G_{24}, which have similar secondary and tertiary structures, are apparently modified when they are part of the composition of an imperfectly matched complex with the corresponding sequence in the D-loop.



The ratios of the rates of the addressed and unaddressed (outside the complex) modifications (f) for $G_{24}+G_{10}$ and $G_{28/29}$ are 58 and 460 respectively, i.e. the level of the nonspecific modification is 2% for G_{10} and G_{24} and less than 0.3% for $G_{28/29}$. The results of the analysis of the modification points of tRNA indicate an enormous influence of the steric structure on the selectivity of the site-specific modification.

Interesting results have been obtained in studies[150,237] on the site-specific modification of the 302 unit DNA fragment by $-NHCH_2RCl-$ and $>CHRCl$ derivatives of 6-14-unit oligonucleotides. It was shown that, by virtue of the formation of an inner hairpin, the 144–149 section is entirely inaccessible for the binding of the reagent $ClRCH_2NH$-pd(TTCCC)rA, whereas the open 261–266 section is modified by this reagent. The modification of this fragment when the latter forms part of an imperfectly matched duplex with an oligo-G-sequence near its 5'-end has also been observed. In addition, the alkylation of G_{179}, which is apparently close to the reagent binding section (261–274), has been noted.[16,149,237–237]

The problem of the selectivity of the site-specific modification has been investigated in greatest detail in a series of studies[240–244] in relation to the modification of the 16S rRNA of *E. coli*, the length of which is equivalent to 1542 nucleotides, by oligonucleotide derivatives carrying the $>CHRCl$ group at the 3'-end. In such a long target, having a fairly complex steric structure, one may encounter, together with sequences fully complementary to the oligonucleotide address, also incompletely complementary fragments. Such fragments may be detected with the aid of a special program for the analysis of nucleic acid sequences—context analysis.[245] For example, according to the data of such analysis, the reagent complementary to the 771–781 region may form imperfectly matched complexes, containing at least five canonical base pairs, also with 12 sections of 16S rRNA. Estimation of the interaction energy showed[240] that at least three out of the twelve imperfectly matched duplexes of the reagent with the target have a stability which does not differ greatly from that of the fully complementary duplex, namely the duplexes with the sequences 442–452, 663–683, and 883–893. It has been demonstrated experimentally that this reagent does indeed modify 16S rRNA along eight sections and that four of these are modified to approximately the same extent (771–781, 442–452, 663–673, and 883–893). This apparently means that the reagent does not interact via all the bases with the fully complementary section, since the complementation section is involved in the formation of the secondary structure. A similar influence of the steric structure on the effectiveness of the site-specific modification has been demonstrated also in the alkylation of 16S rRNA by other reagents[240–242] and in the site-specific modification[235,236] of $tRNA^{Phe}$.

The influence of temperature and the reagent concentration on the selectivity of the modification has been investigated.[243] At 20 °C, the increase in the reagent concentration from 1.3×10^{-1} to 2.5×10^{-6} M entailed the increase in the overall limiting degree of modification of 16S rRNA from 0.05 to 0.62, whereas the modified fraction in the perfectly matched complex fell from 0.38 to 0.22. On raising the modification temperature from 20° to 40 °C at a reagent concentration of 4×10^{-5} M, the overall degree of modification fell from 2.57 to 0.64, but the modified fraction in the perfectly matched complex increased under these conditions from 0.24 to 0.35. Thus, despite the complexity of the model investigated, its modification is likewise determined by the stability of the duplexes formed by the reagent with the NA target. The additional stabilisation of the 16S rRNA · reagent duplex with the aid of an auxiliary effector oligonucleotide [complementary to the section of 16S rRNA immediately adjoining the reagent binding section, carrying at its 5'-end the *N*-(2-hydroxyethyl)-phenazinium residue and stabilising the duplex as a result of the cooperative interaction with the reagent[246]] led to an increase by a factor greater than 2 in the modified fraction of the RNA in the perfectly matched complex but did not affect under these conditions the modification of the imperfectly matched duplexes.[244]

Unfortunately there are virtually no data on the selectivity of the inhibition with the aid of reactive oligonucleotide derivatives of any biochemical processes occurring with participation of nucleic acids. In this connection, one may mention only two studies where the inhibition of the translation of mRNA with the aid of reactive oligonucleotide derivatives was investigated. It has been shown[247] that preliminary incubation of the total mRNA of the cells of the MOPC 21 mouse plasmocytoma with the $ClRCH_2NH$ derivative of an 11-unit oligonucleotide complementary to the sequence of the light chain of immunoglobulin G (coding for the variable section of the protein) leads to the quantitative inhibition of the synthesis of not only the light but also the heavy chain of Ig-G in the RRL.

Similar results have been obtained in a study[221] in which psoralen derivatives of 12-unit oligonucleotides were used to inhibit the synthesis of mouse α- or β-globins in the RRL. The reagent, fully complementary to the α- or β-globin mRNA, inhibited the synthesis of both globins to approximately the same extent. However, the authors of the above studies do not discuss the results from the standpoint of the specificity of the interaction of derivatives of antisense oligonucleotides with mRNA.

V. Conclusion

The data presented in the review show that the problem of increasing the complementary accuracy of the interaction of RNA and DNA with antisense oligonucleotides and their derivatives is a principal one in the study of effect of site-specific interaction on nucleic acids both *in vitro* and *in vivo*. The experimental data available at present on the thermodynamics of perfectly matched duplexes and duplexes with structural anomalies, obtained in the study of the interaction of model oligonucleotides, permit the hope that, by virtue of the destabilising effect of one or several mismatches, extra bases, or loops, a fairly selective interaction may be achieved between the NA target on the oligonucleotide complementary to the selected section.

In the case of site-specific modification, the problem of increasing the complementation accuracy reduces in essence to the exclusion from the reaction sphere of imperfectly matched complexes between the NA target and the 'addressed' reagent. As can be seen from the data discussed, in certain cases highly selective interaction may be achieved in systems *in vitro* either by carrying out the modification at a temperature close to the melting point of the perfectly matched complex or by using fairly low concentrations of the 'addressed' reagent (10^{-8}–10^{-9} M). However, it is then necessary to take into account the fact that, simultaneously with the increase in the selectivity of the modification, the degree of alkylation of the nucleic acid along the selected section will diminish. Furthermore, in systems *in vitro* an increase in the selectivity of the site-specific modification can be achieved by employing effector oligonucleotides stabilising the duplex, which are complementary to the sequence of the NA target adjoining the binding site of the 'addressed' reagent and carrying the *N*-(2-hydroxyethyl)phenazinium residues at one or both ends. Although impressive advances have in fact now been achieved in the application of this approach to increase the selectivity and effectiveness of the site-specific modification of synthetic oligonucleotides and fragments of single-stranded DNA with a weak secondary structure, there are so far insufficient data to postulate that this approach will prove just as productive when applied to high-molecular-mass nucleic acids having a complex steric structure.

Evidently, in inhibition with the aid of antisense oligonucleotides and their derivatives of processes such as translation, transcription, and others, occurring in systems *in vitro* let alone *in vivo*, the accuracy of the effect is determined not only by the differences between the stabilities of the perfectly

and imperfectly matched complexes but also by the steric structure of the NA target, the interaction of the antisense oligonucleotides with other biopolymers involved in the process investigated, and also the mechanism of the inhibition of the given process with the aid of the antisense oligonucleotide. For example, translation is inhibited because mRNA is either cleaved by RNase H or loses the capacity to be translated by the ribosome after the formation of a duplex with the antisense oligonucleotide. Therefore one can hardly assume that the approaches developed for model systems where synthetic oligonucleotides or fragments of single-stranded DNA are used as matrices can be sufficiently effective in increasing the specificity of the inhibition of the above processes.

In our view, the methodology of the approaches used to increase the specificity of the influence on the genetic apparatus requires profound physicochemical and biochemical studies in model systems involving different stages of the processes which ensure the storage, replication, and utilisation of hereditary information (replication, transcription, reparation, translation, etc.) in cells and viruses. The possibility of establishing the primary structure of virtually any nucleic acid allows the creation of specific oligonucleotide and polynucleotide addresses to a great variety of sections of the generic apparatus in living organisms, and the development on this basis of new approaches designed to increase the selectivity of the specific influence, which take into account fully both the characteristics of the steric structure of the given nucleic acid and the imperfect direct repetitions of selected complementation sections, as well as data on the thermodynamics of perfectly and imperfectly matched duplexes.

Thus the problem of the complementation accuracy of the interaction of antisense oligonucleotides and their derivatives with nucleic acids is at present one of the most acute problems in the study of site-specific influences on nucleic acids.

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