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Antisense Oligonucleotides: A New Therapeutic Principle

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I. Introduction

Even today, new drugs are usually not discovered by rational drug design even though this would be the dream of the medically oriented chemist. On average, it is still necessary to synthesize and test about 10 000 new compounds in order to discover a new active substance worth development. In many cases the active substance is to be directed against proteins such as enzymes, receptors, or ion channels, the structure and mode of action of which are usually very complicated and often incompletely understood. On the other hand,

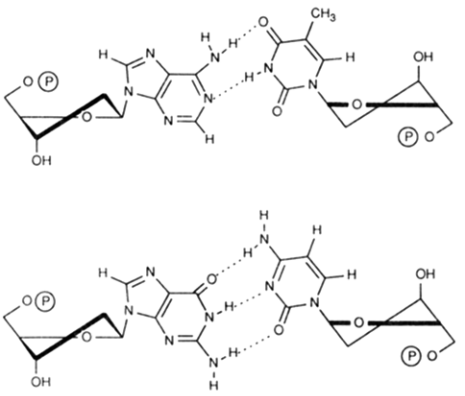

Figure 1. Watson–Crick base pairing.

therapeutic intervention at the level of the nucleic acid appears to offer a number of advantages.

On transcription, every gene gives rise to a relatively large number of copies of messenger ribonucleic acid (mRNA), which is translated into a large number of protein molecules. This is why inhibition of gene expression ought to be more efficient than inhibition of the resulting protein product. There are already a number of drugs on the market whose activity is based on direct interaction with deoxyribonucleic acid (DNA). Many of these compounds, which are mainly used for chemotherapy, intercalate or bind specifically only to DNA. If the recognition step, the binding to the DNA, is followed by a response, according to Hurley and Boyd[1] the DNA should be regarded as a receptor in the pharmacological sense. Classical drugs such as adriamycin, bleomycin, or cisplatin are, however, unable to exhaust the sequence information contained in the nucleic acids and thus do not act specifically on particular genes. It is, however, possible to achieve such sequence-specific recognition of nucleic acids using synthetic oligonucleotides that bind specifically by hydrogen bonding to complementary nucleic acids. These compounds are called antisense oligonucleotides based on their binding to the target sequence (sense strand). Since, statistically, the base sequence of a 17-mer oligonucleotide occurs just once in the sequence of the human genome, extremely selective intervention ought to be possible with antisense oligonucleotides of this length. An additional point in favor of therapeutic use of oligonucleotides is that they occur endogenously in eucaryotic cells.[2] Moreover, the antisense principle is also used in nature to regulate gene expression. In both procaryotic and eucaryotic cells there is known to be a natural method of regulation based on the binding of complementary nucleotide sequences (antisense RNA) to particular nucleic acids.[3-7] Thus, if the nucleotide sequence of the target molecule is known, it is possible to write down directly the chemical formula of the inhibitor, corresponding to the base sequence of the antisense oligonucleotide, which amounts to rational drug design. Unlike rational drug design when the target molecules are proteins, there is no need for X-ray structure and NMR analyses.

However, it ought to be emphasized that antisense oligonucleotides can be used not only to inhibit but also to activate gene expression. This is possible, for example, indirectly by suppressing the biosynthesis of a natural repressor[4] or directly by reducing termination of transcription.[8]

A. Antisense RNA: A Natural Gene Expression Control System

Gene expression in cells is normally controlled by DNA-binding proteins, repressors, and activators. Only recently have regulatory RNA sequences been established as direct repressors of gene expression. This so-called antisense RNA is produced from the corresponding antisense genes by transcription. In the simplest case this can take place by transcription of the complementary (antisense) DNA strand. The antisense RNA then binds, via Watson–Crick base pairing,[9] to the complementary (sense) target nucleic acid. Transcription of both complementary strands in the same region of the DNA was first observed in 1969 by Bovre and Szybalski on phage lambda.[10] However, at that time it was possible only to speculate about a regulatory role of antisense RNA. Not until 1983 did Simons and Kleckner[11] detect in procaryotes an RNA that was complementary to the ribosome-binding site, including the start codon, of a gene and suppressed its expression in a specific manner. Interestingly, 6 years before, Paterson et al. had already directed complementary single-stranded DNA against mRNA and thus achieved suppression of mRNA translation in a cell-free system.[12] They introduced the term HART (*h*ybrid *a*rrested *t*ranslation) for this. As expected, artificial antisense RNA able to inhibit the expression of any desired gene is widely used in fundamental research. However, it is also being investigated extensively with a view to use in therapy as well as in agriculture, especially in crop protection. Since this antisense RNA technology has been dealt with comprehensively in some review articles,[3-7,13] we will focus on synthetic antisense oligonucleotides.

B. Antisense Oligonucleotides

Zamecnik and Stephenson were the first to propose, in 1978, the use of synthetic antisense oligonucleotides for therapeutic purposes.[14,15] They were able with a 13-mer oligonucleotide that was complementary to the RNA of Rous sarcoma virus to inhibit the growth of this virus in cell culture. The specific inhibition is based on the specific Watson–Crick base pairing (Figure 1) between the heterocyclic bases of the antisense oligonucleotide and of the viral nucleic acid. The process of binding of the oligonucleotides to a complementary nucleic acid is called hybridization.

Various cellular processes can be inhibited depending on where the oligonucleotide hybridizes on single-stranded regions of the DNA or mRNA. A simple model describes the inhibition of protein biosynthesis by an antisense oligonucleotide being bound to the mRNA (Figure 2). Although this illustrative model does not always reflect the actual mechanisms, nevertheless it reveals the essential steps in the new principle and the problems associated with it: for the antisense oligonucleotide to be able to inhibit translation it must reach the interior of the cell unaltered. The requirements for this are, on the one hand, stability of the oligonucleotide toward extra- and intracellular enzymes but also, on the other hand, ability to penetrate through the cell membrane. Once it has reached the cytoplasm it must bind specifically and with sufficient affinity to the target mRNA to inhibit its translation into the corresponding protein. In order to meet all these requirements it is necessary for normal oligonucleotides to be chemically modified in a suitable manner. Hence we describe below the synthesis of normal and of modified oligonucleotides and then discuss the properties of these compounds that make some of them good inhibitors, and some of them less effective inhibitors, of gene expression.

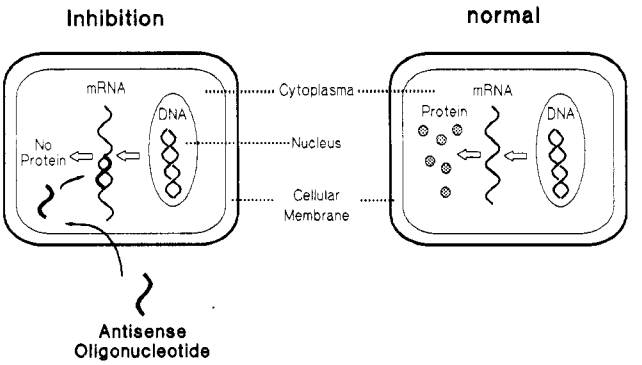

Figure 2. Principle of action of the antisense oligonucleotides.

II. Methods for the Synthesis of Oligonucleotides and Their Analogues

A. Unmodified Oligonucleotides

The preparation of unmodified oligodeoxynucleotides has been the center of interest of many research groups in the past decade, not least because of their use in genetic engineering.[16] The methods and problems of solid-phase and "manual" synthesis have been described in detail in several review articles,[17-27] the most timely written by Sonveaux,[17] so mention will be made here of only the most important points, the chain-extension steps in the conventional methods.

The synthesis via phosphoramidites according to Caruthers,[28] originally introduced by Letsinger[29] as the phosphite triester method, is currently the most efficient method for preparing oligodeoxynucleotides. It entails the 5′-OH group of the growing DNA chain being reacted with a nucleoside 3′-β-cyanoethyl *N*,*N*-diisopropylphosphoramidite with catalysis by 1*H*-tetrazole and the resulting phosphite triester being oxidized immediately with I_2 to the phosphotriester (Figure 3). The coupling yield in the amidite method is >99%,[30] and a synthesis cycle takes about 8 min. It is possible in this way to construct oligomers having up to 175 nucleotides using automatic DNA synthesizers.[31] Mention may be made of the possibility of carrying out 5′-phosphorylation as part of a normal synthesis cycle.[32]

In the past 2 years the H-phosphonate method, which was described for the first time by Todd in 1957,[33] has become reestablished and has in some cases replaced the amidite method, particularly because the synthons are easier to handle and no phosphate protective group is employed.[34-39] This method entails the 5′-OH group of the growing DNA chain being reacted with a nucleoside 3′-H-phosphonate (Figure 4). The condensing agents used are sterically hindered carbonyl chlorides such as adamantoyl or pivaloyl chloride. The resulting phosphite diester is oxidized with *tert*-butyl hydroperoxide or iodine to the phosphotriester only after construction of the chain is complete, in contrast to the amidite method. The H-phosphonate method can also

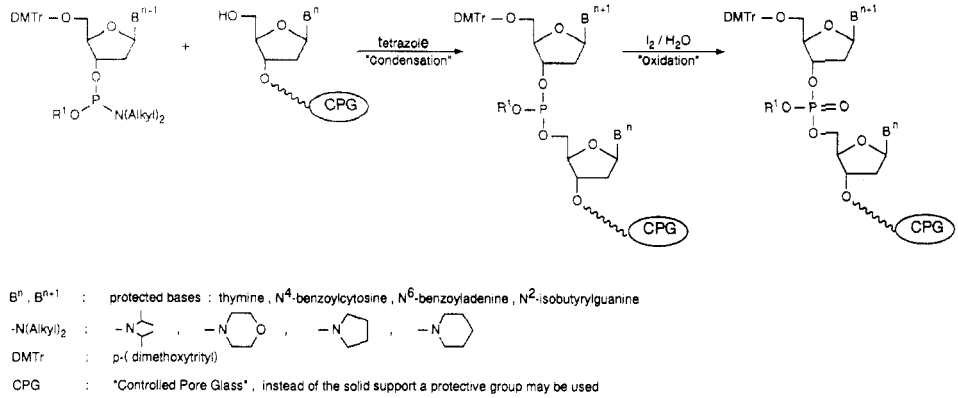

Figure 3. Amidite method.

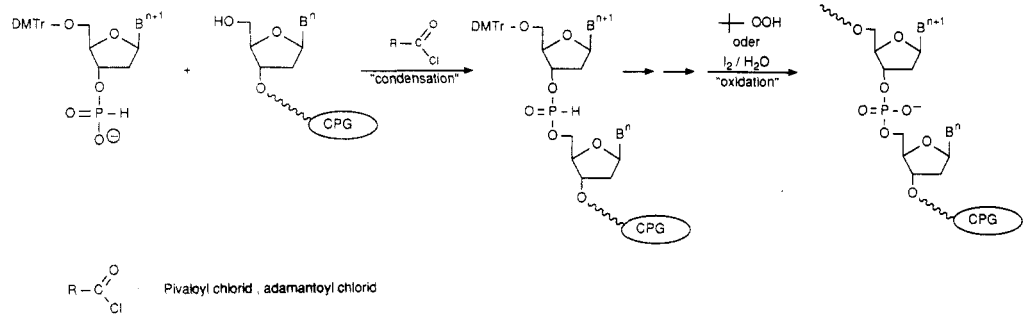

Figure 4. H-Phosphonate method.

be used to prepare oligomers of considerable chain length; e.g., Froehler et al. have reported the synthesis of a 107-mer.[36]

The phosphotriester method,[17,26] which dominated oligodeoxynucleotide preparation for a long time, is still regarded as the method of choice for large-scale synthesis,[26] although the H-phosphonate method has gained ground here, too.[40] In this method the required phosphotriester is obtained in one step from the 5′-OH group of the growing chain and the nucleoside 3′-phosphodiester units (Figure 5). The condensation is brought about with 3-nitro-1,2,4-triazolides of an arenesulfonic acid, preferably 2,4,6-triisopropylbenzenesulfonic acid[41,42] (TIPS) or 8-quinolinesulfonic acid[43,44] (QSNT). A further improvement was achieved by using nucleophilic catalysts, e.g., 4-substituted pyridine *N*-oxides, which, intramolecularly attached to the phosphate protecting group, jack up the coupling yield to 98% and reduce the time taken by a synthesis cycle to 7–8 min.[45]

The synthetic oligodeoxynucleotides are purified by polyacrylamide gel electrophoresis, followed by removal of salts, or by HPLC.[46,47] Capillary gel electrophoresis[48,49] is proving to be extremely useful for the analysis and fractionation of small amounts of oligodeoxynucleotides and may be regarded as the future method of choice. Sequence analysis of purified oligodeoxynucleotides is possible by three methods: The first method is that of Maxam and Gilbert,[50] which is based on chemical cleavage and subsequent gel electrophoresis. The second method is the wandering-spot method,[51] in which the oligodeoxynucleotide is subjected to a partial enzymatic digestion and then fractionated by two-dimensional electrophoresis. A third method has been reported by Ansorge et al. using chemical degradation on oligonucleotides bound to Hybond paper.[52] Sequences of shorter oligodeoxynucleotides can also be analyzed by FAB[53] or by plasma desorption

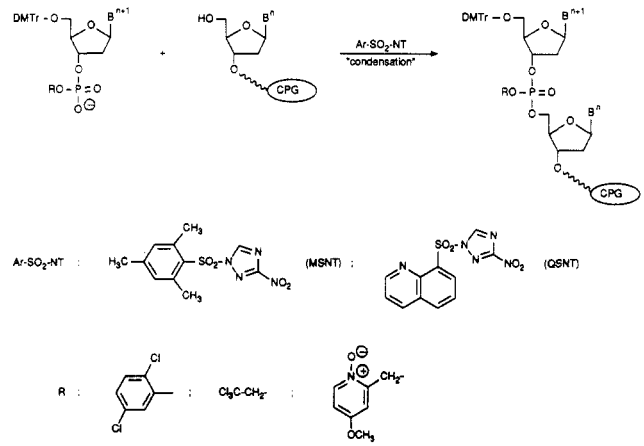

Figure 5. Phosphotriester method.

mass spectroscopy.[54,55] Since these rapid and accurate analytical methods can also be applied to modified compounds,[56] they could well achieve greater importance.

When "normal" oligodeoxynucleotides were used as antisense oligonucleotides, the problems described in section III emerged—i.e., instability to nucleases and insufficient membrane penetration. This and, of course, purely academic interest gave rise to a variety of modifications (Figure 6), which will be dealt with below.

B. Oligodeoxynucleotides with a Modified Internucleotide Phosphate Residue

1. *Methylphosphonates*

In order to improve uptake by cells and extend the biological half-life, Miller and Ts'O have concentrated on eliminating the negative charge on the internucleotide phosphate bridge.[57–59] In the methylphosphonate oligodeoxynucleotides, which they called

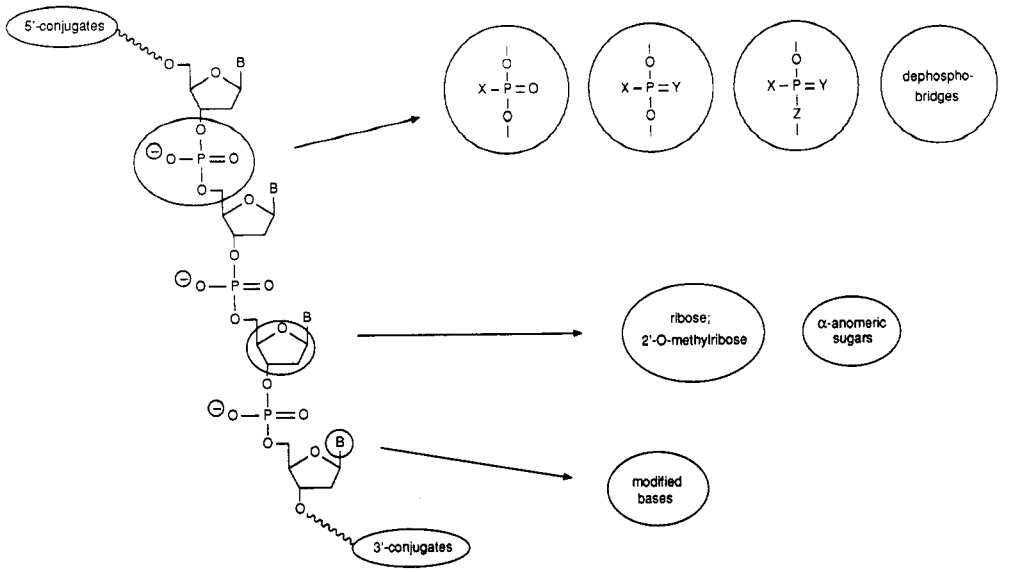

Figure 6. Possibilities for modifying oligonucleotides.

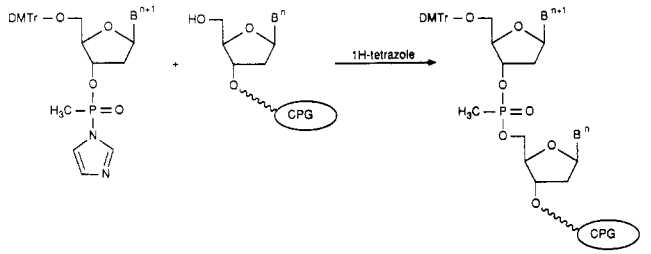

Figure 7. Preparation of oligonucleotide methylphosphonates by the phosphotriester method.

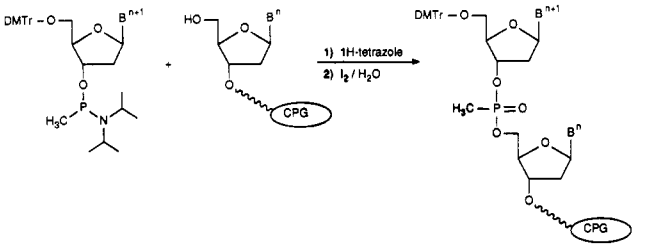

Figure 8. Preparation of oligonucleotide methylphosphonates by the amidite method.

MATAGENES (*ma*sking *ta*pe for *gene* e*x*pression),[59] the negatively charged phosphate oxygen is replaced by a methyl group, which is neutral and sterically undemanding.

The methylphosphonate oligodeoxynucleotides can be obtained in two ways whose chemistry is based on that of the "natural" representatives although the condensation reactions take place very much more slowly than with normal phosphates. The triester method was used first to condense triethylammonium salts of nucleoside 3'-methylphosphonates with 5'-unprotected nucleosides or oligomers using coupling reagents such as 1-(2-mesitylsulfonyl)-3-nitro-1,2,4-triazole (MSNT).[60,61] As an alternative, Agarwal[62] used methylphosphonic acid bis(1,2,4-triazolides), which, additionally activated with benzenesulfonic acid tetrazolides or 1*H*-tetrazole,[63] are employed for the condensation. This procedure, which is based on methylphosphonic dichloridite[64] as combined phosponylating and condensing reagent, derived from the strategy formerly used in the triester method[21] and was eventually optimized by Miller and Ts'O[65] by using 5'-protected nucleoside 3'-methylphosphonic acid imidazolides (Figure 7). The yields in each coupling step are 88–92%, which allows 15-mers to be synthesized in an isolated yield of 4%. In this connection the approach of van Boom et al.[66] should be mentioned in which the methylphosphonate oligodeoxynucleotides were prepared via bis-*O*,*O*-(1-benzotriazolyl) methylphosphonates.

However, a more efficient synthesis is via phosphonoamidites, which were described for the first time by Jäger and Engels[67] after they had previously introduced methylphosphonous dichloridite as central synthon,[68] which was used later on by Köster et al.[69] for the solid-phase synthesis of methylphosphonate oligodeoxynucleotides. However, whereas Jäger and Engels used collidine hydrochloride as activator and obtained only moderate yields (about 80%), Dorman et al.[63] achieved yields of over 90% with imidazole. The synthesis of methylphosphonate oligodeoxynucleotides using methylphosphonoamidites on a solid support[70,71] takes place with yields of 96–97% in each coupling step[71] and may be regarded as the method of choice (Figure 8).

The use of these synthons also allows unmodified oligodeoxynucleotides with only one or with several methylphosphonate bridges at any desired point in the molecule to be synthesized using the usual synthesis cycle.[70] This is of particular interest for the studies of stability to nucleases discussed in section III.D.

Several authors have described the synthesis of methylphosphonate oligodeoxynucleotides via an Arbusov reaction with dinucleoside *O*-methyl phosphites and methyl iodide,[72–75] but this has not proven useful, inter alia, because of the low yields of maximum 70%.[75]

Since the internucleotide methylphosphonate bridge is more base labile than the natural internucleotide linkage,[69,76] milder conditions are necessary for cleavage from the support and deprotection. Whereas the latter is normally carried out with concentrated NH_4OH at 60 °C for 8 h, in the case of the methylphosphonate it is carried out at room temperature for only 2 h and then with ethylenediamine/ethanol (1:1) for 7 h, likewise at room temperature.[65,70] The use of only ethylenediamine/ethanol or *tert*-butylamine in methanol at 0 °C has also been proposed.[63]

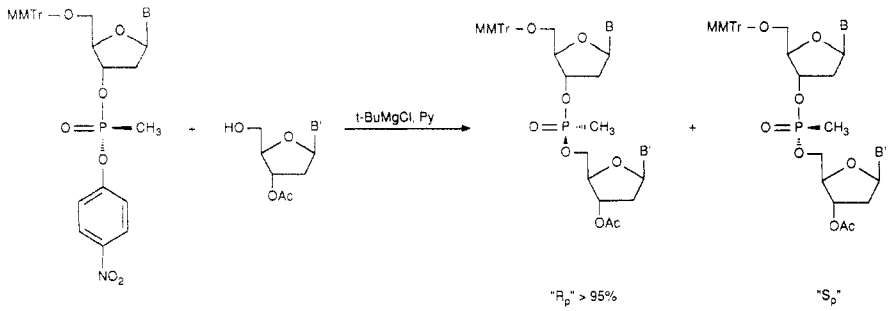

Figure 9. Stereoselective synthesis of oligonucleotide methylphosphonates.

Another problem is posed by the chirality of the methylphosphonate bridge, which can have the R_p or S_p configuration (Figure 9). Attempts were made early on to separate diastereomeric dinucleotides by chromatographic methods[60] and to achieve a configurational assignment. As Katti and Agarwal showed,[77] separation is more effective when chiral protective groups are used, such as 1-menthoxy carbonate in the 3′ position. The configuration of the S_p diastereomer of $dA_{Me}T$ was elucidated by X-ray structural analysis,[78] and the diastereomers of $d(A_{Me}A)$ were individually assigned by ^1H NMR NOE experiments.[79] Similar investigations have recently been undertaken by Engels on $T_{Me}T$.[80] The ^{13}C NMR signals for one diastereomer of $T_{Me}T$ were assigned.[74] None of the syntheses described above takes place selectively. This means that an *n*-meric methylphosphonate oligodeoxynucleotide is obtained as a mixture of 2^n diastereomers, which is an unsatisfactory result for many investigations. A first promising approach to stereoselective synthesis has been introduced by Stec et al.[81,82] This entails the R_p and S_p diastereomers of $T_{Me}T$ being obtained by stereospecific reaction of the P-chiral nucleotide components 5′-*O*-(monomethoxytrityl)thymidine 3′-*O*-(*O*-(4-nitrophenylmethanephosphonate) and 3′-*O*-acetylthymidine (Figure 9). The 5′-OH group is activated by *tert*-butylmagnesium chloride, and the reaction takes place with inversion on the phosphorus. The stereospecificity is >95%, but the chemical yield of ca. 70% means that there is little hope of successful use in solid-phase synthesis. Tetramers with all-R_p or all-S_p configuration are obtained by stepwise reaction. Engels is currently attempting to achieve diastereoselectivity by using chiral amidites, with proline in place of the diisopropylamino group.[83]

The methyl group can be replaced by other alkyl or by aryl moieties. Thus, phenylphosphonate[62] and (difluoromethyl)phosphonate[84,85] internucleotide bridges have been synthesized by the triester method, and allyl- and (dimethoxytrityl)phosphonates have been obtained by Arbusov reactions.[75]

2. *Phosphorothioates/Phosphorodithioates*

Phosphorothioates are among the most obvious and thus probably earliest used analogues of naturally occurring phosphates.[86] For example, the antiviral effect of thiophosphate-substituted polyribonucleotides was described back in 1970 by De Clercq and Eckstein.[87] In phosphorothioate oligodeoxynucleotides one of the phosphate oxygen atoms not involved in the bridge is replaced by a sulfur atom, with the negative charge being distributed unsymmetrically and located mainly on sulfur.[88,89] This substitution results in properties such as stability to nucleases, retention of solubility in water, and stability to base-catalyzed hydrolysis, which makes it exceptionally interesting for use in antisense technology. Just like the unmodified oligodeoxynucleotides that have been discussed, the phosphorothioate analogues can be prepared by the three methods of synthesis: the phosphate triester, phosphite triester, and H-phosphonate methods.

The groups of Reese[90] and van Boom[91] have synthesized the phosphorothioates by the triester method using 2,5-dichlorophenyl phoshorodichloridothioate as phosphorylating and coupling component. The activator used by van Boom, 1-hydroxy-6-nitrobenzotriazole (Figure 10), proved to be superior to 1-hydroxybenzotriazole for this. Van Boom reports the yield for a 10-min synthesis cycle as >90% and was able to synthesize hexadecamers in this way. Standard methods were used for cleavage of the support and removal of the protective groups.[91] The corresponding methylphosphonothioates can be obtained analogously by methylthiophosphonic acid benzotriazolide.[92]

The synthesis of diribonucleoside phosphoromonothioates by oxidation of phosphite triesters with sulfur was described first by Burgers and Eckstein,[93] who carried out the oxidation with a 1 M solution of sulfur in pyridine. This method was easily applicable to the synthesis of the 2′,5′-linked oligoribonucleotide phosphorothioate[94] and of dideoxynucleoside phosphoromonothioates.[95] This was extended to oligodeoxynucleotide synthesis on a solid support in 1984.[96,97a] The standard amidite method is used for this synthesis, the only change being oxidation with 0.4 M S_8 in 2,6-lutidine (60 °C, 1 h) or 5% S8 in CS_2/pyridine at room temperature in place of the usual I_2/H_2O[97] (Figure 11). The yield in the subsequent coupling step remains at the same high level.[96] This method is particularly attractive because it can be carried out without extensive alteration of the normal synthesis cycle and is therefore still regarded as the method of choice.[98] Only for the oxidation with sulfur, which is carried out at 60 °C, must the support be removed from the synthesizer. If KSeCN in acetonitrile is used for oxidation in place of sulfur, phosphoroselenide analogues can be obtained.[97a,99] Another advantage derives from the fact that phosphoromonothioate bridges can be introduced in this way at any desired point in oligodeoxynucleotides.[96,100] This is of interest particularly for studies of enzyme mechanisms[86,96] but also for antisense technology.[98] Subsequent oxidation with I_2/H_2O does not seem to cause loss of sulfur. The deprotection of the thiophosphate group from the *O*-methyl ester is carried out at the end of the synthesis with thiophenol. Only in phosphorothioate diesters is the sulfur easily

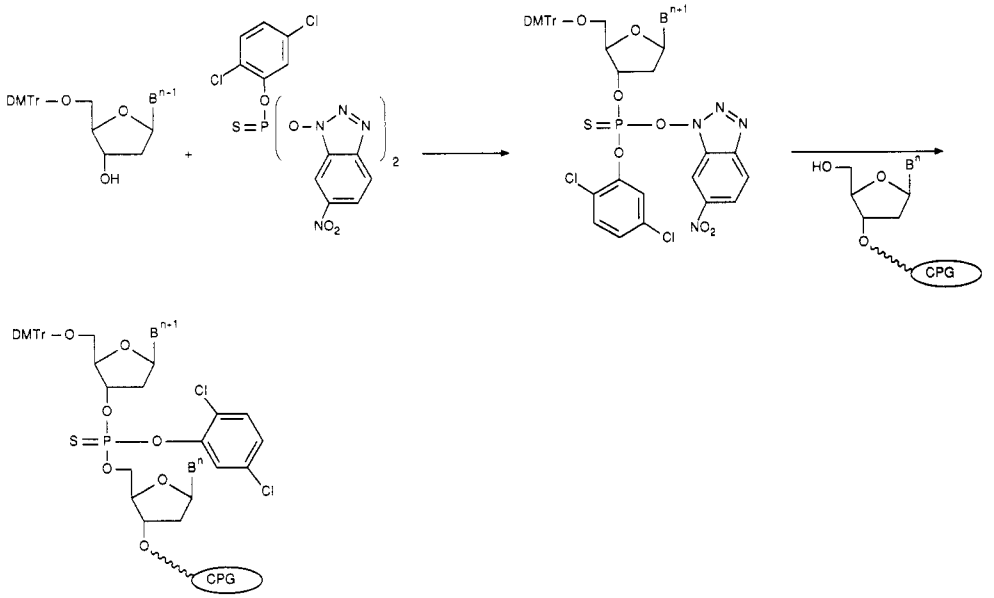

Figure 10. Synthesis of oligonucleotide phosphorothioates by the phosphotriester method.

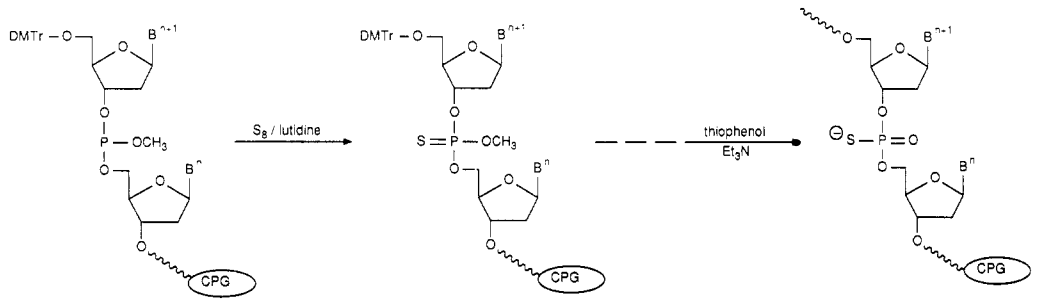

Figure 11. Synthesis of oligonucleotide phoshorothioates by a modification of the amidite method.

replaced by oxygen when oxidation is carried out with 1% I_2 in THF/lutidine/H_2O.[96]

Synthesis of all-phosphorothioate oligodeoxynucleotides is more easily carried out as single-step sulfurization in a modification of the H-phosphonate method.[98] Fujii et al.[101] were the first to describe the oxidation of H-phosphonates with S_8 in pyridine/triethylamine, which was subsequently applied to solid-phase synthesis.[35,98,102] The advantage results from the fact that oxidation with sulfur (0.1 M S in 9:1 CS_2/triethylamine, 2 h) is necessary only once, namely, after synthesis is complete (Figure 12).

The dinucleotide phosphorothioates have, as do the methylphosphonates, a chirality center at the phosphorus. Therefore, they are often used as key substrates for elucidating the stereochemistry of enzymatic reactions.[86,103] The latter can, in turn, be used for configurational assignment: the S_p diastereomers are substrates of nucleases S1 or P1, while the R_p diastereomeric dinucleotides are cleaved by snake venom phosphodiesterase. There is great interest in chromatographic separation of the diastereomeric dimers,[95,96,99,100] spectroscopic characterization,[104] and the possibility of stereospecific synthesis. A first attempt at the synthesis of pure R_p and S_p diastereomers was carried out by Stec et al., who reacted P-chiral phosphoranilidate dimers with NaH/CS_2 by the Wadsworth–Emmons method to give the phosphorothioates.[105,106] Fujii et al.[101] published, in 1986, a method for the stereoselective preparation of the R_p diastereomeric dimers T_ST and dA_ST. This entailed the corresponding aroylphosphonates being reacted with *tert*-butylamine in the presence of DBU and sulfur directly and exclusively to the R_p isomer with a yield of ca. 60% (Figure 13). Cosstick and Williams[107] have supplemented this by describing a synthesis that is based on work by Ohtsuka[108,109] and results in the preferential formation of the S_p diastereomers (Figure 13). This involves the condensation of a β-cyanoethyl S-protected nucleoside 3′-phosphorothioate with a 5′-unprotected nucleoside using 1-(mesitylenesulfonyl)-5-(2-pyridinyl)tetrazole (MSPY). After deprotection an 8:2 excess of the S_p isomer is obtained, but the overall yield of 46% is low.

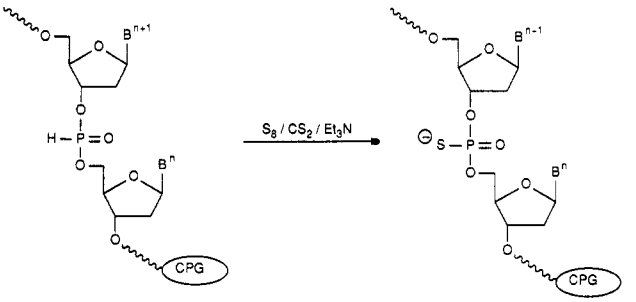

Figure 12. Synthesis of oligonucleotide all-phosphorothioates by a modification of the H-phosphonate method.

The chirality problem can be avoided if the second phosphate oxygen, which is not involved in the bridge, is also replaced by sulfur. Caruthers et al.[110,112] have described the synthesis of such phosphorodithioates. In this, a phosphorothioamidite is condensed with a second

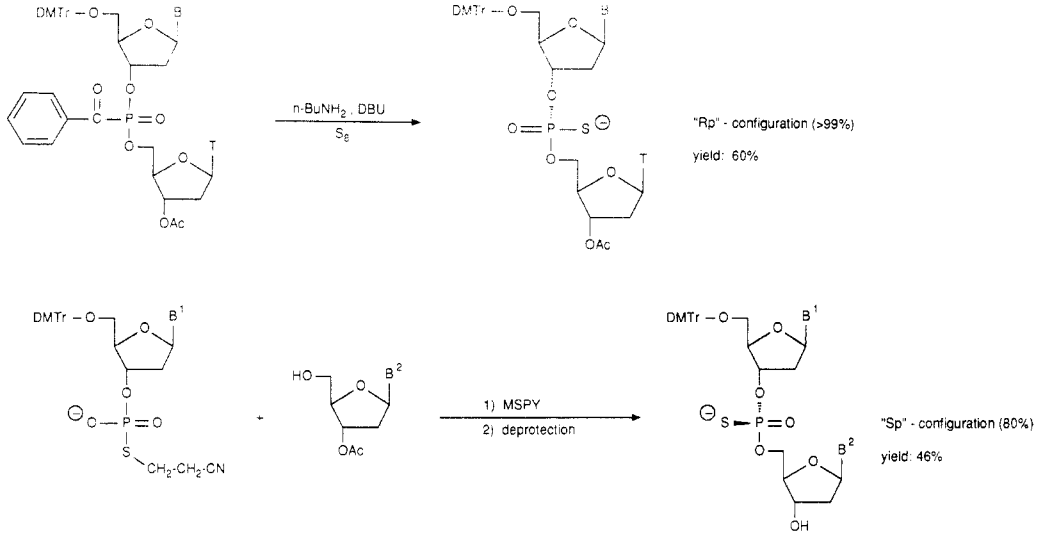

Figure 13. Stereoselective synthesis of dinucleoside phosphorothioates.

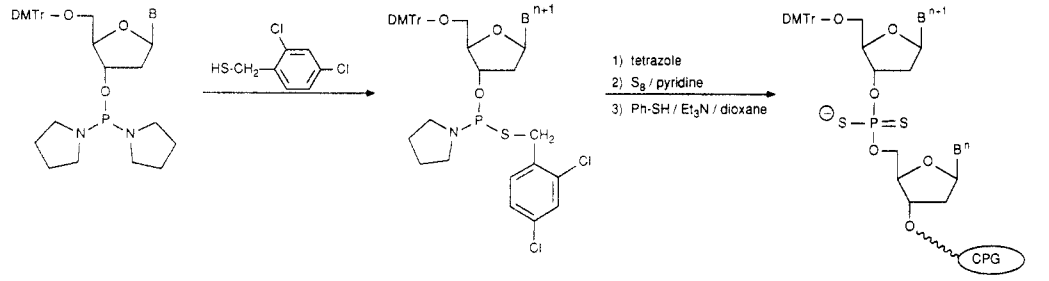

Figure 14. Synthesis of phosphorodithioates via thioamidites.

nucleoside, with tetrazole catalysis, and the resulting intermediate is oxidized with 5% sulfur in pyridine/CS_2 to the phosphorodithioate. This synthesis can also be carried out on a solid support (Figure 14). To ensure high coupling yields (>98%) the phosphorothioamidite must be supplied twice to the solid support in the coupling step. The deprotection of the oligonucleotide phosphorodithioate is carried out in two steps: first the *S*-(2,4-dichlorobenzyl) protective group is eliminated with thiophenol/triethylamine/dioxane, and then the bases are deprotected with concentrated NH_4OH as usual. No hydrolysis to the phosphates or phosphorothioates is observed during this. The synthons used must be in the form of *N,N*-dimethyl- or pyrrolidinylphosphoramidites because the *N,N*-diisopropyl group has proved to be too inert. The advantage of this procedure is that the phosphorodithioate bridge can be incorporated at any desired point in the molecule.

3. Phosphoramidates

The phosphoramidates are, like the thiophosphates, an easily obtainable group of nucleotide analogues which can be prepared in various ways from three- and five-valent phosphorus intermediates.

The method of phosphorylating amines by condensation with phosphate diesters in the presence of triphenylphosphine and CCl_4 has often been used to prepare dinucleoside phosphoroamidates[105,106,113] (Figure 15). However, the yields are moderate and vary between 24% (T_NT, *n*-butylamine[113]) and 53% (T_NT, aniline[105]). Yields of 70% (T_NT, *n*-butylamine) are reached only on solid supports.[113]

Other possible syntheses comprise addition of alkyl and aryl azides to phosphite triesters,[113-116] but these

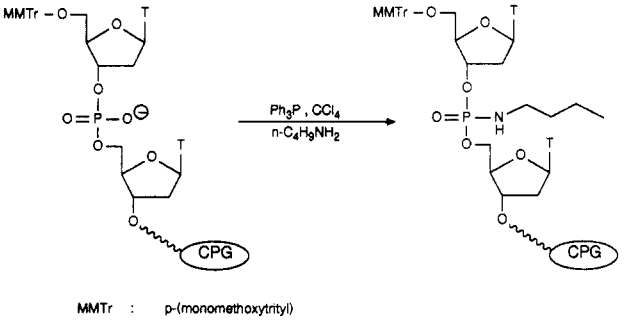

Figure 15. Synthesis of phosphoroamidates by "Appel" condensation.

reactions take place very slowly at room temperature[113,114,116] (several days), and even under more drastic conditions (dioxane, reflux, 4 h) only "moderate" yields (87%[115]) are obtained.

Even less favorable is the preparation of phosphoramidates by nucleophilic substitution of phosphate triesters by alkylamines,[117-119] the yields scarcely exceeding 70% in the most favorable cases.[117]

By contrast, oxidation of dinucleoside H-phosphonates in the presence of amines gives the corresponding dinucleoside phosphoramidates[73,121,122] in high yields.[120] This reaction can easily be extended to polymer-bound di- and oligonucleotide H-phosphonates.[35,102,122,123] The oxidizing agents used for the coupling, which can be carried out with primary and secondary amines, are CCl_4[121,123,124] and I_2[35] (Figure 16).

Besides the described oxidation of H-phosphonates, which results in all-phosphoramidate oligodeoxynucleotides, the oxidation of phosphite triesters with iodine in the presence of alkylamines, which was first described by Nemer and Ogilvie,[73] is particularly at-

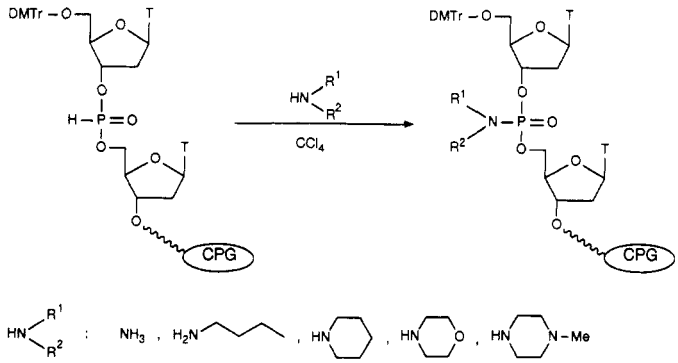

Figure 16. Synthesis of phosphoroamidates by oxidation of H-phosphonates.

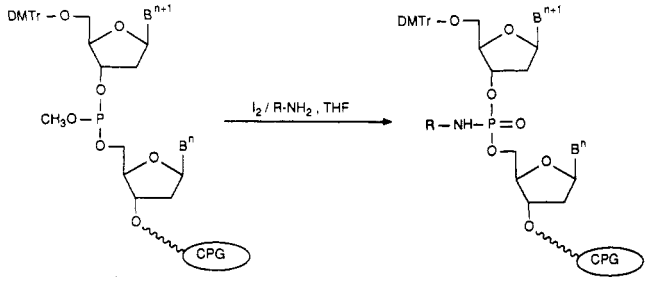

Figure 17. Synthesis of phosphoroamidates by oxidation of dinucleoside phosphite triesters with I_2/alkylamine.

tractive[113] because it can be carried out without expensive alteration in the amidite synthesis cycle.[113] This entails oxidation with 0.1 M I_2 in 2:1 THF/alkylamine, resulting in selective elimination of the methyl protective group used (Figure 17). The yields vary between 50 and 90%.[113] Once again it is possible to incorporate phosphoramidate bridges at any desired point in the oligonucleotide. Deprotection and cleavage of the support are carried out by using *tert*-butylamine in methanol.

None of these methods results in the preferential formation of one diastereomer, but the mixtures in the case of the dimers can easily be separated by chromatography and characterized by spectroscopy.[113] In contrast to the thioates, the diastereomeric phosphoramidates cannot be differentiated by digestion with nuclease P1 or snake venom phosphodiesterase; on the contrary, they are absolutely inert to these enzymes.[113,117] *N*-Alkylphosphoramidates are suitable as functionalizing groups, via the alkyl radical, and can be used to produce oligodeoxynucleotide conjugates, e.g., with intercalating agents.[113,114] These are discussed separately in section II.F.

4. Phosphate Esters

The P(O)-alkyl derivatives, like the alkanephosphonates, differ from the oligodeoxynucleotides in lacking the negative charge on the oxygen in the latter. DNA phosphate triesters have been of interest for a long time as products of alkylating agents,[125] but they acquired additional significance as potential antisense oligodeoxynucleotides.[57,126]

Oligodeoxynucleotide phosphate triesters can be synthesized in a variety of ways. Specific phosphate triesters are produced as intermediates in the phosphate triester method already discussed, as well as in the amidite method. In these cases, the triester serves to

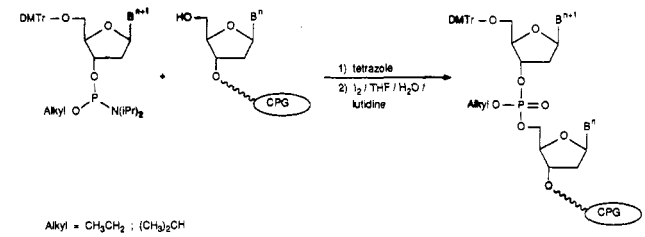

Figure 18. Synthesis of oligonucleotide phosphotriesters by the amidite method.

protect the phosphate and must, accordingly, be easily cleaved. This is why the most suitable groups are those such as *o*-chlorophenyl or 2,5-dichlorophenyl in the triester method and methyl or cyanoethyl esters in the amidite method. However, if the synthesis is to terminate on the triester stage, this restriction does not apply and, accordingly, other esters can be used. The amidite method is widely used to synthesize phosphate triesters from nucleoside dimers and oligomers. Mainly employed for this are the 5′-protected nucleoside 3′-(*O*-isopropyl *N*,*N*-diisopropylphosphoramidites) and their *O*-ethyl analogues[127-132] (Figure 18). However, sterically more demanding esters such as 1,1-dimethyl-2,2,2-trichloroethyl esters[117] have also been prepared. The synthesis is efficient and, moreover, provides the opportunity to replace the I_2/H_2O oxidation by S_8/pyridine and thus obtain phosphorothioate triesters. It is, of course, possible to incorporate both groups at any desired point in normal oligodeoxynucleotides.[129,132] The cleavage of the support and the deprotection of the bases is carried out with 25% NH_4OH at room temperature for 48 h. The ethyl and isopropyl phosphate triesters are stable under these conditions, whereas methyl and cyanoethyl esters are completely removed.[132]

Stec et al.,[133] in 1987, developed a method that combines some advantages of the classical amidite method with some of the H-phosphonate method. This employs 5′-protected nucleoside 3′-*O*-phosphorodimorpholidites as synthons, with hydrolysis to the H-phosphonate per each synthesis cycle (yields between 90 and 99%), and oxidation is then necessary only once, at the end of the synthesis. Alternatively, alcohols can be used for alcoholysis, and the resulting phosphite triesters are oxidized at the end of the synthesis (Figure 19). This method is attractive owing to its versatility.

For the sake of completeness, it may also be mentioned that phosphorothioates can be reacted with 2,4-dinitrofluorobenzene, and the intermediate phosphorofluoridates trapped with alcohols to give triesters.[99]

The problem of the chirality of the phosphorus that has already been mentioned also occurs with the phosphate triesters. Considerable efforts have been made to separate the diastereomers arising due to this chirality center and to characterize them (^{1}H NMR, UV).[99,130,134] The synthesis of ^{18}O-chiral dinucleotides is also based on a separation of this type.[135] It was, of course, interesting in this connection to find a method that allows configurational assignment at the phosphorus irrespective of the nature of the triester. Such a method is based on the stereoselective reaction of a P-chiral phosphothionotriester with *m*-chloroperbenzoic acid to give an *O*-triester on the one hand and with KSeCN as a strongly carbophilic nucleophile to give a

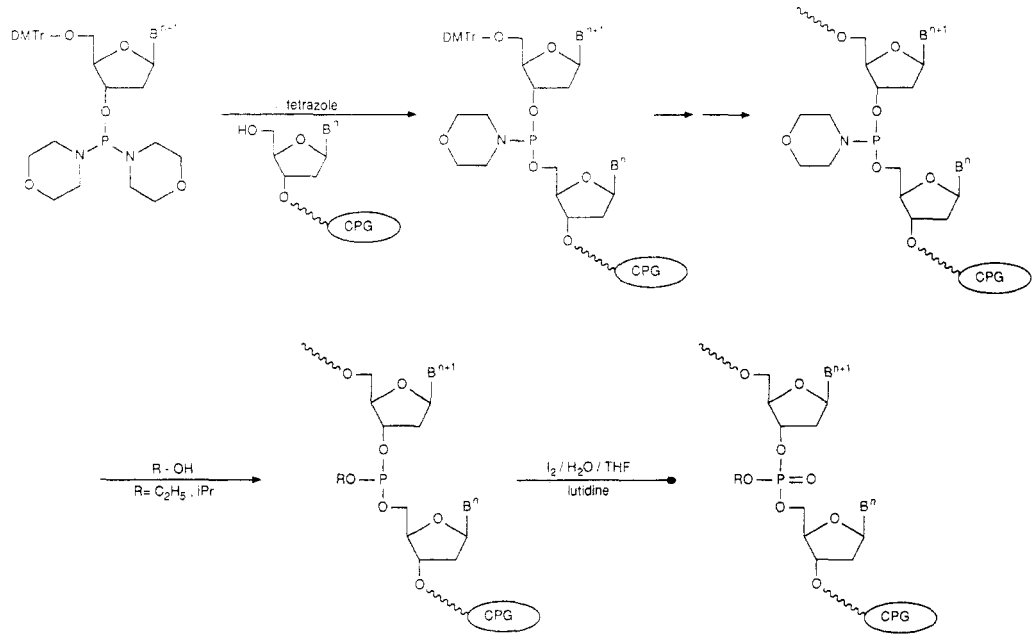

Figure 19. Preparation of oligonucleotide phosphotriesters by synthesis with phosphodimorpholino amidites, alcoholysis, and oxidation.

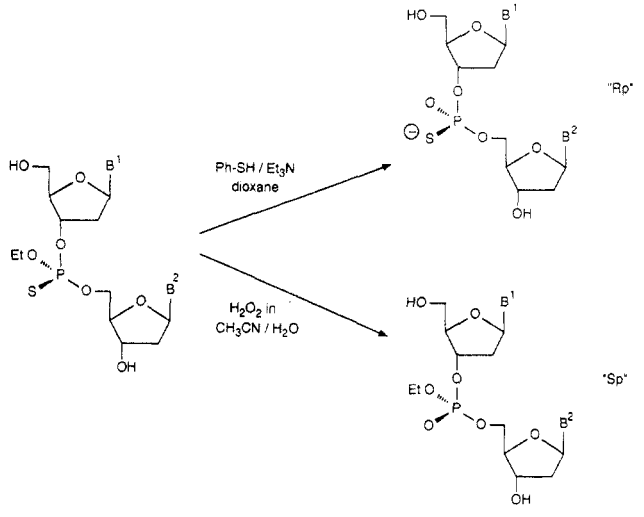

Figure 20. Assignment of configuration of phosphotriesters by stereoselective conversion of thiono triesters to thioates or phosphotriesters.

phosphorothioate on the other hand.[128] An alternative to *m*-chloroperbenzoic acid is H_2O_2 in acetonitrile/water, while an alternative to KSeCN is thiophenol/triethylamine in dioxane[129,132] (Figure 20). Since the phosphorothioates have been thoroughly investigated (see section II.B.2), configurational assignment is not difficult. The dinucleoside monophosphate triesters are resistant to nuclease digestion by snake venom phosphodiesterase and nuclease P1.

As is the case with the oligonucleotide analogues already discussed, stereoselective syntheses are in short supply for phosphate triesters, too. It is worth mentioning the selective formation of S_p diastereomers found by Ohtsuka and Ikehara when the otherwise unselective triester method is carried out with (arylsulfonyl)-5-(2-pyridinyl)tetrazoles as coupling reagents.[108,109,136]

Alkyl esters functionalized in the side chain are suitable as linkers to give oligodeoxynucleotide conjugates, which have been utilized with the alkylating oligodeoxynucleotides as discussed in section II.F.

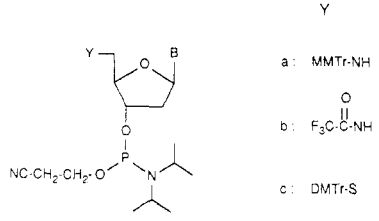

Figure 21. Monomers for incorporating (a) 5′-NH-bridged phosphoramidates, (b) 5′-NH_2 nucleosides at the 5′ end, and (c) 5′-SH-nucleosides at the 5′ end.

5. Replacement of Phosphate Oxygens Involved in the Bridge

Besides the modifications discussed, all of which related to replacement of oxygen atoms not involved in the bridge on the phosphate group, there is also the possibility of replacing one of the two oxygen atoms involved in the bridge. Potential candidates are analogues with NH–, CH_2–, and S–P bridges. This type of linkage results in DNA segments having a number of interesting properties because the chirality problem no longer applies. However, only a few of the conceivable modifications have actually been carried out.

(*a*) *Bridged Phosphoramidates.* The best known of these compounds are the bridged phosphoramidates. These can be prepared most straightforwardly by the amidite method. Bannwarth[137] synthesized the monomers necessary for this (Figure 21) and was able to incorporate them, using the standard method, at any desired point in normal oligodeoxynucleotides, with the coupling yield being >96%. Monomers suitable for incorporation at the 5′ ends of oligodeoxynucleotides have been described by Sproat et al.[138]

Another way of obtaining this class of compounds comprises the azide addition onto phosphite triesters which has already been described. Mag and Engels[139] synthesized and characterized (2D ^1H NMR) dinucleoside phosphoramidates in this way, and followed the reaction by IR spectroscopy, specifically of the azide band. There was initial formation of a phosphite imine, followed by a Michaelis–Arbusov-type transformation

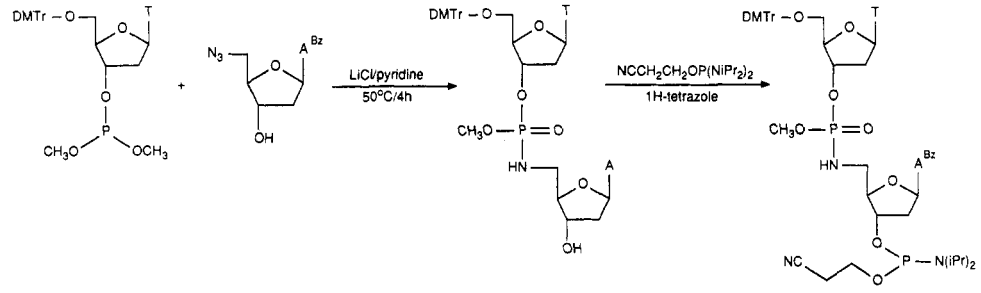

Figure 22. Synthesis of bridged phosphoramidates.

into the phosphoramidate, whose rate is increased by LiCl (Figure 22). These dimers can be converted into amidite units by phosphitylation and reacted further.[139] Trimeric bridged phosphoramidates were synthesized, based on the principle of the azide reaction, for the first time by Letsinger.[116] He was also able to prepare tetramers by a modified triester method.[140]

These compounds can be synthesized not only chemically but also enzymatically. Once again, it was Letsinger[141] who, in a template-controlled polymerase reaction, was able to incorporate 5′-amino-5′-deoxythymidine phosphate via the triphosphate into a DNA fragment. Also worthy of mention is the work by Orgel[142,143] and Shabarova,[144] who were able, likewise in a template-controlled reaction, although without enzyme, to bring about the self-condensation of activated 3′-amino-3′-deoxynucleosides (as 5′-phosphorimidazolides).

The chemical property of the bridged phosphoramidates that must be emphasized is their sensitivity to acids.[145] In 80% acetic acid at room temperature, all the phosphoramidate linkages are cleaved in 6 h, whereas unmodified DNA is not attacked.[137] This can be utilized for cleaving DNA fragments at specific positions under mildly acidic conditions.

(*b*) *Bridged Phosphorothioates.* Although the synthons for 3′-*O*-5′-*S*-thiophosphate oligodeoxynucleotides, namely, the 5′-(*S*-(triphenylmethyl)-thio)-2′,5′-dideoxyribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidites), can be obtained[146] (Figure 21), these have been incorporated only at the 5′ end of oligodeoxynucleotides.[146] Cosstick and Vyle[147] used a 5′-(monomethoxytrityl)thymidine 3-*S*-thiophosphoramidite to prepare oligonucleotides containing 3′-thiothymidine on a solid support. The intermediate thiophosphites were efficiently oxidized with tetrabutylammonium periodate.

(*c*) *Bridged Methylenephosphonates.* Another modification comprises replacement of one of the oxygen atoms in the bridge by a methylene group. However, this has been achieved to date only in dimers. Thus, Morr et al.[148,149] were able to synthesize the 3′-methylenephosphonate analogues of d(GC) in 80% yield by a method based on triester chemistry (Figure 23).

C. "Dephospho" Internucleotide Analogues

An obvious way of obtaining nonionic oligodeoxynucleotides is to replace the phosphodiester bridge by an entirely different group. Of course, a plethora of replacements is conceivable, but some structural criteria have to be met to ensure that there is still efficient hybridization with the target sequence. This is why the

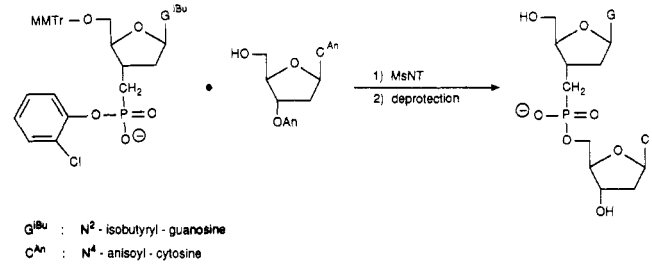

Figure 23. Synthesis of a methylene-bridged phosphonate dinucleoside.

studies described below are primarily aimed at replacement groups that do not change the natural 5′–3′ distance to drastically.

1. Siloxane Bridges

The structures of phosphate and siloxane bridges are very similar because in both cases the central atoms have tetrahedral geometry. Synthesis was initially confined to dimers. This entailed 5′-protected nucleosides being reacted with dichlorodiphenylsilane[150] or dichlorodimethyl- or dichlorodicyclohexylsilane[117] in pyridine and the resulting intermediate being trapped in situ with 3′-protected nucleosides. This resulted in the 3′,5′ dimers in ca. 50% yield. A major problem was the formation of almost 30% of the symmetrical 3′,3′ dimers. On the other hand, the yield of the 3′,5′ compound was considerably increased with imidazole in DMF. Thus, Ogilvie[152] obtained almost 80% 3′,5′ dimers with di-*tert*-butyldichlorosilane and 74% with dichlorodiisopropylsilane and bis((trifluoromethyl)-sulfonyl)diisopropylsilane. He was able to synthesize hexamers using suitable protective groups such as 5′-*O*-dimethoxytrityl and 3′-*O*-levuloyl (the siloxane bridge is sensitive to both bases and acids) (Figure 24).

2. Carbonate Bridges

Oligodeoxynucleotides with carbonate bridges have been described up to the trimers.[153] Whereas phosgene was initially used for the condensation,[154] this was subsequently replaced by activated carbonic esters, with (trichloroethoxy)carbonyl chloride proving most suitable.[153,155] The coupling yields were, however, only ca. 50% because only inadequately protected bases could be employed. It proved to be extremely difficult to remove protective groups in the presence of the carbonic diester.

3. Carboxymethyl Ester Bridges

Polynucleotide analogues (poly-U and poly-T) with carboxyl ester bridges were synthesized as early as 1968 by Jones,[156,157] whose assumption was that these would

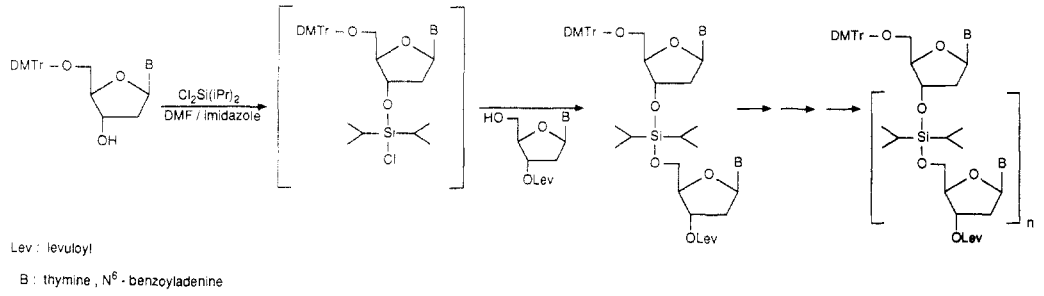

Figure 24. Preparation of siloxane-bridged oligonucleotide analogues.

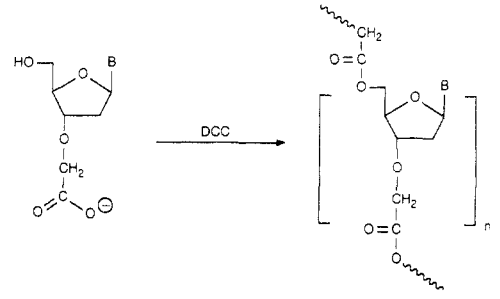

Figure 25. Preparation of poly(3′-*O*-carboxymethyl-2′-deoxynucleoside).

be flexible enough to keep the nucleotide units apart at their natural distance. The synthesis was carried out by polymerization of 3′-*O*-(carboxymethyl)thymidine in pyridine with dicyclohexylcarbodiimide (DCC) and the 5′-O-protected unit as terminator. In 1975 he prepared poly-dA[158] and poly-dC[159] analogues in the same way, with protection being unnecessary for adenine but necessary for cytosine as the 4-*N*-phenoxyacetamide (Figure 25). The resulting oligomers had a mean chain length of 13 bases.[157] In 1971 Jones[159] replaced the polymerization reaction by a stepwise synthesis using suitable protective groups that could be eliminated under nonbasic or only mildly basic conditions because of the sensitivity of the carboxymethyl group to bases (the half-life at pH 7.5 is 7 h[158]). The 5′-OH group was protected with dimethoxytrityl, 2′- and 3′-hydroxyl groups were blocked as isopropylidene and anisylidene compounds, and the carboxyl group was in the form of the 2-cyanoethyl ester. Cytosine required 4-*N*-(dimethylamino)ethylene protection, while the other bases were not acetylated under the reaction conditions. He was able to perform specific syntheses of dimers,[161] trimers,[160] and tetramers.[162]

4. Acetamidate Bridges

Oligodeoxynucleotide analogues with carboxymethyl bridges have two disadvantages: the bridge is unstable under physiological conditions, and the solubility of the compounds is low, which makes it difficult to test their biological activity. It appears possible to solve both problems by replacing the 5′-ester oxygen by an amide, and the acetamidate bridge ought also to provide a suitable internucleotide distance. The dimers were synthesized, in exact analogy to the preparation of the carboxymethyl esters, by condensation of 3′-*O*-(carboxymethyl)-5′-*O*-tritylthymidine with 5′-amino-5′-deoxythymidine and DCC.[163] However, polymerization proved to be far more difficult: condensation of 5′-amino-5′-deoxy-3′-*O*-(carboxymethyl)thymidine with DCC yielded no polymer, only the 3′,5′-bridged lactam as the main product[163] (Figure 26). Polymerization of

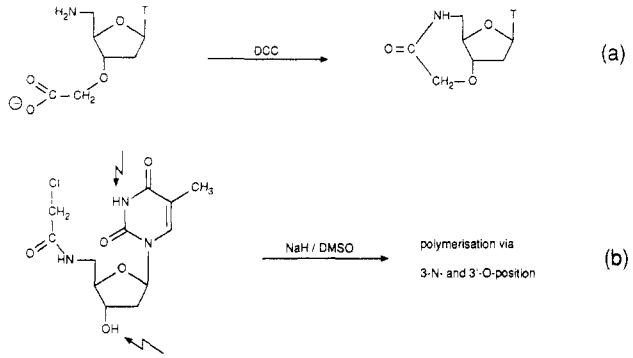

Figure 26. Unsuccessful attempts to prepare acetamidate-bridged polythymidine.

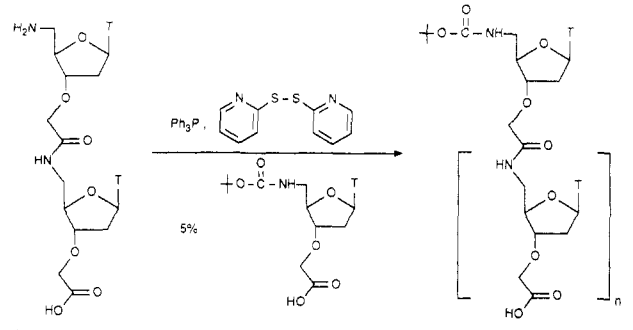

Figure 27. Preparation of acetamidate-bridged oligonucleotides.

5′-(chloroacetamidyl)-5′-deoxythymidine with sodium hydride in DMSO was equally unsuccessful, because alkylation took place not only at the 3′-O but also at the N^3 position of thymidine[164] (Figure 26). On the other hand, when the corresponding 5′-(chloroacetamidyl)-5′-deoxy-4-*N*-acylcytidine was employed, polymerization was successful, but most of the product was lost on deprotection of the base, so that the overall yield was only ca. 1%. Because the lactam formation mentioned above was certainly due to the favorable entropy for intramolecular ring closure, Jones[164] successfully used an appropriate dimer for the polymerization with the aid of triphenylphosphine and 2,2′-dipyridyl disulfide (Figure 27). It was possible to isolate a 54% yield of the highly disperse polymer, whose mean chain length was reported as 10–13. Although the acetamidate bridge is stable over a somewhat larger pH range, unfortunately other desired properties such as stability in water and good hybridization characteristics were not obtained.[163,164] An additional difficulty in the investigations was that the polymer was also adsorbed onto glass and plastic surfaces.

5. Carbamate Bridges

Carbamate bridges were supposed, like the acetamidate bridges, to be superior to the carbonate ana-

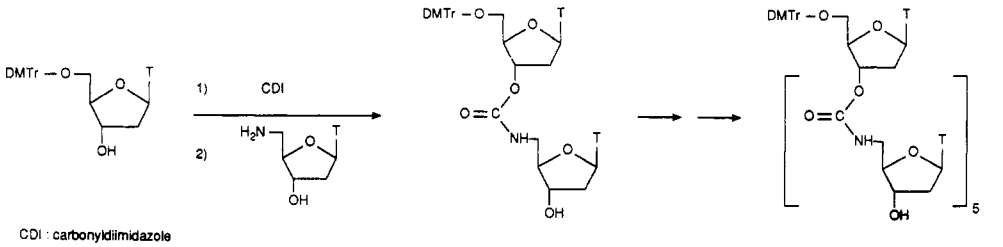

Figure 28. Synthesis of carbamate-bridged oligonucleotides.

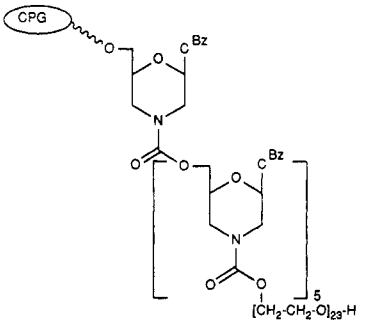

Figure 29. Carbamate-bridged oligonucleotide analogues of the morpholine type.

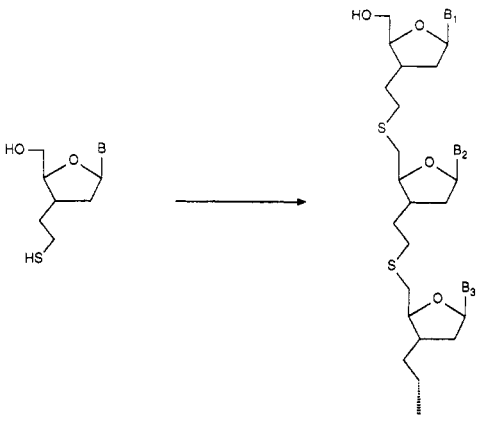

Figure 30. Thioether-bridged oligonucleotide analogues.

logues by having greater pH stability. The first 3′-*O*-5′-*N*-carbamate-bridged dimer of thymidine was synthesized in 1975 by Jones, who obtained it in 38% yield by condensation of the 3′-*O*-(2,2,2-trichloroethyl) carbonate of 5′-*O*-tritylthymidine with 5′-amino-5′-deoxythymidine.[163] Mungall and Kaiser described, in 1977,[165] the synthesis of a trimer in which they used *p*-nitrophenyl esters in place of the trichloroethyl ester. The dinucleoside carbonate that was initially obtained was derivatized with *p*-nitrophenyl chloroformate and again condensed with 5′-amino-5′-deoxythymidine. Not until 1987 (10 years later) was the synthesis of a hexamer described by Coull et al.[166] (Figure 28). They used for this 1,1′-carbonyldiimidazole (CDI) as carbonyl synthon. 5′-*O*-(Dimethoxytrityl)thymidine was alternately reacted with CDI in THF and then, after workup, with 5′-amino-5′-deoxythymidine in pyridine. The hexamer was obtained in 40% yield. Like the compounds mentioned above, its solubility in water as well as in organic solvents is low, and it adsorbs onto glass surfaces. It, like the acetamidate analogues, does not enter into base pairing with the natural compounds.[166] The work of Stirchak et al.[167a] contrasts with these results. They synthesized a corresponding cytosine oligomer from dimer blocks which they condensed with bis(4-nitrophenyl) carbonate in DMF/triethylamine. The final study to be mentioned here is one by Summerton[167b,c] which shows the way to an entirely new class of compounds. Ribonucleosides were used as the starting material for the synthesis of *N*-trityl-6′-(*p*-nitrophenyl) carbonate protected monomer units of the morpholine type. Coupling in DMF without catalyst was applied several times and resulted in the formation of polycarbamate compounds. The solubility of the product in water was improved by terminal conjugation via a carbamate with polyethylene glycol (Figure 29).

6. *Thioether Bridges*

The synthesis of thioether-bridged oligodeoxynucleotides as neutral, nonhydrolyzable analogues from 3′-deoxy-3′-(2-mercaptoethyl)nucleoside synthons was reported by Kawai and Just[168] (Figure 30), but no experimental details were given.

7. *"Plastic" DNA*

Besides replacement of the phosphate group, there is also the possibility of replacing sugar and phosphate residues by a synthetic polymer and thus obtaining "plastic" DNA with new and interesting properties. A large number of such analogues have already been prepared. These include poly(*N*-vinyl), poly(meth-

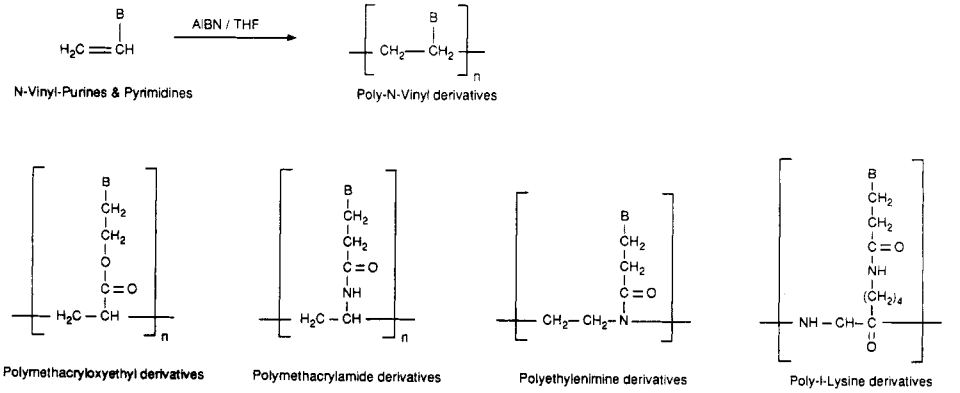

Figure 31. Examples of "plastic" DNA.

acryloxyethyl), poly(methacrylamide), poly(ethylenimine), and poly(amino acid) derivatives, to mention just a few (Figure 31). The synthesis and use of these compounds have been reviewed in several excellent articles, some of recent date.[169-171] Hence, only the poly(*N*-vinyl) derivatives are to be dealt with by way of example at this point, these having been synthesized and very carefully tested for their biological and antiviral activity by Pitha.[171] They were prepared from the *N*-vinyl monomers[172] by radical polymerization in DMSO,[173] DMF,[174] or THF.[175] It was possible in this way to obtain poly(1-vinyluracil), -(9-vinyladenine), -(1-vinylcytosine), and -(9-vinylhypoxanthine). The solubility in water of all of them was poor in the neutral range but good at basic pH. The mean chain length was about 300,[175] so it is appropriate to refer to these molecules as polymers.

D. Oligodeoxynucleotides with Modified Nucleoside Units

1. α-Anomeric Nucleoside Units

In virtually all naturally occurring nucleosides and nucleotides the glycosidic linkage is in its β-anomeric form. On the basis of Dreiding stereomodels Sequin[176a] postulated that the secondary structure of oligo-α-deoxynucleotides based on the β-DNA structure ought to resemble that of the natural β-anomeric DNA. He also predicted that such α-anomeric oliodeoxynucleotides ought to form a helix with a complementary β strand, and the two strands ought to have parallel polarity. Both postulates were later confirmed by Imbach.[176b] The first synthesis of α-anomeric dimers was in 1973 by Holy[177] (βU-αU, βG-αU). α-Anomeric nucleosides were prepared first by Robins[178] in 1969 and subsequently by others.[179] In 1974 Sequin[180] published the synthesis of the four isomeric dithymidine monophosphates βT-βT, αT-βT, βT-αT, and αT-αT, which he obtained by phosphotriester chemistry. The α-anomers proved to be astonishingly stable to nucleases, and Imbach's group took up this modification to use it in antisense oligonucleotides.[181] They synthesized hexameric α-anomeric oligodeoxynucleotides, initially consisting only of αdC and αT,[182] but then from all four α-anomeric deoxynucleotides,[183] using the classical triester method. The customary protective groups were employed, except that guanosine was used as 2-*N*-palmitoyl-6-*O*-(diphenylcarbamoyl)-α-2′-deoxyguanosine. No particular differences from the chemistry of the β-anomers were noticed during the synthesis. The coupling yields were between 80 and 99%. The hexamer consisting of αdC and αT was characterized by ^{1}H NMR spectroscopy,[182] from which it emerged that both thymine and cytosine assume an anti conformation and that the preferred conformation at the sugar is $C_{3'}$-endo (Figure 32). However, the ^{1}H NMR data did not allow the polarity of a hybrid with the complementary oligo-β-deoxynucleotide to be determined. Later on[184] it was possible to show by ^{1}H NMR that an α,β hybrid had parallel polarity. Subsequent investigations on oligo-α-deoxynucleotides with all four bases[185] confirmed that they are present in the anti conformation, but the ring conformation of the sugar was $C_{3'}$-exo. The oligo-α-deoxynucleotides displayed the expected stability toward phosphodiesterases

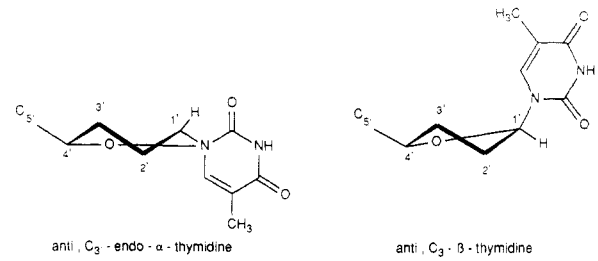

Figure 32. Preferential conformations of α- and β-thymidine.

both from snake venom and from calf spleen. Finally, in 1988 Imbach et al.[186,187] reported the successful solid-phase synthesis of α-anomeric oligodeoxynucleotides by the amidite method. This synthesis was completely analogous to that of the β-anomers except for the use of the 2-*N*-palmitoyl in place of the 2-*N*-isobutyryl protective group on deoxyguanosine. Thereby, α-deoxynucleoside 3′-*O*-((methyl) diisopropylphosphoramidites) were used. The mean coupling yield for a 12-min synthesis cycle was 99% for a pentadecamer and 98.3% for an eicosamer. A similar approach has been reported by Thuong et al.,[188] but they used cyanoethyl-protected phosphoramidites. Imbach[189] and Thuong and Hélène[188,190-193] have reported the synthesis of α-anomeric oligodeoxynucleotides and their linkage with intercalating agents and photo-cross-linkers. This is dealt with in more detail in section II.F.

2. Base-Modified Oligodeoxynucleotides

Apart from the natural bases adenine, guanine, cytosine, and thymine, also incorporated in oligodeoxynucleotides have been other, less common or even purely synthetic bases. These efforts were usually aimed at testing the effect of other modifications, such as methylation of bases, on the action of DNA-modifying enzymes, examining alterations in hybridization characteristics, or even introducing, via reactive groups, covalent bonds between hybridizing DNA strands. In particular, interest has been directed at nucleosides that hybridize nonspecifically, such as inosine, which is located in the "wobble" position of the anticodon in some transfer RNAs. DNA probes with base modifications of this type can be used as an alternative to oligonucleotide mixtures for locating sequences that are ambiguous owing to the degeneracy of the genetic code. Deoxyinosine (Figure 33) has been incorporated in oligodeoxynucleotides for this reason. This was carried out both by the amidite method[194] and by the phosphotriester methods.[195,196] Hypoxanthine, the base of inosine, needs no protection during this. Seela succeeded in synthesizing some isosteric purine 2′-deoxyfuranoside analogues such as 2′-deoxytubercidin and 8-aza-7-deaza-2′-deoxyadenosine (Figure 33) and in incorporating them into oligonucleotides via the 3′-phosphoramidites.[197,198] The amidite method has also been used to introduce 2′-deoxynebularine and 2′-deoxyxanthosine (Figure 33) into oligonucleotides,[199] it being necessary to protect deoxyxanthosine with the 6-*O*-(2-(*p*-nitrophenyl)ethyl) group which was introduced by Pfleiderer[200] into nucleoside chemistry. These nucleosides were also intended to act as nonspecific hybridization partners.

Enzymatic incorporation of base-modified nucleosides into oligodeoxynucleotides is also possible, such as the incorporation of 2-amino-2′-deoxyadenosine (Figure 33)

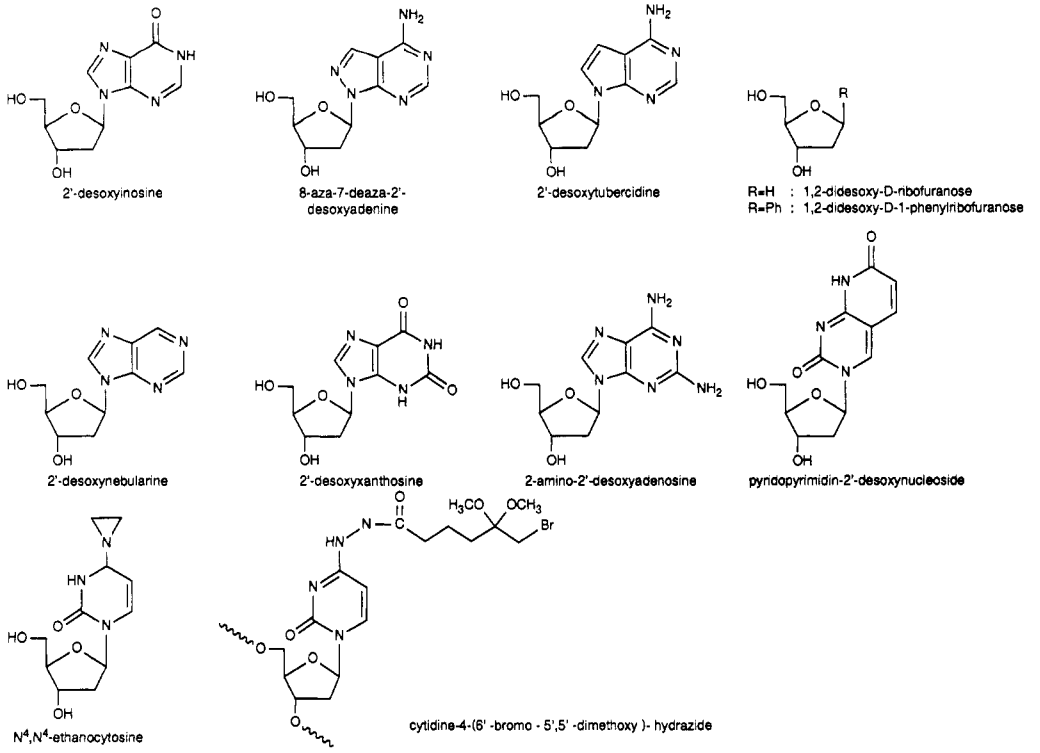

Figure 33. Base-modified nucleosides.

via the triphosphate in a reaction catalyzed by Klenow polymerase.[201,202] However, the modifications have not been confined to isosteric compounds, and in some cases drastic alterations have been made. Millican et al.[203] used 1,2-dideoxy-D-ribofuranose and 1,2-dideoxy-1-phenylribofuranose units (Figure 33), which they introduced into oligonucleotides via phosphotriester synthesis. As expected, the duplexes are less stable with such compounds than those between completely complementary strands. Interesting properties are also shown by the fluorescent 3-(β-D-2′-deoxyribofuranosyl)-2,7-dioxypyrido[2,3-*d*]pyrimidine (pyridopyrimidinedeoxynucleoside) incorporated likewise by phosphotriester chemistry into oligodeoxynucleotides by Ohtsuka[204] (Figure 33). Whereas all the previously mentioned nucleoside analogues could be used in the synthesis without altering the customary reaction conditions, particular care was necessary with the deprotection of oligonucleotides containing pyridopyrimidine (NH_4OH, room temperature, 24 h).

Also of interest for use in antisense oligonucleotides are units that have groups able to react with the target sequence. Ideally, this reaction ought to take place only after the hybridization has occurred. Matteucci and Webb achieved such "hybridization-triggered cross-linking" by incorporating N^4,N^4-ethano-5-methylcytosine (C^e) (Figure 33) via its 3′-phosphoramidite.[205] However, because C^e is not stable to the normal deprotection conditions (NH_4OH, 60 °C, 5 h), 9-fluorenylmethoxycarbonyl was used to protect the exocyclic amino groups, since it can be eliminated under mild conditions (0.5 M DBU, 18 h, room temperature). It was possible in this way to incorporate C^e at any desired point in 20-mers. A modification of this method is reported by Benkovic et al.[206] They prepared the 4-triazole derivative of 5′-(dimethoxytrityl)thymidine, which they incorporated into oligonucleotides and then reacted it with aziridine to N^4,N^4-ethanol-5-methylcytosine (C^e). A similar approach was taken by Bartlett and Summerton,[207] who introduced 6-bromo-5,5-dimethoxyhexanohydrazide in the C^4 position of cytidine, which then became able to alkylate guanosine and thus to cross-link.

Finally, mention may be made of the synthesis of *O*-methyl- and *O*-alkyl-modified bases and their incorporation in oligodeoxynucleotides.[208,209] This was carried out without special precautionary measures in the usual synthesis cycles. These compounds are mainly used for mechanistic studies.

E. Oligoribonucleotides and 2′-Modified Derivatives

1. Oligoribonucleotides

The methods for synthesizing oligoribonucleotides, which originated in the 1960s,[210] have been reviewed by Ohtsuka and by Reese,[211] and thus only a few recent developments will be discussed here. The main problem in the synthesis is in finding a suitable protective group for the 2′-hydroxyl group, which must be compatible with that in the 5′ position. The 2′ protective group must be stable under the conditions of synthesis, especially when the 5′ protective group is removed, and must be amenable to elimination at the end in such a way that there is no possibility of isomerization or cleavage of the phosphate diester bridge.

The usual procedure[212,213] of using two protective groups with different acid stabilities has recently been refined by Reese,[214] who used the 1-(2-chloro-4-methylphenyl)4-methoxy-4-piperidinyl group (Ctmp) to protect the 2′ position (Figure 34). The 9-phenyl-9-xanthenyl group (pixyl) was introduced in the 5′ position, being somewhat more labile than the 4,4′-dimethoxytrityl group. The 2′-acetal protective group is completely stable under the conditions necessary to eliminate the pixyl group, but can itself be easily re-

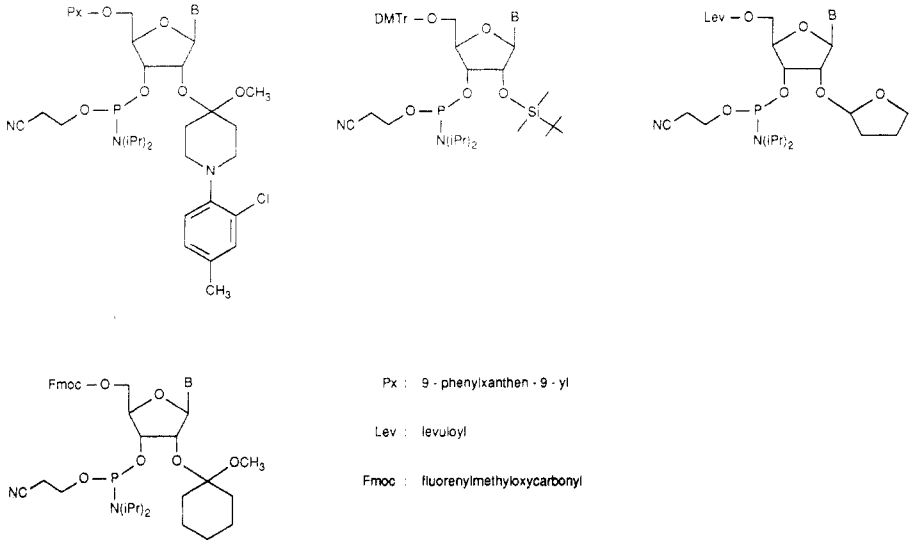

Figure 34. Synthons for the solid-phase synthesis of oligoribonucleotides with various protective groups.

moved at pH 2 in water. Reese was able, using amidite chemistry, which has also become widely used in RNA synthesis, to prepare 19-mers with mean coupling yields of 95%.[213]

Another approach is the use of *o*-nitrobenzyl as the 2′ protective group, which can be removed by photolysis at pH 3.5. In combination with acid-labile 5′ protective groups it has been employed for oligoribonucleotide synthesis by the amidite methods,[215] the H-phosphonate methods,[216] and the phosphate triester methods.[217] Ogilvie[218] used the *tert*-butyldimethylsilyl group, which can be cleaved with fluoride ions, to protect the 2′ position with great success. Thus, he was able to synthesize a 77-mer by the amidite method. Finally, mention may be made of two interesting developments: protection of the 2′ position with acid-labile groups such as the tetrahydrofuranyl and 4-methoxytetrahydropyranyl groups and the use of the levuloyl group in the 5′ position, which can easily be removed by hydrazinolysis[219] (mean coupling yield/synthesis cycle ca. 90%), and the Fmoc protective group,[220] which can be removed under mild basic conditions with 0.1 M DBU in acetonitrile (mean yield/synthesis cycle ca. 96%) (Figure 34). Under these conditions there is probably some elimination of cyanoethyl groups, which results in side reactions.

Another important aspect of the synthesis of oligoribonucleotides is the use of enzymes. A rapid and efficient method for small amounts of unmodified oligoribonucleotides is in vitro transcription using SP6 or T7 RNA polymerase and synthetic DNA.[221] Small segments can be linked together using T4 RNA ligase,[222] the smallest substrates being nucleoside 3′,5′-diphosphates. This method enabled relatively large RNA fragments to be synthesized quite early on.[211]

2. 2′-Modified Oligoribonucleotides

The instability of oligoribonucleotides under physiological conditions led to the synthesis of their 2′-modified analogues. These modifications were confined to the 2′-*O*-methyl derivatives, which also occur naturally and whose monomers were synthesized in 1965 by Furukawa et al.[223] This class of compounds is attractive for antisense applications because ribonucleosides are much less costly as starting materials than their deoxy analogues and because the thermal stability of the hybrids of 2′-*O*-methylribonucleotides with complementary RNA is much greater than that of corresponding DNA–RNA duplexes.[224] The synthesis of 2′-*O*-methylribonucleotides and oligodeoxynucleotides differ only slightly. The former can be prepared just as well by the phosphotriester methods,[224] the amidite methods,[225] and the H-phosphonate methods,[226] the only limiting factor being the availability of monomeric units. Nor did the coupling yield differ in any case from that of the deoxy analogues, although the use of bulky protective groups, such as the *tert*-butyldimethylsilyl group, in the 2′-*O* position results in a drastic reduction in the yields.[228] Sproat et al.[227] employed 5-(4-nitrophenyl)-1*H*-tetrazole (0.1 M in acetonitrile) as activator in the amidite method and achieved yields of >99% with coupling times of 6 min, whereas this time was far too short with 0.5 M 1*H*-tetrazole alone. We have had good results with the protocol of Pon.[229]

The synthesis of 2′-*O*-methylribonucleotide phosphorothioates to inhibit HIV, via corresponding H-phosphonates, has been described and is entirely analogous to the deoxynucleoside phosphorothioates.[226]

F. Oligodeoxynucleotide Conjugates

Oligodeoxynucleotides can be covalently linked to a wide variety of molecules. In the context of antisense technology, this has usually been done to improve transport and hybridization properties of the oligonucleotides. Conversely, conjugates can also be regarded in such a way as to introduce a specificity into otherwise unspecific DNA-binding molecules by covalently linking them to a selectively hybridizing oligonucleotide. In the following, conjugates are differentiated according to the position of their linkage to oligonucleotides and according to their function. This covalent linkage is usually brought about at a 5′ end with phosphorylating reagents and at a 3′ end via modified linkers.

1. Conjugation via the 5′ End

Covalent linkage from the 5′ end of oligodeoxynucleotides is particularly attractive because it can be carried out in the solid-phase synthesis in a similar

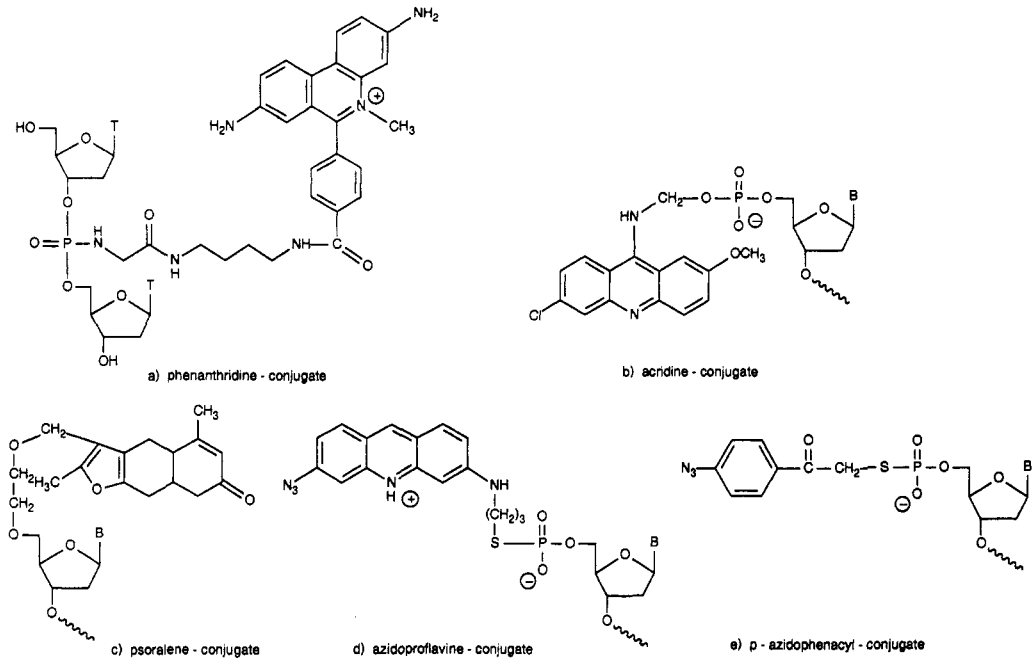

Figure 35. Conjugates of oligonucleotides with intercalating agents and cross-linkers.

manner to the 5′-phosphorylation of oligonucleotides that we have described[32] provided that the group which is to be linked can be derivatized as a phosphoramidite, H-phosphonate, or phosphate and withstands the coupling and deprotection steps.

(*a*) *Intercalating Agents.* The idea of linking an intercalating compound to a "recognition sequence", namely, an oligonucleotide, was described first by Letsinger[230] but was achieved only for a dithymidylate linked via a phosphoramidate bridge to phenanthridine (Figure 35). There have been a number of papers in which phosphotriester or amidite chemistry has been used for covalent attachment of intercalating agents, usually acridine derivatives, to the 5′ end of modified and unmodified oligodeoxynucleotides.[231-234] Thus, 2-methoxy-6-chloro-9-((5-hydroxypentyl)amino)acridine has been linked via its phosphoramidite to oligodeoxynucleotides, oligodeoxynucleotide methylphosphonates, and α-anomeric oligodeoxynucleotides[188,235,236] (Figure 35). The same intercalating agent had previously been incorporated by using phosphotriester chemistry.[237,238] The work of Jäger et al. should be mentioned once again in this connection; they replaced the I_2/H_2O oxidation in the amidite method by an I_2/1,5-diaminopentane oxidation and thus were able to introduce an alkylamino linker via the resulting phosphoramidate and, via the former by alkylation, an acridine derivative at any desired point in the molecule.[113]

(*b*) *Cross-Linkers.* The introduction of cross-linkers into oligodeoxynucleotides via modified bases has been discussed in a previous section (II.D). Cross-linkers can also be linked to the 5′ position by a variety of methods.[232-234] Thus, psoralen derivatives (Figure 35) have been incorporated in oligonucleotides and oligonucleotide methylphosphonates via amidite chemistry[239] and phosphotriester chemistry.[240] Hélène et al. introduced azidophenacyl[241,243] (Figure 35), proflavine,[242] and azidoproflavine[190] (Figure 35) to the 5′ position by first converting the oligonucleotide into the 5′-thiophosphate, which could then be alkylated via a bridge

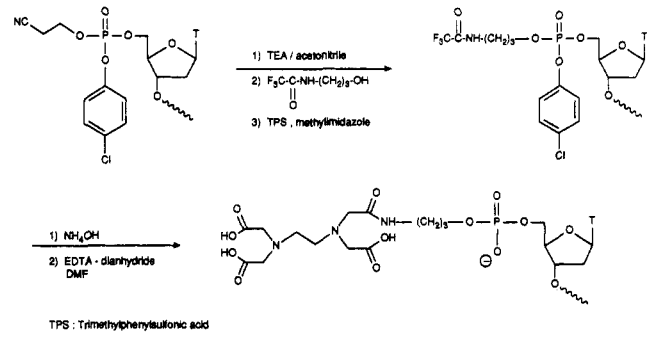

Figure 36. EDTA derivatization of oligonucleotides.

with the particular cross-linker (3-azido-6-((3-bromopropyl)amino)acridine[190] or *p*-azidophenacyl bromide[241,243]).

(*c*) *Artificial Endonucleases.* The term "artificial endonucleases" is intended to comprise those conjugates whose nuclease component is able as such to cleave DNA nonspecifically and acquires a specificity by covalent linkage to the oligonucleotides. This class of compounds includes, in particular, metal complexes such as EDTA–Fe(II), *o*-phenanthroline–Cu(I), or porphyrin–Fe(II), but nonspecific nucleases have also been linked to oligonucleotides for this purpose.

Boutorin et al.[244] derivatized an oligothymidylate with EDTA by first introducing an alkylamino group by condensation with aminopropanol and then, after deprotection, acylating it with EDTA dianhydride (Figure 36). Nuclease activity (local production of hydroxyl radicals) is achieved by incubation with Fe(II). Orgel et al. followed a similar procedure but they used ethylenediamine as linker in place of aminopropanol.[245,246] Ethylenediamino bridges have also been employed by Miller and Ts'O for the EDTA derivatization of oligodeoxynucleotide methylphosphonates.[247]

The work of Dervan should also be quoted at this point: he achieved EDTA linkage by modification of thymine in the 5 position.[248,249] This entailed thymidine-5-propionic acid 2-aminoethylamide being synthesized first and then acylated with EDTA. The ad-

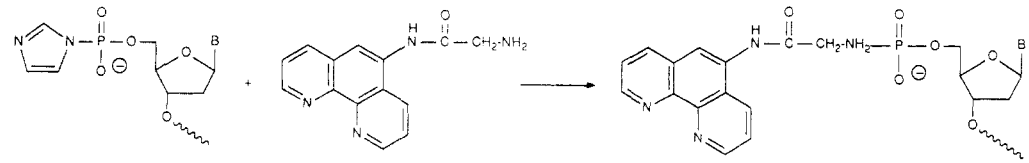

Figure 37. Phenanthroline derivatization of oligonucleotides.

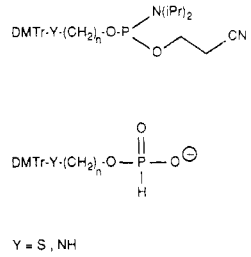

Figure 38. Synthons for the amino- and thiofunctionalization of oligonucleotides.

vantage of this synthon is that it can be incorporated at any desired point in the oligonucleotide.

The conjugation with phenanthroline was carried out in a similar manner to the EDTA derivatization. Chen and Sigman[250] converted their 5′-phosphorylated oligonucleotide initially into the imidazolide, which they condensed with 5-(glycylamido)-1,10-phenanthroline (Figure 37). Hélène et al.[251] chose derivatization via a 5-hexamethylene–thiophosphate linker which was alkylated with 5-(iodoacetamido)-1,10-phenanthroline.

Derivatization with porphyrins and enzymes in the 3′ position is described in section II.F.2.

(*d*) *Lipophilic Carrier and Peptide Conjugates.* The transport of oligodeoxynucleotides into cells has been facilitated by linking them to lipophilic carriers and peptides. Goodchild et al.[252a] described the incorporation of long-chain alcohols as phosphate esters in the 5′ position of oligonucleotides. This can take place at the end of the solid-phase synthesis by use of an appropriate phoshoramidite. Several groups have now synthesized cholesterol conjugates of oligonucleotides;[253] however only one example has been reported in the literature up to now by Letsinger et al.[252b]

Universal methods for functionalizing oligonucleotides with amino or mercapto groups have been described by Sinha and by Connolly,[254] who used H-phosphonate and amidite chemistry to attach aminoalkyl and mercaptoalkyl linkers to the 5′ end (Figure 38). A large number of derivatives, such as dyes or other nonradioactive markers, could be introduced via these linkers. Simple linkage with polylysine via a maleimide has also been described. Polylysine is intended as a carrier for oligodeoxynucleotides.[255]

The synthesis of nucleopeptides via the phosphate esters of the side chains of tyrosine and serine has been described by van Boom.[256] Because of the lability of the serinyl–nucleotidyl phosphodiester bond to bases, special protective groups are required for the exocyclic amino groups. Van Boom used the di-*n*-butylformamidino and the 2-nitrosulfenyl groups, which can be eliminated under milder conditions. Diribonucleosidephosphoro(P–N)amino acid derivatives were first described by Juodka and Smrt[119] via the synthesis of phosphoramidates.

2. Conjugation via the 3′ End

(*a*) *Intercalating Agents.* The synthesis of conjugates of intercalating agents with oligonucleotides via the 3′ position has been extensively investigated by Hélène et al. The intercalating agent mostly used was 2-methoxy-6-chloroacridine, which has already been described. Whereas 5′ derivatization is primarily carried out in solid-phase synthesis using amidite chemistry, that in the 3′ position mainly employes triester chemistry (Figure 39).[257-262] 3′ conjugation of modified oligonucleotides such as methylphosphonates and methyl esters[263] has also been described. Gautier et al.[189] have reported the linkage of α-anomeric oligonucleotides with oxazolopyridocarbazole via the 3′ end using an aminoalkyl linker.

(*b*) *Alkylating Oligonucleotides.* The idea of specific and irreversible modification of DNA by cross-linking was put into practice as much as 20 years ago by Grineva et al.[264] They used for this a diribonucleotide that was functionalized at the 3′ end via an acetal linkage with an aromatic (2-chloroethyl)amino group (Figure 40). The neighboring group participation of the nitrogen means that the (chloroethyl)amino groups are particularly susceptible to alkylation and thus cross-linking (cf. mustard gas). This mode of substitution was subsequently extended to oligonucleotides with a 3′-ribonucleoside residue and has been widely used in antisense technology.[265-269] The alkylation reaction appears to be "hybridization triggered" (cf. section II.E.2), because no self-alkylation takes place. Alkylating groups have also been attached in the 5′ position via phosphoramidate linkages (Figure 40).[266]

(*c*) *Artificial Endonucleases and Enzyme Conjugates.* Of course, it is also possible to attach DNA-cleaving groups at the 3′ end of oligonucleotides, although but few examples of this are known. Hélène et

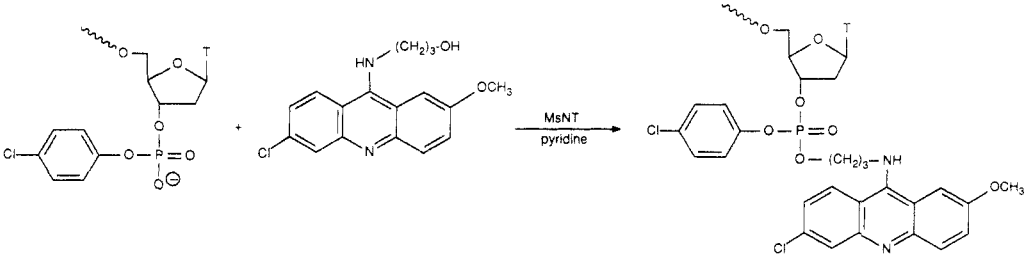

Figure 39. 3′-Derivatization with intercalating agents using phosphotriester chemistry.

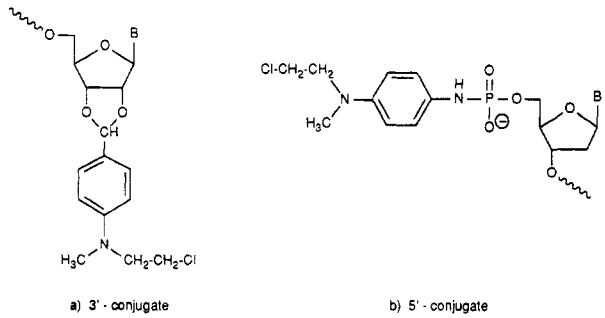

Figure 40. Alkylating oligonucleotides.

al.[270,271] carried out porphyrin derivatization of oligonucleotides in the 3′ position. This entailed a methylpyrroporphyrin ethyl ester being initially reacted with amino-1-hexanol as linker and then coupled using triester chemistry to the 3′ position of an oligothymidylate (dT_7). After metalation with Fe(II) the conjugate displayed nuclease activity.

Oligonucleotide–enzyme conjugates have also been synthesized with a view to converting nonspecific nucleases into specific endonucleases by attaching a selectively binding oligonucleotide. Two examples of this type of modification are known from the literature. Schultz et al.[272,273] succeeded in covalently bonding staphylococcal nuclease to a 15-mer oligonucleotide. This was performed very elegantly via a disulfide exchange. First a 3′-*S*-thiopyridyl oligonucleotide was synthesized, and a lysine was replaced by a cysteine in the nuclease by site-specific mutagenesis, which produced a unique free mercapto group. The two molecules then reacted to give the desired conjugate (Figure 41). Jablonski et al.[274] synthesized an oligonucleotide conjugate with alkaline phosphatase. Linkage took place via a 2′-deoxyuracil-5-aminoheptyl unit with glutaraldehyde. The enzyme activity was retained in both cases.

(*d*) *Oligonucleotide–Peptide Conjugates.* To improve the transport properties of oligonucleotides Lebleu et al. synthesized oligonucleotide–polylysine conjugates.[255,275,276] Oligonucleotides were first provided with a 3′-terminal ribose unit using T4 RNA ligase. This ribose was oxidized with periodate, condensed with a 6-amino group of polylysine, and reduced with $NaBH_3CN$ to the *N*-morpholine conjugate (Figure 42). It is simpler to synthesize chemically a "chimeric" oligodeoxyribonucleotide that contains at the 3′ end a ribonucleoside, the solid support (controlled pore glass, CPG) being loaded, for example, with 5′-*O*-(dimethoxytrityl)-N^4-benzoylcytosine 2′(3′)-succinate, the free 2′(3′)-OH group being acetylated and then the standard synthesis being carried out.[277]

On the other hand, the successive solid-phase synthesis of oligodeoxynucleotides with a 3′-peptide unit, as described by Haralambidis et al.,[278] is somewhat more general. After the peptide chain has been synthesized on the support by the Fmoc method, a 4-hydroxybutyryl linker is attached, and then the oligodeoxynucleotide synthesis is carried out. The conjugate is deprotected at the end of the solid-phase synthesis.

(*e*) *Terminal Transferase.* Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotides at the 3′ end of DNA molecules.[279] Both single- and double-stranded DNA with at least three phosphate groups and a free 3′-OH end act as substrates. Terminal transferase can be used for 3′-homopolymer tailing and accepts not only the naturally occurring deoxynucleoside triphosphates but also triphosphates with modified bases and biotin-linked bases.[280] The cofactor required for purine bases is Mg^{2+}, and that for pyrimidine bases is Co^{2+}.[281] 3′-Radioactive labeling using ^{32}P-labeled 3′-deoxynucleosides[282] and 2′-deoxyribonucleosides[283] is also possible.

III. Properties of the Antisense Oligodeoxynucleotides and Resultant Problems

For the antisense oligonucleotide principle to be put into practice the nucleic acid derivatives employed must comply with the following requirements:[284-287] (1) The complex formed between the oligonucleotide and its complementary target sequence must be sufficiently stable under physiological conditions. (2) The interaction between the oligonucleotide and its target sequence must be specific. The specificity is determined by the defined base sequence and the resulting interaction with the complementary nucleic acids. (3) The oligonucleotide must have a sufficiently long half-life under in vivo conditions for it to be able to display its desired action in the cell. It must therefore be resistant to enzymes that degrade nucleic acids (nucleases). (4) The oligonucleotide must be able to pass through the cell membranes to reach its site of action. Uniform distribution of the oligonucleotide will normally be desirable; i.e., it should not accumulate either in particular organs such as the liver or in particular cell compartments such as the lysosomes.

Whereas the requirements for specificity and binding affinity are satisfactorily met by the unmodified oligonucleotides, adequate stability to nucleases and sufficient passage through membranes can be achieved only by modification of the oligonucleotides. However, the derivatization often has an adverse effect on specificity and binding affinity. Thus, the development of antisense oligonucleotides involves walking a tightrope, with all four basic requirements needing to be met at all times.

A. Physicochemical Properties

1. The T_M Value

The physicochemical properties are one of the major determinants of the efficiency of antisense oligonucleotides. The affinity of the binding between the oligonucleotide and its target sequence is characterized by the melting temperature T_M of the double-stranded

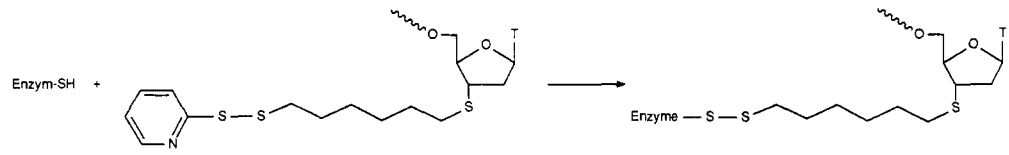

Figure 41. Synthesis of an enzyme–oligonucleotide conjugate by disulfide exchange.

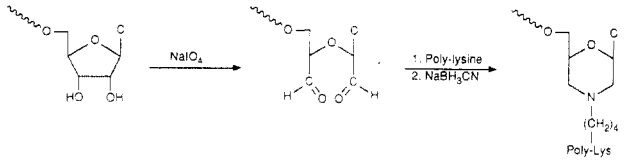

Figure 42. Synthesis of a 3′-polylysine conjugate.

nucleic acid that is formed. T_M is the temperature at which 50% of the double strand has dissociated into its two single strands. T_M depends on the concentration of the oligonucleotide and on the properties of the solvent. To estimate T_M for unmodified oligonucleotides with a length of ca. 12–20 bases the rule of Wallace[26,288] can be used, which holds true for double-stranded DNA with perfect base pairing at high salt concentration

$$T_M = n(2\ °C) + m(4\ °C) \quad (1)$$

where n = number of dA·T base pairs and m = number of dG·dC base pairs.

The stability increases with the proportion of dG·dC base pairs as a result of the three hydrogen bonds formed by this pair. Thus, under physiological conditions (37 °C, low salt concentration), at least 12 base pairs are required to achieve reasonably stable hybridization of the oligonucleotide with the target sequence. A somewhat more accurate prediction of T_M is obtained with the free energy parameters and using the nearest-neighbor model,[289] the accuracy being about 5 °C for oligonucleotides. In practice, a 20-mer oligonucleotide with average base pairing may be assumed to have a T_M of 54 °C (DNA duplex in 0.1 M NaCl).[290] The model of Damle[291] can be used to calculate from T_M the thermodynamic parameters δH and δS for the binding of an oligonucleotide to polynucleotides, as follows

$$1/T_M = \delta S/\delta H + 2.3R/\delta H \log C_M \quad (2)$$

where C_M = concentration of free oligonucleotide at $T = T_M$.

Watson–Crick base pairing permits the formation of double strands between DNA and DNA, DNA and RNA, and RNA and RNA, with the stability of the double strand decreasing in the sequence RNA·RNA > RNA·DNA > DNA·DNA.[224] The extent of double-strand formation can be checked by gel migration analysis[292] because the double-stranded molecule moves different from the corresponding single strands on polyacrylamide gel electrophoresis.

2. Effect of the Internucleotide Phosphate Modification on the Binding Affinity

Modification of an oligonucleotide may result in an increase or decrease in T_M. If the internucleotide group is altered by introduction of a new substituent into the phosphate center, the factors that determine the T_M are (1) the electronic nature (charge), (2) the steric requirement, and (3) the absolute stereochemical arrangement of the substituent. The charge repulsion will be less with uncharged internucleotide linkages (phosphate esters, methylphosphonates, and dephospho compounds) than for the natural phosphodiesters and thus the duplex should be stabilized. There has been some experimental confirmation of this,[292–294] but there are discrepancies with some other investigations.[134,295,296] There are various reasons for this. First T_M depends on the ionic strength of the buffer used.[292] It is an important point that this dependency of the T_M on the ionic strength is found only with the phosphodiester compounds and not with the phosphotriester analogues.[294] Second, the stoichiometry of the hybridization is often different from 1:1 so that the hybrid molecules undergoing dissociation are not always equivalent. Sarin et al. have also reported that the melting curve for oligonucleotide methylphosphonates was broadened when they had been insufficiently purified.[295] The stability of the complexes formed from the nonionic homooligomeric dA methylphosphonates with poly(rU) was greater than that of the corresponding natural phosphodiesters.[294] The stoichiometry of complex formation in this case is 2rU·1dA and 2T·1dA. By contrast, Tidd et al. found a 1:1 complex on sequence-specific hybridization of a 9-mer methylphosphonate with a 20-mer oligodeoxyribonucleotide.[296] However, only a small proportion (10–20%) of the stereoisomers was at all capable of producing this hybrid population with a T_M of 34 °C. Most of the stereoisomers were in dissociated form at 25 °C. The depression of the T_M values was less pronounced in an investigation by Sarin et al. on 20-mer methylphosphonates.[295] Whereas the unmodified 20-mer had a T_M of 55 °C, this was reduced to 53 °C for the same sequence with four phosphonate residues and to 51 °C with 18 phosphonate residues.

When Stein et al. compared the T_M values for phosphorothioates with the unmodified oligonucleotides, they made the interesting observation that A·T pairs depress the T_M values considerably more than do G·C base pairs.[235,297] The position of the phosphorothioate residue in a partially modified oligonucleotide also appears to influence the T_M. A phosphorothioate in the 5′ position in front of a pyrimidine residue lowers T_M more than when in front of a purine residue.[298,332]

The fact that the binding affinity of an antisense oligonucleotide crucially determines its efficiency in a biological assay is evident from the fall in biological activity as the assay temperature increases. Modified antisense oligonucleotides are often more active at 25 °C than at 37 °C.[299–302] The observation that the activity of oligonucleotides increases with their chain length is in the same direction.[290,303–305] This correlation disappears above a particular chain length, which is about 30 bases for natural oligonucleotides. The explanation for this is obvious because the T_M of large natural DNA molecules ranges from 80 to 95 °C, irrespective of their particular length, although it depends on the G·C content, which, in turn, is species dependent.

3. Approaches To Improve the Binding Affinity

Hélène et al. covalently linked intercalating agents to the oligonucleotide with the aim of increasing T_M. Attachment of an acridine residue to the 3′ end of $(T)_{12}$ increased T_M from 33.5 to 47.3 °C.[261] The length of the alkyl chain connecting the acridine to the antisense oligonucleotide had a crucial effect on duplex stability. Another possibility for increasing T_M comprises introduction of modified bases that form more stable Watson–Crick base pairs with the complementary bases owing to additional hydrogen bonds. For example, diaminopurine (DAPu) forms three hydrogen bonds

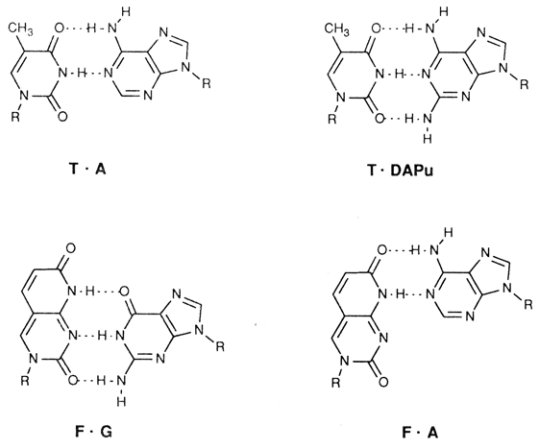

Figure 43. Enhancement of base pairing using modified bases (DAPu = diaminopurine, F = pyridopyrimidine).

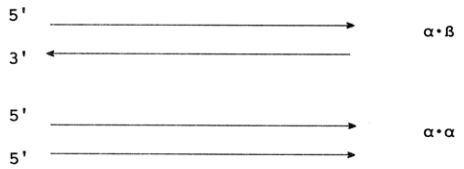

Figure 44. Hybridization of two oligonucleotides in the antiparallel orientation as α·β duplex (top) and in the parallel orientation as α·α duplex (bottom).

with thymine, whereas the natural partner adenine is able to form only two hydrogen bonds.[201] The pyridopyrimidine bases (F) publicized by Inoue et al. similarly pair with guanine more strongly than does natural cytosine and thus stabilize the duplex[204] (Figure 43).

Whereas introduction of a phosphate residue at the 3′ end of the antisense oligonucleotide has no noticeable effect on T_M, the introduction at the 5′ end markedly stabilizes the double helix.[306] The improved base pairing may also be the reason why cleavage of the 5′-phosphates of the duplexes of d(GGAATTCC) with *EcoRI* is about 8 times, and of d(GGTTAACC) with *HpaI* is 30 times, faster compared to that of the corresponding 5′-hydroxy derivatives.[96,307] In this context it is surprising that attachment of relatively large molecules, for example, nucleases, to the 3′ end of oligonucleotides only slightly lowers their T_M values.[308] It is also possible, interestingly, to raise the T_M values by altering the sugar. For example, Inoue et al. have reported a 9-mer 2′-deoxyoligonucleotide to have on hybridization with a complementary oligoribonucleotide a T_M of 41 °C. As an oligoribonucleotide or a 2′-*O*-methyloligoribonucleotide the same sequence shows a T_M of 50 or 54.3 °C, respectively.[224] A drastic positive change in T_M was found by Imbach et al. for oligonucleotides that have the α-anomeric nucleosides incorporated in place of the natural β-anomers.[182,183] For example, a α-d($G_2T_{12}G_2$)·RNA duplex melts at 53 °C, while the corresponding β-anomeric duplex melts at only 27 °C. This surprising effect was explained, on the basis of investigations by NMR spectroscopy, by proposing that hybridization of α-oligonucleotides with β-DNA results in a parallel arrangement of the strands, whereas the natural double helix consists of antiparallel strands.[184,185] (Figure 44). In this case the 2′-deoxyribose unit is in the $C_{3'}$-endo conformation (Figure 32). Remarkably, the B-type DNA is retained even when the strands are arranged in parallel, and the base pairing

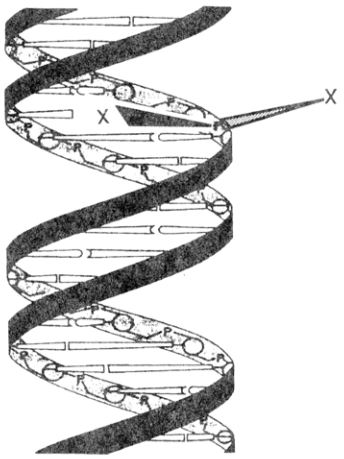

Figure 45. Assignment of the stereochemistry of a phosphate substituent in double-stranded DNA.

is consistent with a Watson–Crick model. The stabilization of the hybridization appears to be less favorable for purine-rich α-anomeric oligonucleotides than for pyrimidine-rich sequences.[309]

Some dephospho modifications lower T_M very greatly. For example, it was impossible to determine T_M for oligonucleotides with diisopropylsiloxane[152] or carbamate[166] bridges. Surprisingly, Summerton reported a dramatic increase in T_M when the carbamates are expanded to a morpholine ring[167b] (Figure 29). For p-$(dC)_6$·p$(dG)_6$, T_M increases from <30 to 64 °C when the natural DNA·DNA duplex is changed to the carbamate·DNA duplex. However, it should be pointed out that homooligomers of guanylate tend to aggregate, which means that T_M determination is no longer possible.[297]

A promising approach is that of Letsinger et al., who used cationic oligonucleotides, which ought to have an increased binding affinity for natural anionic polynucleotides.[122,310] The binding of the positively charged oligonucleotide can be controlled by the salt concentration and the pH. The binding of a cationic 10-mer deteriorates (T_M = 32.5, 27.5, 15 °C) with increasing NaCl concentration (0, 0.1, 1 M) while, conversely, that of the natural oligonucleotide improves (T_M = <10, 22, 38 °C). This effect is desirable because the ionic strength in the cell is low.

B. Stereochemical Problems

Elimination of the negative charge on the phosphate center ought, by its nature, to increase T_M. However, this does not occur in every case. With some phosphate triester oligonucleotides, which are not subject to the charge repulsion of the phosphate diester backbone, the T_M values are greatly lowered. This can be explained by stereochemical considerations. The introduction of a substituent on the phosphorus generates a new center of chirality. In principle, the substituent X that has been introduced can point into the interior of the DNA double helix or outward into the surroundings (Figure 45), and the latter is considerably more sterically favored. Bower et al. used NMR and UV spectroscopy to show, with methylphosphonate oligonucleotides, that DNA duplexes with substituents directed inward into the wide groove are generally less stable than those whose methyl groups project into the solvent.[293] At

various positions in the self-complementary oligonucleotide d(GGAATTCC) a phosphate diester linkage was replaced stereospecifically by a methylphosphonate residue. Although the R_pR_p isomeric duplexes have similar T_M values to the parent molecule, the T_M of the S_pS_p isomers was lowered markedly by ca. 7–11 °C. The steric effect of the methyl group increased with its proximity to the center of the molecule. In the case of the monophosphorothioate oligonucleotide d[GG(S)-AATTCC], LaPlanche et al. found the following sequence of T_M values by comparison with the parent molecule mentioned above: unmodified $\approx S_pS_p > R_pR_p$.[104] In the case of the ethyl phosphotriester d-[GGAA(Et)TTCC], too, the melting temperature of the R_pR_p duplex was ca. 11 °C lower than that of the S_pS_p diastereomer and the parent compound.[134]

However, the situation becomes more complicated when more than one phosphate residue is modified. Modification at n centers will result in 2^n diastereomers. At a chain length of 21 bases, corresponding to 20 internucleotide linkages, this amounts to 1 048 576 isomers. It is clear from the stereochemical effects discussed previously that not all isomers will bind equally well to the target sequence. Tidd employed affinity purification to separate a mixture of 9-mer methylphosphonates into strongly binding and weakly binding fractions.[296,311] This entailed the hybrids consisting of the 9-mer and a complementary 20-mer oligonucleotide being separated out by binding to a weak ion exchanger and the enriched 9-mer being recovered by thermal dissociation. Hybridization experiments with the two fractions revealed that the T_M of the strongly binding fraction was 5 °C higher, and that of the weakly binding fraction was 2.6 °C lower, than that of the initial mixture. Improved fractionation would be achieved in an affinity chromatography in which the complementary oligonucleotide is covalently attached to the support.

In contrast to the methylphosphonates, the chirality of the phosphorus in the methyl phosphate triesters apparently has no effect on the strength of hybridization.[312] The reason given for this is the preference of the methylphosphonates to form nonhelical conformations. For example, the melting temperature of a methyl phosphate triester 12-mer oligonucleotide is 55 °C, whereas that of a methylphosphonate 12-mer of similar sequence is only 32 °C. The T_M of an unmodified oligonucleotide is ca. 40 °C. Like the α-anomeric oligonucleotides, which likewise bind more strongly than the normal oligonucleotides, the arrangement of the strands in the hybrid molecule has also been found to be parallel for the hexathymidine pentaphosphate pentamethyl ester.[313] This hexamer hybridizes with itself via T·T base pairings. It would be interesting in this connection to know whether the stable carbamate duplexes[167b] likewise hybridize in parallel orientation.

C. Specificity

1. Specific Effects

One of the basic requirements to be met by antisense oligonucleotides is absolutely specific binding to the target nucleic acid, whose base sequence must be known. Statistically, the sequence of a 17-mer oligonucleotide occurs just once in the human genome. When it is remembered that not all the genes in the cell are switched on at the same time, but that only about 10–20% of the genes are being transcribed into the corresponding mRNA at a particular point in time, it is clear that oligonucleotides with 15–20 bases offer the opportunity of extremely selective intervention in gene expression. This high specificity and the universality are the reasons for the attractive advantages of the antisense oligonucleotide strategy. However, in order to meet the requirements for stability and passage through membranes, the structure of the intrinsically highly specific natural DNA must be modified. This may have an adverse effect, and in rarer cases a positive effect, on the specificity of the antisense oligonucleotides.

An instructive example of the high specificity of unmodified antisense oligonucleotides is found in the study by Holt et al.[314] about the part played by the nuclear protooncogene *c-myc* in the regulation of cell growth and differentiation. The antisense oligonucleotide directed against the *c-myc* mRNA contained 15 bases. A search for homologous sequences in the GenBank databank revealed a number of potential target sequences with up to 13 bases identical with the total of 15. Remarkably, the 15-mer antisense oligonucleotide was able to inhibit specifically the proliferation of HL-60 cells employed and to induce differentiation. Related oligonucleotides of the same length but with 2–12 mispaired bases were unable to influence the growth of HL-60 cells. Comparably high specificities were found by Anfossi et al. in studies of *c-myb* protooncogene expression.[315] The experimental procedure for examining the specific action of the antisense oligonucleotides was as follows: the oligonucleotide that is complementary to the antisense oligonucleotide, which is also called the sense oligonucleotide, did not inhibit gene expression, in contrast to the antisense oligonucleotide.[316] However, an excess of the sense oligonucleotide was able to abolish the action of the antisense oligonucleotide. This rules out a general toxic effect of the antisense oligonucleotide as the principle of action. In the inhibition of β-globin synthesis by antisense oligonucleotides, Goodchild et al. found specificity for the β type whereas earlier experiments had also shown nonspecific inhibition of α-globin synthesis.[290] In addition, a 19-mer antisense oligonucleotide brought about specific suppression (100%) of the expression of a functional *Torpedo* acetylcholine receptor in the oocyte test. By contrast, the expression of the homologous cat muscle acetylcholine receptor was inhibited by only 47%.[317] This finding also indicates that there is no nonspecific toxic effect by the antisense oligonucleotide.

2. Nonspecific Effects

Nonspecific effects of antisense oligonucleotides have also been reported. Unmodified oligonucleotides appear to cause, when injected into *Xenopus* embryos, a nonspecific breakdown of the mRNA at the injection sites.[318] Marked specificity problems have been found with the phosphorothioates. Zamecnik et al. investigated phosphorothioate antisense oligonucleotides for their antiviral activity against HIV (human immunodeficiency virus).[102] At an oligonucleotide concentration of 4 μg/mL they found an effect only with those antisense oligonucleotides complementary to the viral RNA.

However, at the higher dose of 20 μg/mL, a phosphorothioate oligonucleotide that had no binding site on the viral RNA had an effect similar to that of the oligonucleotide with the designed binding site. Hence it appears that the phosphorothioate oligonucleotides can act by different mechanisms depending on the used doses (cf. section IV.D). It is even more surprising that homooligomeric phosphorothioate oligonucleotides are likewise active against HIV[319] with the effectiveness of a 14-mer homooligonucleotide decreasing in the sequence dC > T > dA. Corresponding phosphorothioates of the 2′-*O*-methyloligoribonucleotides showed a similar lack of specificity in the HIV test, in which the inosine derivative was more effective than the cytidine derivative.[226]

The antiviral activity of the methylphosphonate oligonucleotides is less than that of the phosphorothioates, but the action of the former is generally more specific. An 8-mer methylphosphonate antisense oligonucleotide that had one phosphodiester linkage and was complementary to the splice acceptor site of HSV-1 (herpes simplex virus type 1) had a sequence-specific anti-HSV action, whereas a similar oligonucleotide without a viral binding site had no antiviral activity.[320] In another experiment by Miller et al., methylphosphonate antisense oligonucleotides against the mRNA of VSV (vesicular stomatitis virus) were tested for inhibition of viral protein biosynthesis.[321] They synthesized three different methylphosphonate oligonucleotides against the initiation regions of mRNA of the N, NS, and G proteins of VSV. The N oligonucleotide specifically inhibited N protein synthesis. By contrast, the NS oligonucleotide suppressed the synthesis of both the NS protein and the N protein. The diminished specificity was attributed to the formation of partial duplexes between the NS oligonucleotide and the mRNA for the N protein. When it is remembered that the oligonucleotides used were only 9 bases long, this explanation seems somewhat unsatisfactory. A methylphosphonate oligothymidylate that was also tested as a control exerted only a weak inhibitory effect on viral protein synthesis.[247,321]

With regard to the specificity of antisense oligonucleotides that contain a bound intercalating agent such as acridine, it may be thought initially that there will be a reduction, because aminoacridines intercalate as such into DNA and, as part structures in other derivatives, have enzyme-inhibitory properties.[322] However, the inhibition of influenza virus investigated by Hélène et al. was found to be highly specific.[262] A heptanucleotide with a 3′-terminal acridine unit selectively suppressed the cytopathic effect of influenza virus type A, whereas it had no effect on type B viruses. The target sequence was the 3′ terminus of the type A virus, which differs in four of twelve bases from the type B virus. This high specificity of the intercalating oligonucleotides was confirmed in another experiment in which growth of *Trypanosoma brucei* parasites was specifically prevented.[236] However, in another study by the same group on some intercalating antisense oligonucleotides there were stated to be nonspecific effects on transcription.[260] The observed side effect was ascribed to the binding of the antisense oligonucleotides to RNA polymerase. This suggests a parallel to the inhibition of reverse transcriptase by nonnucleotidic acridine-containing derivatives.[322]

3. Control of Specific Hybridization

The specificity of the corresponding antisense oligonucleotides can be controlled, within limits, by altering the heterocyclic bases. Thus, the hybridization of oligonucleotides containing diaminopurine nucleosides appears, at least at high salt concentrations, to be more specific than that of normal adenine-containing oligonucleotides.[201] By contrast, incorporation of inosine reduces the specificity. Inosine pairs with all four natural bases in the sequence dC > dA > T ≈ dG.[194] As specified, a reduction in specificity appears undesirable, but it may be an advantage, for example, when the intention is to direct only one antisense oligonucleotide against a family of very similar target sequences (e.g., a family of parasites[323]) that are homologous apart from only a few positions in the base sequence.

There are some simple experimental modalities for checking the specific hybridization of antisense oligonucleotides to their target sequences in vitro. If the oligonucleotides have a free 3′-hydroxyl group that can act as a primer for DNA polymerases, sequencing by the Sanger method[324] is possible. This primer function, for which methylphosphonates and phosphorothioates are also suitable, can be used to check the correctness of the binding site.[325] Nonspecificity can be estimated qualitatively from the sequencing quality that can be achieved. Another possibility comprises examination of the protein products if the antisense oligonucleotides bind in the coding region of the mRNA. The translation stop brought about by hybridization can be used to prepare specifically shortened proteins whose size can be determined by gel electrophoresis.[304] Finally, there is also the possibility of specific cleavage of the mRNA on the points on the RNA·DNA double strand using RNase H. However, this type of cleavage is not possible with some modified oligonucleotides (cf. section IV.E).

D. Stability to Nucleases

1. Stability of Unmodified Oligonucleotides

Another major requirement to be met by antisense oligonucleotides is their stability both under assay conditions and in vivo following therapeutic use. It has long been known that natural nucleic acids are subject to catabolism in the serum and in cells. For example, the stability of an mRNA in the cell is a crucial factor determining the extent of expression in the relevant protein. Even oligonucleotides that occur endogenously in eucaryotic cells are subject to rapid breakdown.[2] The enzymes responsible for the degradation of nucleic acids (DNA, RNA), the nucleases (DNases, RNases), differ in their specificities. The most important references are nucleases specific for double strands and single strands—exo- and endonucleases—and the highly specific restriction endonucleases. The nucleases that degrade single strands, especially the exonucleases, are important in the context of antisense oligonucleotides: unmodified oligonucleotides are degraded within a few hours in calf serum. Under optimal conditions the enzymatic cleavage may be complete after only 15 min.[326] On examination of the breakdown of a 5′-labeled oligonucleotide (20-mer) it becomes evident that the nu-

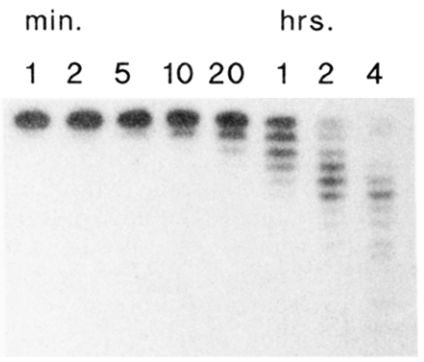

Figure 46. Kinetics of exonucleolytic cleavage of a radiolabeled 20-mer oligonucleotide in fetal calf serum (12% polyacrylamide gel).

cleolytic cleavage in serum is mainly brought about by exonucleases (Figure 46). This is indicated by the ladder-like breakdown pattern, because cleavage by endonucleases would give rise to smaller fragments even after a shorter incubation time. We were able to show by further investigations on oligonucleotides whose 5′ and 3′ ends were each protected against nucleases that a 3′-exonuclease activity is responsible for the degradation of oligonucleotides in calf serum. This 3′-exonuclease activity corresponds to that of snake venom phosphodiesterase, which degrades oligonucleotides with a free 3′-hydroxyl group from the 3′ end. Oligonucleotides whose 5′ end has been protected by modification are not substrates of spleen phosphodiesterase. These findings are consistent with the results obtained by other groups on variously modified oligonucleotides.[191,314,326,327]

To improve the cell culture test on unmodified oligonucleotides, the nucleases in the serum that is used are commonly inactivated by heat treatment. However, care is necessary with this because some batches of fetal calf serum apparently contain heat-resistant nucleases, which may cause the results to be wrongly interpreted. In addition, the rate of oligonucleotide degradation in each cell culture depends on the cell type used. Whereas a 20-mer oligonucleotide suffers extensive breakdown within 3 h in HeLa cells, it is virtually stable overnight in a culture of chicken embryo fibroblasts.[328] The half-life of a 5′-phosphorylated oligonucleotide (15-mer) in a culture of HL-60 leukemia cells using a heat-inactivated calf serum was 1–2 days.[314] Wickstrom has reported a systematic study on the stability of oligonucleotides on various subcellular extracts and culture media.[326] Oligonucleotides are virtually stable for 2 h in rabbit reticulocyte lysate or Dulbecco's modified medium containing 5% fetal calf serum. By contrast, they are completely degraded within 2 h in a postmitochondrial cytoplasmic HeLa cell extract and in 15 min in bovine calf serum. The different results with reticulocyte lysate and with HeLa cell lysate suggest that breakdown depends on the cell compartment. There is rapid degradation of oligonucleotides in some cell compartments, such as the liposomes, while it takes place distinctly more slowly in other parts of the cell, for example in the cytoplasm.[326] Unmodified oligonucleotides are relatively stable inside human T lymphocyte cells, too.[316] In summary, it can be said that oligonucleotides with a natural phosphodiester linkage are unsuitable for in vivo studies and need to be stabilized to nucleases by modification.

2. Stabilization of Oligonucleotides to Nucleolytic Degradation

An obvious approach to stabilization is to modify the phosphate center, which is where the nucleolytic attack occurs. An apparently slight change is to replace an O atom by an S atom. De Clercq and Eckstein described phosphorothioate polynucleotides with increased resistance to nucleolytic breakdown long ago.[87] In a number of publications, Eckstein has reported phosphorothioate analogues of nucleotides as tools for investigating biochemical processes and the practical use of this class of compounds.[86,329–331,333] Phosphorothioates have also been used for studies on RNA[334,335] and 2′–5′-linked oligoadenylates.[336–339]

Phosphorothioate antisense oligonucleotides are stable to snake venom phosphodiesterase and spleen phosphodiesterase, which are exonucleases.[102,319] The half-life of unmodified oligonucleotides under HIV assay conditions is 17 h, whereas no significant breakdown of phosphorothioate oligonucleotides was found after 1 week.[319] The cleavage of phoshorothioate-containing DNA duplexes by specific endonucleases such as *Eco RI* is usually stereospecific, with one diastereomer being cleaved slowly and the other usually being completely resistant.[97a,130] The phosphorothioate groups of both diastereomers of d[AA(S)AA] are resistant to staphylococcal nuclease, DNase I, and DNase II.[333]

The nonionic methylphosphonate oligonucleotides were used as antisense oligonucleotides for the first time by Miller and Ts'O.[76] In the case of dithymidylate whose internucleotide linkage was in the form of methyl- or phenylphosphonate, it was found that both compounds are stable to spleen phosphodiesterase but one diastereomer in each case is slowly hydrolyzed by snake venom phosphodiesterase.[62] Methylphosphonates are stable to endonucleases such as S1. Oligonucleotides whose backbone is constructed of alternate methylphosphonate and phosphate diester internucleotide linkages are stable to spleen phosphodiesterase and S1 but are slowly hydrolyzed at the phosphodiester linkage by snake venom phosphodiesterase.[340]

A third class of phosphate-modified oligonucleotides comprises the phosphoramidates, which, like the phosphorothioates, are stable to snake venom phosphodiesterase, spleen phosphodiesterase, and S1 endonuclease.[102,341] A fourth class is found in the phosphate triesters, which have been little used to date as antisense oligonucleotides, possibly because of their potential alkylating properties. Isopropyl phosphate triesters are, as expected, not cleaved by endonucleases.[128] Interestingly, ethyl phosphate triesters are deethylated after being taken up by the cell and then undergo nucleolytic breakdown.[76,342] However, phosphate triester oligonucleotides appear to have a mutagenic potential.[343]

The oligonucleotides that have been changed to the α-anomers in the sugar moiety are very stable to both endo- and exonucleases. Slow cleavage is found only on incubation with snake venom phosphodiesterase.[183] The stability in *Xenopus* oocytes of the α-anomeric oligonucleotides ($t_{1/2} > 8$ h) is considerably better than that of the β-anomers ($t_{1/2} = 10$ min).[191] According to Sproat[227] the 2′-*O*-methyloligoribonucleotides are completely resistant to RNA- and DNA-specific nucleases,

but are cleaved with varying efficiency by the less specific RNA/DNA-recognizing nucleases. Compared with DNA, the rate of hydrolysis of 2′-*O*-methyloligoribonucleotides is ca. 100 times slower with micrococcal nuclease, ca. 10 times slower with P1 nuclease, and about the same with snake venom phosphodiesterase. The partial instability to nucleases makes additional stabilization of 2′-*O*-methyloligoribonucleotides necessary, for example, as described, as combination with phosphorothioate residues.[226] Open-ring sugar analogues of adenosine have been found by Hakimelahi et al. to be stable to snake venom phosphodiesterase and spleen phosphodiesterase.[344] However, it is unclear how far such drastic changes in the molecule still allow good hybridization with mRNA. Cyclic oligonucleotides whose synthesis has been described by van Boom[345] had to be protected from endonucleases although they were stable to exonucleases.

The same stabilization toward exonucleases can be achieved by incorporating two successive blocked internucleotide linkages. Stec et al. have reported oligonucleotides that have two successive phosphorothioate residues and are not cleaved by snake venom phosphodiesterase.[97a] A 15-mer oligonucleotide protected similarly by 3′,5′-terminal phosphorothioates has a half-life of more than 1 month in 50% human serum, compared with 2–3 days for the normal 15-mer. This means that the terminally modified oligonucleotide is almost as stable as the corresponding all-phosphorothioate in the serum.[297] A third phosphorothioate internucleotide linkage, however, appears to confer no further stabilization to exonucleases.[97b] Shibara et al. reported a 20-mer 2′-*O*-methyloligoribonucleotide in which five internucleotide linkages at the 5′ end and three at the 3′ end were in the form of phosphorothioate and which was almost as active as a corresponding all-phosphorothioate against HIV-1 in vitro.[226] They attributed this effect to an increased resistance to exonucleases, because the corresponding oligonucleotide with phosphodiester linkages did not inhibit growth of HIV. However, the situation in vivo is more complicated because of the presence of endonucleases. For example, in *Xenopus* embryos breakdown of normal oligonucleotides, terminally protected oligonucleotides, and all-phosphorothioates is relatively rapid, with $t_{1/2}$ < 30 min.[346] By contrast, in *Xenopus* oocytes the phosphorothioates are considerably more stable ($t_{1/2}$ > 3 h) than the oligonucleotides without or with only partial sulfur modification.

Oligonucleotides with terminal modification by two adjacent methylphosphonate groups are much more stable to nucleases than are those with only one methylphosphonate group.[70,252,295] The explanation of this is that snake venom phosphodiesterase can jump over an internucleotide bridge as it were and liberates a dinucleotide methylphosphonate.[252] An observation that can be interpreted in the same way has also been made with phosphate triesters.[130]

Of course, many other derivatizations at the 3′ or 5′ end of oligonucleotides also protect against nucleolytic breakdown, examples being intercalating agents,[193,235,236,259] polylysine,[255,347] or poly(rA).[348] Even a single 5′-phosphate residue protects an oligonucleotide against breakdown by spleen phosphorodiesterase[349,350] because this enzyme requires a free 5′-hydroxyl group on the oligonucleotide to cleave it. In vivo, oligonucleotides of this type are, of course, instantly dephosphorylated by phosphates and then degraded. Finally, mention may be made of the dephosphooligonucleotides such as carbamates,[165] siloxanes,[151,152] or "plastic" DNA,[351] which are completely nuclease resistant but which have unsatisfactory hybridization and solubility properties.

E. Penetration through Membranes

1. Mechanism of Cellular Uptake of Oligonucleotides

It is essential for the activity of a therapeutic of any type whatever that its bioavailability is satisfactory. Thus, the activity of the antisense oligonucleotides is crucially affected by how well they reach their site of action unmetabolized. The protein biosynthesis apparatus of the cell is located in the cytoplasm and comprises 55% of the cell volume. Thousands of enzymes bring about there the biosynthesis of sugars, fatty acids, nucleotides, amino acids, and proteins. The mRNA produced in the nucleus by transcription of the DNA is translated into the corresponding protein on the ribosomes in the cytoplasm. In order for the antisense oligonucleotides to be able to act to stop translation by hybridization, they must pass through the plasma membrane into the interior of the cell. The plasma membrane is a natural barrier to many large or negatively charged molecules. It might therefore be supposed that this membrane barrier would form a bottleneck in the antisense oligonucleotide concept.

Surprisingly, however, cellular uptake of the oligonucleotides takes place better than would have been expected from a polyanionic compound of this size. The uptake of radiolabeled oligonucleotides takes 15 min to a few hours depending on the type of cells used and the experimental conditions. Goodchild reported that the process of uptake is energy dependent because it is inhibited by dinitrophenol, an inhibitor of ATP synthesis.[328] The active transport of the oligonucleotides takes place by endocytosis, which, according to all the evidence, is receptor mediated.[352] Affinity chromatography on oligo(T) cellulose was used to isolate an 80-kDa surface protein that might be responsible for oligonucleotide transport. The membrane receptor hypothesis is supported by the finding that phosphorothioate oligonucleotides, but not methylphosphonate oligonucleotides, inhibit the uptake of fluorescence-labeled oligonucleotides with normal phosphodiester linkages.[235] However, the introduction of a fluorescence label may also change the penetration characteristics of oligonucleotides. Also worthy of note is the receptor-mediated uptake of the DNA of bacteria phage *lambda* in white blood cells, which possibly presents a general mechanism of uptake of exogenous DNA.[353]

However, there is also the possibility, especially for modified oligonucleotides, of nonspecific binding to the membrane. The membrane-bound oligonucleotides might then gradually be internalized by the process of membrane reorientation.[354] Passive uptake into the cell appears to be confirmed for methylphosphonate oligonucleotides.[76,126,301] It appears necessary, and not least for this reason, to define the term "cellular uptake". In his early work between 1971 and 1974 on the uptake of polynucleotides by animal cells, Schell left two possibilities open.[355,356] According to his definition, cellular

uptake comprises both penetration of the polynucleotides into the interior of the cell and their irreversible binding to the cell surface. Zamecnik et al. were able, however, by electroporation experiments and investigations with the electron microscope to demonstrate clearly that the oligonucleotides are located inside the cell.[252]

It has been estimated that in cell culture in the micromolar concentration range the intracellular concentration of oligonucleotide is ca. 7.5–10% of the concentration outside.[327,328] Studies of the penetration of tritium-labeled oligonucleotide to HeLa cells showed that the radioactivity was mainly on the outside of the cells after a very short incubation time, whereas the label was located exclusively in the nucleus after 15 min.[328] Although only 6% of the oligonucleotides were broken down to free nucleosides outside the cell, it is difficult to say how much of the intracellular radioactivity is attributable to the free nucleoside. Investigations on nuclear hsp70 transcripts[357] and on antisense oligonucleotides against the splice sites of HSV and HIV[320,327] also support the possibility of penetration as far as the nucleus, because both transcription and splicing of the RNA take place in the nucleus (cf. section IV.C). The time course of the cellular uptake of oligonucleotides is linear in the initial phase and then reaches a plateau that usually lasts from 2 to 3 h.[316] With acridine-labeled oligonucleotides in HL-60 cells, this plateau phase is not reached until after 50 h. The concentration that can be reached inside the cell is inversely proportional to the length of the oligothymidylate used in the range from 3 to 20 bases.[352]

The cellular uptake of methylphosphonates is also time dependent in a similar manner.[126] At least the small methylphosphonate oligonucleotides appear to be taken up very efficiently by animal cells, and no dependence on the chain length was found in the range from two to nine bases.[76] Interestingly, *E. coli* B cells do not take up any methylphosphonate oligonucleotides with more than four bases, whereas *E. coli* ML 308-225 displayed no problems with the cell uptake of these oligonucleotide derivatives.[301] It has been found that the trinucleotide ethyl phosphate triesters are very effectively internalized by hamster fibroblasts, but once inside the cell they are deethylated and metabolized.[342] Surprisingly, phosphorothioate oligonucleotides do not penetrate as well as unmodified oligonucleotides. Stein et al. used flow cytometry to show that uptake of a 5′-acridine-labeled 20-mer homothymidylate phosphodiester was more efficient that that of a similarly labeled 7-mer phosphorothioate homothymidylate.[235] This process of uptake appears to be energy dependent because there is no uptake in dead cells. Moreover, uptake of oligonucleotide is, in contrast to that of free acridine, temperature dependent.

3′-Derivatization of oligonucleotides appears to have a beneficial effect on both the ability to penetrate and the stability to nuclease. In addition, when an acridine label is used it provides a simple detection method for following the cellular uptake of such oligonucleotides. Thus, the uptake of a 9-mer antisense oligonucleotide into live *Trypanosoma brucei* parasites can be detected in <2 h by means of the green fluorescence.[236]

With regard to the interpretation of individual results, it appears important to mention the coupling between penetration and breakdown by nucleases. The rate of degradation of an oligonucleotide depends on its location in the various compartments in the cell.[326] However, its location is determined by its penetration behavior, and the latter in turn is determined by the type of modification of the oligonucleotide. It is not experimentally straightforward to investigate the distribution of the oligonucleotides in various cell compartments, such as the cytoplasm, endoplasmic reticulum, mitochondria, Golgi apparatus, lysosomes, or nucleus, and the breakdown in these compartments, but it appears to be extremely important. For example, it would be of little benefit if antisense oligonucleotides were endocytosed in the cells and then mainly broken down or stored packed in lysosomes. This would mean that, despite a high intracellular concentration, they would be enveloped in a membrane and not available for inhibiting protein biosynthesis. Problems of this type were recognized and discussed early by Pitha in investigations on "plastic" nucleic acids.[171,351,358,359] Moreover, distribution of "plastic" nucleic acids in vivo is nonuniform. Thus, the nucleic acid analogues are found to accumulate mainly in the liver, spleen, thymus, and bone marrow, whereas the lungs and kidneys purge themselves of these compounds. The blood–brain barrier seems to be effective for these polymers because the concentration in the brain is near the detection limit.[171] Accumulation of "plastic" nucleic acids in particular organs may prove to be an advantage or disadvantage depending on the case. The results obtained on this class of substances are certainly not representative of antisense oligonucleotides because they have very different structures and, moreover, much higher molecular weights. Nevertheless, some of the experience collected by Pitha et al. should be taken into account in subsequent testing of antisense oligonucleotides.

Only two research groups have reported on the penetration characteristics of antisense oligonucleotides in vivo. Miller and Ts'O found after injection of methylphosphonate oligonucleotides into the tail vein of mice that there was distribution over all the organs and tissues excepting the brain.[247] Application of these methylphosphonates in the form of a cream to the HSV-infected ear of a mouse prevented lesions caused by HSV. This finding indicates that the methylphosphonate oligonucleotides are able to penetrate the mouse skin. Antisense oligonucleotides with alkylating groups also penetrate into all organs and tissues, reported by Vlassov.[253]

2. Deliberate Improvement in the Penetration of Oligonucleotides through Membranes

Various ways have been employed to improve the penetration of antisense oligonucleotides, the most important being incorporation in liposomes or covalent bonding to nonspecific or specific carriers. Another that may be mentioned is lipophilic modification of the oligonucleotides, but the effectiveness of this has not yet been evaluated.[252]

(*a*) *Liposomes.* Liposomes are microscopic particles composed of mono- or multilamellar lipid bilayers. They enter the cells by phagocytosis or endocytosis. Review articles deal with the use of liposomes as carriers of antimicrobial[360] and antiviral[361] products. The in-

creased antiviral activity of 2′,5′-oligoadenylates encapsulated in liposomes was reported by Lebleu in 1985.[362] Although this class of compounds operates not by the antisense oligonucleotide strategy but by activation of RNase L, it is subject to the same problems of pentration and stability to nucleases. The reported rate of homoadenylate (tetramer) incorporation in liposomes is 0.8%, resulting in an intracellular concentration of the oligonucleotide of about 20 nM. However, the actual concentration of free oligonucleotide present in the cytoplasm remains unclear. It is probable that the release of oligomer from the liposomes depends on partial breakdown of the liposomes by phospholipases or other enzymes and does not necessarily result from the low intravesicular pH.[362] We are not aware of any publications on the incorporation of unmodified antisense oligonucleotides into liposomes. However, antisense oligonucleotides with lipophilic modifications can be incorporated to the extent of ca. 50% in liposomes.[253]

One disadvantage of conventional liposomes that has hindered widespread use to date is their short half-life in serum. The cause of this is the high rate of uptake of the liposomes by the reticuloendothelial system (liver and spleen). There has recently been a description of a new generation of liposomes that still have a reticuloendothelial system/blood ratio of 0.7 after 24 h.[363] Also of very great interest are targeted *immunoliposomes*: an antibody incorporated in the liposomes ensures specific attack on those cells having the corresponding surface antigen. They have been used, inter alia, for targeting antiviral nucleosides on HSV-infected cells.[364] Furthermore, virus-infected cells sometimes appear to alter their surfaces in such a way that selective fusion with liposomes becomes possible.[365]

(*b*) *Poly-L-lysine.* A systematic investigation of the cellular uptake of polynucleotides has been published by Schell.[355,356] His findings included stimulation of the uptake of homooligoribonucleotides in tumor cells by poly-L-lysine, and he suggested two mechanisms for this. On the one hand, poly-L-lysine can form a complex with the wall of the intact cell, which, because of the net positive charge, makes the latter penetrable by polyanions. On the other hand, poly-L-lysine is able to form complexes with the polynucleotides themselves, and these complexes have high affinity for the cellular membrane.[355] Polycations were suggested to have a helix-stabilizing effect by Glaser and Gabbay in 1968.[366] It is possible that the effects of poly-L-lysine go beyond those mentioned here. For example, poly-L-lysine is able, by interacting with the cellular receptor of HSV-1, to block the binding of the virus to the receptor.[367] In this connection, polylysine of molecular weight 50 000 proved to be toxic in the micromolar range.

Lebleu et al. used poly-L-lysine to investigate the antiviral activity of modified 2′,5′-oligoadenylates[368] and antisense oligonucleosides.[255,347] A 15-mer oligonucleotide or a mixture of this oligonucleotide with poly-L-lysine was not active against VSV. However, if the antisense oligonucleotide was covalently bonded via the 3′ end to poly-L-lysine, it had a specific inhibitory effect on VSV growth in cell culture.[255] The antiviral effect in the 100 nM range that was observed is impressive because most antisense oligonucleotides had previously been effective only in the micromolar range. The stabilization of the antisense oligonucleotides to 3′-exonucleases caused by covalent bonding to the poly-L-lysine is scarcely able by itself to explain the good activity of these oligonucleotides. It is probably a combination of stabilization to 3′-exonucleases and an improvement in penetration, which may be promoted by the helix-stabilizing component of the polycation.

Unfortunately, there are some problems with the use of poly-L-lysine conjugates. Poly-L-lysine has a cytotoxic effect on almost all cell lines at concentrations of 2 μM.[347] In addition, poly-L-lysine–oligonucleotide conjugates tend to aggregate, which may give rise to experimental difficulties.[369] However, the most serious point is that although poly-L-lysine conjugates have good effects on L929 cells, they confer no protection whatever against VSV infection in other cell types such as HeLa or LM fibroblasts.[347]

(*c*) *Specific Carriers.* The remaining question is the extent to which other protein conjugates able to penetrate through the membrane in a specific manner can be used to improve the passage of oligonucleotides through membranes. A possible example is a receptor-mediated uptake process as has been described for methotrexate coupled to MBSA (maleylated bovine serum albumin).[370] This conjugate is recognized by "scavenger" receptors that are mainly present on macrophages. It has therefore been proposed for the treatment of leishmaniasis. Also of interest is the finding by Vestweber and Schatz that a double- or single-stranded 24 bp DNA that is bonded by the 5′ end to a mitochondrial precursor protein is able to penetrate into mitochondria.[371] The essence of their study is the proposal that oligonucleotides should be coupled to "neutralized" bacterial toxins in order to introduce them into the cytoplasm. By neutralization this meant mutation of the toxins with the aim of eliminating the toxicity while retaining the penetrating ability. The introduction of the antisense oligonucleotides using nonpathogenic viral coat proteins represents another possible method.

IV. Mechanism of Action

Most antisense oligonucleotides to date have been designed with the aim of inhibiting translation. Only a few have been aimed at inhibiting transcription.[231,260,312,372] The mechanism of action of an antisense oligonucleotide accordingly depends on the target sequence it seeks (cf. section VI). Although the structural requirements to be met by an effective antisense oligonucleotide have not yet been clearly defined, there are concrete theories about the mechanism of action.

A. Inhibition of Translation

A possible mechanism of translation inhibition comprises the antisense oligonucleotide binding to the point on the mRNA where translation is started by the mRNA and a number of necessary initiation factors becoming bound to the ribosomes (Figure 47). In this way the RNA·DNA duplex may result, by a direct steric effect, in preventing the ribosomes and important initiation factors binding on. In the case of eucaryotic mRNA, for example, interference with the interaction between the initiation factor 4F and the mRNA is conceivable.[373,374] If the protein biosynthesis apparatus has already gone into action on the mRNA, however,

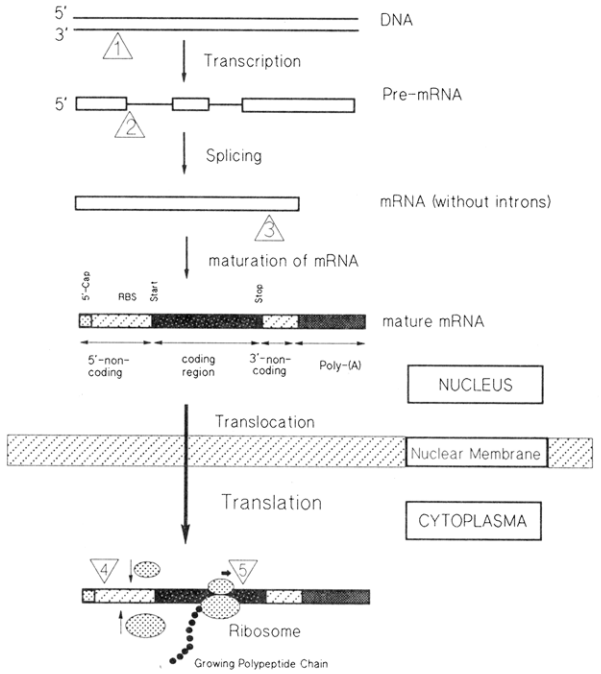

Figure 47. Mechanisms of action and target sequences of antisense oligonucleotides. [Inhibition of transcription (1), of splicing (2), of polyadenylation or translocation (3), of initiation of translation (4), or of ribosome movement along the mRNA (5).] (RBS = ribosome binding site.)

a hybridizing oligonucleotide might block the necessary translocation of the ribosomes along the mRNA. This idea has most similarity with the experiments by Dobberstein et al., who used antisense oligonucleotides against coding regions to prepare selectively shortened polypeptides of predictable length.[304] This process can be imagined to be such that the presence of an antisense oligonucleotide on the mRNA holds up the progress of the ribosomes for a time until they finally drop off. When the ribosomes dissociate from the blocked point, the growing polypeptide chains are released as polypeptidyl-tRNAs, which are subsequently hydrolyzed to the free polypeptides. All the positions investigated within the coding region were similarly effective on inhibition, irrespective of the particular reading frame. Moreover, there is only 1–2% reading over of the blockade.[304] It is not entirely clear whether, and to what extent, the ribosomes can strip the antisense oligonucleotides off the mRNA, assisted by, for example, an unwinding activity,[375] and this presumably depends on the nature of the oligonucleotide modification in the individual case. This translation inhibition due to hybridization as described above corresponds to the originally postulated mechanism action (HART).[12] However, recent findings indicate that some additional mechanisms apply, which are discussed below.

B. Inhibition of Transcription

The mRNA is produced in the cell nucleus by the enzyme RNA polymerase from the four ribonucleoside 5′-*O*-triphosphates. This entails one DNA strand acting as template. Initiation of RNA synthesis comprises the following steps: RNA polymerase recognizes specific start sequences on the DNA (promoters) and initially binds to them in the closed form (Figure 48). The closed complex is then converted into an open complex by a particular section of the double-stranded DNA

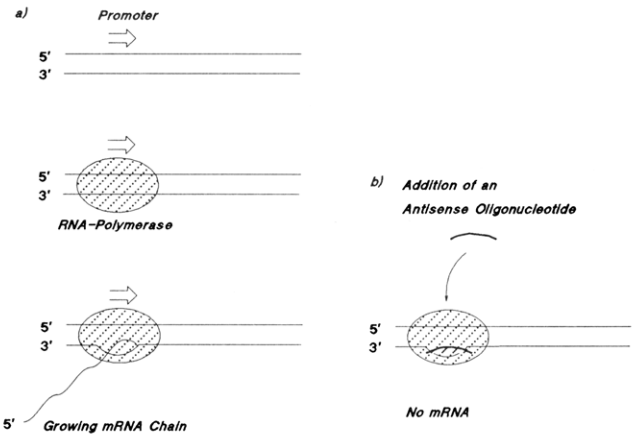

Figure 48. (a) Formation of the open promoter/RNA polymerase complex (normal transcription). (b) Inhibition of transcription by the antisense oligonucleotide binding to the open complex.

being unwound. This open complex is the stage at which the antisense oligonucleotides attack. Hélène et al. used antisense oligonucleotides having acridine derivatives bonded at the 3′ end to inhibit transcription of the β-lactamase gene.[260] The antisense oligonucleotides were hexamers to nonamers and were complementary to the transcribing strand of the β-lactamase promoter. The hexanucleotide acted specifically on the β-lactamase promoter but did not inhibit the *lac* promoter. However, both antisense oligonucleotides were found to have nonspecific effects when they were incubated with the RNA polymerase before addition of the promoter. This suggests that the oligonucleotides have nonspecific effects due to direct binding to the RNA polymerase.[231] It is worthy of note in this context that HIV reverse transcriptase is inhibited by nonnucleotidic aminoacridine derivatives.[322]

The methylphosphonate antisense oligonucleotides have been studied taking the example of blockade of transcription of the *lac* operon in *E. coli*.[312] In the noninduced state, the *lac* repressor protein binds to the *lac* operator DNA sequence and thus prevents access by RNA polymerase. Induction with isopropyl β-D-thiogalactopyranoside (IPTG) converts the *lac* repressor into a nonbinding form, and thus RNA polymerase is able to start transcription. If induction is followed by addition of an antisense oligonucleotide that is complementary to the *lac* operator sequence (repressor binding site), the result is specific inhibition of β-galactosidase synthesis in *E. coli*. It may be concluded from this that the antisense oligonucleotide has replaced the coding strand at the specified point in the DNA duplex.[312]

Hogan et al. have reported an in vitro test of the inhibition of transcription of the human *c-myc* gene by a 27-mer oligonucleotide.[372] This unmodified oligonucleotide presumably binds specifically, with the formation of a triplex structure (cf. section V.B), to a regulatory DNA sequence 115 bp upstream from the start of transcription. Astonishingly, the transcription is suppressed in the nanomolar range.

C. Inhibition of Posttranscriptional Processes

In eucaryotic cells, the primary transcript, the so-called pre-mRNA, is subject to a number of maturation processes before the mature mRNA is translocated into the cytoplasm and transcribed there. In the nucleo-

plasm, noncoding regions (introns) are cut out of the pre-mRNA (splicing), the 5′ end of the mRNA is modified to stabilize it (5′ cap structure), and various bases are altered. The polyadenylation of the mRNA at the 3′ end might be linked with the export process. Antisense oligonucleotides may intervene in any one of these processes (Figure 47).

Miller and Zamecnik showed that growth of HSV[58,320] and HIV[376] can be efficiently inhibited by oligonucleotides that bind to the splice sites of the viral precursor mRNA. In eucaryotic cells there are a few known examples of alternative splicing where two different mature mRNA species that encode different proteins can be formed from the pre-mRNA depending on the cell. The extent to which phenomena such as alternative splicing or transsplicing can be utilized, for example in a specific missplicing reaction,[252] will emerge in the future. There is already evidence that antisense oligonucleotides can prevent transport of mRNA from the nucleus to the cytoplasm: the formation of double-stranded RNA, especially in the region of the polyadenylation site,[376] prevents translocation of the mRNA into the cytoplasm.[4,190,377]

D. Non-Sequence-Specific Mechanisms

The phosphorothioate antisense oligonucleotides attracted early attention due to their exceptionally good in vitro activity against HIV in the range 0.5–1 μM.[102,284,319] However, a phosphorothioate oligonucleotide which has no binding site on the viral RNA also inhibits HIV growth at high dosage (20 μM), although there is no activity at 4 μM. This similarity of the effect of a nonspecific oligonucleotide at 20 μM to that of a specific antisense oligonucleotide indicates that there is a mechanism differing from that conceived for antisense oligonucleotides.[102] Equally surprising is the activity of homopolymeric phosphorothioates. Thus, the phosphorothioate of $(dC)_{28}$ inhibits the de novo synthesis of HIV DNA.[319] Experiments on this have shown that it is likely that the phosphorothioate $(dC)_{28}$ neither interferes with the interaction between HIV and the CD_4 receptor nor has an antiviral effect by induction of IFN-γ. De Clercq and Eckstein reported, in 1970, that the anti-VSV effect of phosphorothioate homopolymers in vitro and in vivo was greater than that of the normal phosphodiester homopolymers and was accompanied by interferon induction.[87] The inhibition of the DNA polymerase of Rauscher leukemia viruses by homopolynucleotides[378] also points to a mechanism of action based on inhibition of HIV reverse transcriptase. Only recently has the mechanism entailing direct inhibition of HIV reverse transcriptase been confirmed.[379] The phosphorothioate of $(dC)_{28}$ proved to be a competitive inhibitor of DNA synthesis with a K_i one two hundredths of that of $(dC)_{28}$. If it is assumed that the phosphorothioate reaches the cytoplasm, it is probable that inhibition of HIV growth at low concentration (<4 μM) is based on nonspecific reverse transcriptase inhibition on which is superimposed, at higher concentration (>25 μM), a mechanism based on specific hybridization.

However, the mechanism of action on HIV is intrinsically more complicated than that on normal mRNA. HIV is a retrovirus whose genetic material is RNA which is transcribed into DNA by HIV reverse transcriptase (Figure 49). Each of the steps 1–3 shown in the figure are catalyzed by HIV reverse transcriptase, which acts as an RNA-dependent DNA polymerase, an RNase H, and a DNA-dependent DNA polymerase. The stages that are shown separately for clarity in fact take place simultaneously. Mölling et al. detected the RNase H activity of HIV reverse transcriptase,[380] and it has been localized to the C-terminus of the holoenzyme.[381] The double-stranded DNA is incorporated as a provirus, with the aid of viral integrase, into the genome of the affected cells. It is evident from this that there are a large number of possible interventions which may be based on different mechanisms. A new mechanism is immediately evident from Figure 49, because viral RNase H degrades the RNA part of the RNA·DNA double strand. This is exactly the situation arising after hybridization of an antisense oligonucleotide onto the viral RNA. This means that it will be possible to attack the virus with its own weapons by using a suitable oligonucleotide to induce the viral RNase H to degrade viral RNA. In principle, the antisense oligonucleotide can be directed against a de novo infection or against chronically infected cells. De novo infections have always been investigated in the publications that have appeared so far. Matsukura et al. have now reported on phosphorothioate antisense oligonucleotides that act sequence specifically against cells chronically infected with HIV.[382] The nonspecific effect of sense oligonucleotides or homopolymers that is found on inhibition of de novo HIV infection does not occur in this case.

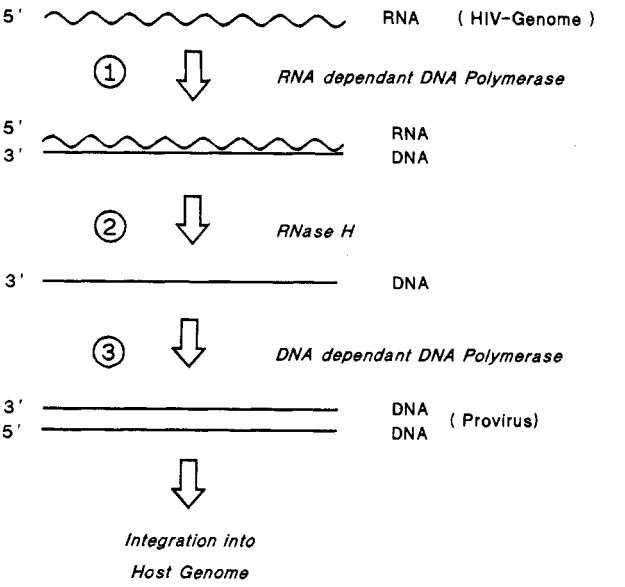

Figure 49. Reverse transcription of the HIV genome.

E. RNase H Mechanism

The excellent hybridization properties of the α-anomeric oligonucleotides were mentioned in Section III.A. However, it was sobering to find that these α-oligonucleotides are unable, despite their high binding affinity, to inhibit translation into proteins.[187] Normal β-oligonucleotides with lower melting temperatures were very effective in the rabbit reticulocyte lysate assay used. There appears to be a simple explanation for this. Hybrids of RNA and β-DNA are substrates of RNase H, but those of RNA and α-oligonucleotides are not. The inhibition of protein synthesis in these assays can

be attributed mainly to the RNase H activity.

Our attention was drawn for the first time to the possibly crucial importance of RNase H for the activity of antisense oligonucleotides by the papers of Häuptle et al.[304] and of Minshull and Hunt.[383] The latter demonstrate, using single-stranded cDNA, which is equivalent to synthetic oligonucleotides in this respect, that inhibition of translation in the cell-free wheat germ system is mainly due to RNase H. The authors noticed the following inconsistency: translation in *Xenopus* oocytes can be effectively blocked with short synthetic antisense oligonucleotides but this fails with antisense RNA transcripts, even when long. This suggested the presence of RNase H in the cytoplasm of the oocytes, because it cuts RNA·DNA but not RNA·RNA.[384] These results are consistent with the earlier work of Häuptle et al.[304] who attributed the ineffectiveness of antisense oligonucleotides in the cell-free reticulocyte assay to the RNase H activity in this lysate being too low. Cleavage with RNase H yields 5′-phosphates and 3′-hydroxyl derivatives.[385] After a lengthy controversy, the RNase H of reverse transcriptase has now also been shown to have endonuclease activity,[386] the products being mainly mono-, di-, and trimers with a 3′-hydroxyl group.[362]

RNase H occurs not only in HIV or *E. coli* but also ubiquitously in plant and animal cells. Thus, this would represent no restriction on the strategy. Since RNase H is involved in DNA replication, it is required in every dividing cell. However, although this process takes place in the cell nucleus, this does not mean that RNase H does not play a part in vivo in the antisense oligonucleotide strategy.[303] Although protein biosynthesis takes place in the cytoplasm, consideration must be given to the nuclear mechanisms discussed previously, such as inhibition of mRNA transport or of splicing or breakdown of pre-mRNA. In addition, RNase H also appears to occur in the cytoplasm.[374] As early as 1969, Stein and Hausen described an enzyme from calf thymus that cleaves the RNA part of RNA·DNA hybrids.[387]

For the conventional test systems studied, it should be remembered that although RNase H occurs in *Xenopus* oocytes and eggs, wheat germ extracts, homogenized sea urchin eggs,[388] and extracts of Krebs-2 cells,[389] it is absent from or present in insufficient quantities in the reticulocyte system.[304] The RNase H level in the test system used by various authors is often unclear. In addition, the RNase H content may vary between batches. Freshly prepared reticulocyte lysates may still contain 1–2% of the RNase H activity of live cells, which is enough for quantitative cleavage of the target mRNA.[374] Thus, the reader is often left in the dark about the extent to which the test system is subject to an RNase H dependent mechanism. An elegant procedure in this respect is that of Walder and Walder,[374] who employed a competitive inhibitor of RNase H, namely, poly(rA)·poly(T), in addition to the enzyme. This made it possible to demonstrate that those antisense oligonucleotides that hybridize entirely at the 5′ end of the mRNA exert under these conditions a significant effect, which is independent of RNase H, on translation initiation. The inhibition of translation otherwise took place by the RNase H mechanism because this does not depend on a position on the target mRNA.

Further support for this principle of action was found by the selective elimination of mRNA by RNase H in vivo. Dash et al. stated that injection of antisense oligonucleotides (15- to 30-mers) into *Xenopus* oocytes was followed by complete, sequence-specific degradation of both coinjected and exogenous mRNA.[390] The mechanism of action of antisense oligonucleotides in *Xenopus* oocytes can be regarded as taking in two stages.[357] In the first stage, RNase H cuts the mRNA in the region of DNA·RNA duplex. In the next step the cleaved fragments after their dissociation are digested by exonucleases. The degradation of internally cleaved mRNA is generally much faster than of intact mRNA because the latter is protected from exonuclease degradation by a 5′ cap or by particular structures at the 3′ end.[391,392] The only reasonable explanation of the observed high effectiveness of unmodified antisense oligonucleotides at inhibiting translation is on the basis of mRNA degradation by RNase H. This is because hybridization of the antisense oligonucleotides onto the target mRNA is a physical interaction that involves an association/dissociation equilibrium for both molecules and thus, in principle, cannot be 100% effective.

However, a major mechanism based on RNase H entails the risk of a certain lack of specificity, because even brief interactions of only 5–6 base pairs may be recognized as a DNA·RNA substrate.[357,388] This fact might also explain the nonspecific action of antisense oligonucleotides at higher concentration.[388] On the other hand, a 10-mer is the shortest antisense oligonucleotide that brings about cleavage of H4 mRNA in *Xenopus* oocytes.[318] A nonspecific mRNA breakdown may occur in *Xenopus* eggs, probably at the injection site because the local concentration here is transiently very high.

The current view is that it appears worthwhile to employ for the antisense oligonucleotide strategy those oligonucleotides whose duplex with RNA is an RNase H substrate. Methylphosphonate oligonucleotides do not meet this condition.[311,393] Although methylphosphonate oligonucleotides inhibited translation of DHFR mRNA much less well than normal oligonucleotides, this effect was not attributed to unsuitability as an RNase H substrate.[393] The reasons for this statement were the observed dependence of the inhibition of translation on the position of the binding site on the mRNA, as well as the absence of a reduction in intact DHFR-mRNA. However, the authors were unable to give a plausible explanation of the ineffectiveness of the methylphosphonates. On the other hand, Tidd's experiments with methylphosphonates support the RNase H model.[311] The absence of action of translation inhibition by the α-anomeric oligonucleotides is generally explained by the RNase H mechanism.[187,191,347] α-DNA·β-RNA hybrids are not substrates but inhibitors of the RNase H of *E. coli* or *Drosophila*.[181] By contrast, phosphorothioate antisense oligonucleotides give rise to the expected cleavage with RNase H after hybridization on RNA.[297,346,394] The substrate activity may be less[394] or more[297,394] than with unmodified oligonucleotides. For example, the RNase H activity on the duplex poly(rA)·$(T)_{40}$ is much less than on the corresponding phosphorothioate complex.[297] One possible explanation of the increased activity is provided by the lower stability of the phosphorothioate

hybrids (local melting or increased flexibility) during the cutting process. On the other hand, the cut duplex might dissociate more rapidly because of the lower stability; another alternative is a combination of the two effects. Terminally modified antisense oligonucleotides appear to behave almost like phosphodiesters,[346] with the length of the internal phosphodiester region being crucial for the suitability as a substrate. Finally, it might be conceivable that a predictable site-specific cleavage of RNA[225,395-397] could be induced by using suitably modified or chimeric oligodeoxynucleotides/oligoribonucleotides.

F. Antisense Oligonucleotides with Interactive Groups

There are in principle two different types of interactive groups on oligonucleotides. The interaction of one category of these compounds with the complementary RNA or DNA is based on chemical modifications. This includes the chemical cross-linker and photo-cross-linker oligonucleotides as well as the artificial endonucleases. The second category comprises oligonucleotides with intercalating residues that cause no change in the target nucleic acid. Category I derivatives appear particularly attractive in combination with those oligonucleotides that, after hybridization, do not allow cleavage by RNase H of the mRNA in the double strand (α-anomeric, methylphosphonate, 2′-*O*-methyloligonucleotides) and, for this reason, often have little or no activity.

1. Antisense Oligonucleotides with Intercalating Residues

The mechanism of action of antisense oligonucleotides with intercalating residues can be imagined to be such that, by reason of its special sequence, the oligonucleotide binds specifically to the target nucleic acid, with the intercalating group providing the antisense oligonucleotide with an additional binding energy which is, however, nonspecific.[261] It should be noted that the specific component is distinctly more important. The noncovalent binding of these groups is generally based on intercalation between two adjacent base pairs but may also take place by binding to the periphery of the nucleic acid. The intercalation is reversible and distorts the sugar–phosphate backbone of the double strand.

Acridine and its derivatives are the intercalating agents that have been longest known and they were also the first to be incorporated in oligonucleotides. Hélène's group reported in 1983 that duplex molecules having an aminoacridine group bonded via a phosphate residue at the 3′ end of one strand have increased stability.[258] This was followed by further investigations[189,399,400] in which the intercalating unit was bonded via the 3′ or 5′ end of normal phosphodiesters,[236,238,259,260,262,401] phosphorothioates,[235] phosphotriester/methylphosphonates,[263] or α-anomeric oligonucleotides.[237]

In general, the advantages of intercalating oligonucleotides may be said to be the following: (1) The attachment of an intercalating residue to oligonucleotides increases their binding affinity for the target nucleic acid. (2) The 3′-derivatized oligonucleotides are more stable toward 3′-exonucleases. (3) The attachment of intercalating residues usually improves the ability of the oligonucleotides to penetrate owing to increased lipophilicity. (4) The binding of acridine-modified oligonucleotides to oligoribonucleotides is much stronger than to oligodeoxyribonucleotides.[238] (5) Apart from a few exceptions,[259,260] the high specificity of the antisense oligonucleotides is retained.

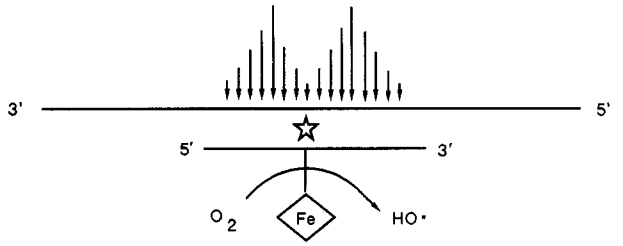

Figure 50. Intensities of cleavage by an artificial endonuclease (EDTA/Fe bonded via C^5 to thymidine) according to Dreyer and Dervan.[248]

2. Antisense Oligonucleotides for Specific Modification of the Target Nucleic Acids

Irreversible inactivation of the target nucleic acid may occur either in a catalytic process with cleavage of the internucleotide linkage (artificial endonucleases, ribozymes in sections V.A) or in a stoichiometric reaction with the target nucleic acid (chemical cross-linkers and photo-cross-linkers). Characterization of the products of the irreversible modifications allows the binding site of the oligonucleotide on the target nucleic acid to be checked.

(*a*) *Artificial Endonucleases.* This class of antisense oligonucleotides provides selective cutting of the target sequence and makes the mechanism based on endogenous RNase H activity redundant. Vlassov and coworkers were the first to report phosphate ethyl esters of homooligothymidylates with an EDTA unit (ethylenediaminetetraacetate) at the 5′ end.[244] Some further work with the EDTA group on phosphodiesters[233,245,248,249] and on methylphosphonates[59] have been published. In the presence of the reducing agent DTT (dithiothreitol) and Fe(II) and after the oligonucleotide has hybridized onto the target nucleic acid, cleavage of the latter is brought about by the EDTA unit. The chemical reaction is based on the local production of hydroxyl radicals. The extent of the reaction depends on the concentrations of oligonucleotide and salts and on the temperature.[233] After hybridization of a 16-mer oligonucleotide with a 5′-EDTA residue to a complementary 37-mer oligonucleotide, the cuts took place mainly within four nucleotide residues on either side.[245] Dreyer and Dervan incorporated the EDTA via C^5 of thymidine approximately in the middle of a 19-mer antisense oligonucleotide that had complementarity with pBR322.[248] After hybridization of this oligonucleotide to the appropriate heat-denatured fragment of pBR322, strand breakage was induced by addition of Fe(II), DTT, and O_2. Cleavage took place within 16 bases on either side (Figure 50).

Oligonucleotides modified with 1,10-phenanthroline[250,300,402] bring about, in the presence of Cu^{2+} and 3-mercaptopropionic acid, a hybridization-dependent cleavage of the target nucleic acid.

Schultz achieved selective RNA cleavage by linking staphylococcal nuclease, an extracellular enzyme composed of 149 amino acids, via the 3′ end to an oligonucleotide.[272,308] Surprisingly, hydrolysis took place

mainly at just one phosphodiester linkage. About 50% of the M1 RNA substrate that was used was cleaved after addition of the cofactor Ca^{2+}. It is evident that this strategy can function only in vitro, because it is not possible in vivo to eliminate Ca^{2+} using chelating reagents. Presumably, the oligonucleotide–nuclease conjugate would hydrolyze itself as a single strand in the presence of the cofactor.

A particular type of reaction is shown by the iron/porphryin system,[270,271] which oxidizes the heterocyclic bases, induces cross-linkages, and additionally causes strand breakages.

(*b*) *Chemical Cross-Linkers.* The cross-linking of DNA with oligonucleotides by attached alkylating agents have been investigated for some years by the Russian group of Knorre and Vlassov. Selective modification of nucleic acids at arbitrary positions had already been proposed by Grineva in their institute in 1967.[403] The derivatives that have been investigated most are those of (*N*-(2-chloroethyl)-*N*-methylamino)-benzylamide.[268,269,398,403-405] All four bases of the DNA are alkylated by 2-chloroethylamines, the reactivity decreasing in the sequence G > A ≈ C > T. The reaction takes place in two stages. Initially an ethylimmonium cation is formed by intramolecular cyclization, and it then attacks easily accessible nucleophiles. The position of the nucleotide which is attacked in the opposite strand depends on the type of attachment (3′ or 5′ end) of the reactive group on the oligonucleotide.[405] Oligonucleotides with alkylating radicals at the 5′ end react with the first nucleotide next to the complementary base, while those with alkylating groups at the 3′ end mainly attack the third nucleotide after the complementary base. However, there was also found to be reaction with a base that is 80 nucleotides further away in the sequence but is apparently in the spatial vicinity of the binding site.[405]

Webb and Matteucci have reported hybridization-dependent cross-linking using oligonucleotides that contain the reactive nucleoside 5-methyl-N^4,N^4-ethanocytosine.[205] This dispenses with the cyclization of the chloroethylamine derivatives, which is the rate-determining step with aromatic chloroethylamines. However, investigation of the ethano derivatives showed that the rate of alkylation with these compounds is much too slow to be useful.[205]

With regard to antiviral applications, mention should be made of the theoretical study of Summerton, which was submitted in September 1973 and finally published in revised form in 1978.[406] This article makes proposals on the treatment of viral infections based on chemically cross-linking oligonucleotides.

(*c*) *Photo-Cross-Linkers.* The cross-linking that can be achieved by irradiation, usually with long-wavelength light, represents an elegant method for controlling gene expression at a particular instant. Besides time-limited activation, another possibility is local activation, which might be achieved with fiber optic systems.[407] On the other hand, the distance tranversed by the radiation through organs is limited. Thus, photo-cross-linkers will be used mainly for eyes and skin and, with extracorporal irradiation, for the blood, too.

The most widely investigated derivatives are psoralen derivatives, which on irradiation with light of wavelength around 365 nm cause cross-linking to bases only of single-stranded DNA but not double-stranded DNA.[302] In the case of photoreaction with the DNA bases, alkali treatment results in strand breakage at the cross-link, which allows it to be located. Kean et al. found that the graded reactivity of methylphosphonate oligonucleotides with a 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen residue at the 5′ end depends on the base in the complementary strand.[341] Thus, inhibition by psoralen paired with U is about 10 times better than that paired with C. Besides the intermolecular reaction, when there are two consecutive thymidine residues at the 5′ end, there must also be expected to be a intramolecular reaction.[341] Pieles and Englisch have reported that in the case of an 18-mer oligonucleotide with a trimethylpsoralen unit at the 5′ end the cross-linking obtained at 350 nm is photoreversible at 254 nm.[239] The mechanism of the photo-cross-linking is thought to be as follows: initially the bonded psoralen residues intercalate in the double strand. On irradiation with light at 360 nm two bases, preferentially thymine, react to give a cyclobutane adduct. Psoralen groups have been linked to phosphodiester[239,408,409] and methylphosphonate[59,302,341] oligonucleotides.

The group of *p*-azidophenacyl derivatives generate on irradiation (>300 nm) the very reactive nitrene radicals. They have been tested in combination with α-anomeric oligonucleotides.[193,243] In contrast to the psoralen derivatives, the azidoproflavines can also enter into reactions at double-stranded regions.[190,242] The limitations arising from this will be discussed in relation to the example of triplex formation (section V.B).

V. Modification of the Principles

Although the two principles described below are closely related to antisense technology, they form a separate class from the mechanistic viewpoint. Neither triplex formation nor ribozymes have been investigated with a view to therapeutic use as intensively as have the antisense oligonucleotides.

A. Ribozymes

Up until a few years ago it was generally believed that enzymes are always proteins. The works of Cech, Altman, Symons, Szostack, Uhlenbeck, and others, who have carried out intensive studies on catalytic nucleic acids in recent years,[410] have taught us better. Ribozymes are catalytic nucleic acids that often, as part of the mRNA structure, catalyze the self-splicing of the primary transcript. The ribozyme activity originally discovered by Cech in 1981[411] in *Tetrahymena thermophila* has not remained an isolated instance. Ribozyme activities in *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, plants, and eucaryotes suggest that they occur universally. Particularly of interest for therapeutic use are the hammerhead structures that occur in the viroids of plants. Forster and Symons attributed the self-cleavage activity of this satellite RNA to a hammerhead-like structure of the active site composed of only 55 nucleotides.[412] The hammerhead structure can be divided into two RNA fragments, a ribozyme and a substrate portion. Koizumi et al. synthesized two model oligonucleotides (21-mers) that contained the consensus sequences of the self-cleavage domains.[413] The substrate is cut in the presence of Mg^{2+}, resulting in a cleavage product with a 2′,3′-cyclophosphate resi-

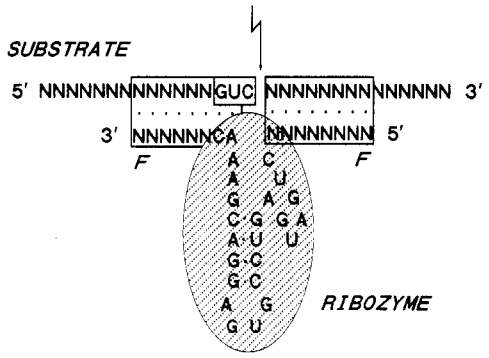

Figure 51. Consensus sequence of a ribozyme (F = flanking antisense region) according to Haseloff and Gerlach.[415]

due at the 3′ terminus. In another study, the same authors prepared ribozymes designed to cut any desired RNA sequences at predictable positions.[414] Thus, ribozymes might be formally included in the class of artificial endonucleases, with the consensus sequence GAAAC forming the RNase part and the two flanking sequences forming the antisense oligonucleotide part. Haseloff and Gerlach showed that ribozymes can be directed against any desired RNA sequences by flanking the conserved catalytic domains by appropriate antisense sequences (Figure 51).[415a] In most natural hammerhead RNAs the base triplet 5′ to the cleavage site is GUC. Alteration of GUC to GUA or GUU does not affect cleavage efficiency, whereas a change to CUC, AUC, or UUC results in a lower cleavage efficiency.[415a] Cleavage is strongly reduced for a GUG sequence 5′ to the cleavage site.[415b] Uhlenbeck prepared a 19-mer oligoribonucleotide by in vitro transcription with T7 RNA polymerase on a synthetic DNA template and used it for specific cleavage of a 24-mer RNA substrate prepared in a similar way.[416] The smallest ribozyme hitherto known is a 13-mer oligo*ribo*nucleotide.[417] By contrast, an analogous oligo*deoxyribo*nucleotide has no catalytic activity. However, it may be expected that certain ribonucleotide residues will be replaceable, at least in the antisense portion, by deoxyribonucleotide residues without loss of ribozyme activity.

Although the ribozyme strategy may appear very attractive at first sight, there are some fundamental problems. The rate constants for RNA catalysis are several orders of magnitude smaller than those of protein-catalyzed cleavage. In addition, the optimal cleavage temperatures are ≥37 °C.[417] It is likely that the dissociation and rehybridization of the ribozyme onto a new substrate molecule, which is necessary after cleavage of the substrate, take place too slowly for a catalytic action at low temperature. If the chosen flanking sequences are too long, dissociation becomes the determining factor, in which case only equimolar amounts of substrate are cleaved.[346] However, it is an advantage by comparison with artificial endonucleases that the ribozyme can become active only after hybridization.

B. Triplex DNA

In general, there are two possible ways to influence gene expression by binding an oligonucleotide to double-stranded DNA. On the one hand, the double-stranded DNA may partially melt and bind a complementary oligonucleotide with the formation of a duplex (cf. section IV.B). Another possibility comprises an

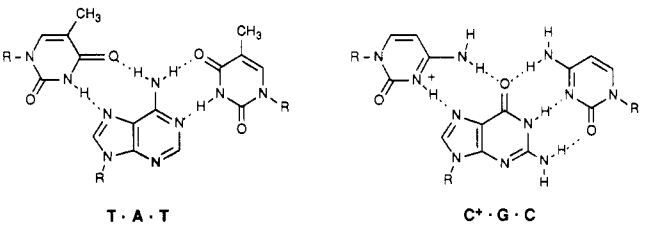

Figure 52. Triple base pairing according to Hoogsteen.

oligonucleotide binding to the double-stranded DNA with the formation of a DNA triplex structure. The antisense oligonucleotides that have been discussed previously belong to the first type, whereas the second type might be called "anti-gene oligonucleotide".[418] Felsenfeld et al. found, in 1957, the first triple helix in which two poly(C) strands hybridized with a poly(A) strand in the presence of $MgCl_2$.[419] Moreover, at pH 6.2 poly(U) forms with poly(G) a triplex structure in which one cytosine is evidently protonated. Figure 52 depicts both types of base triples. The Watson–Crick base pairing is supplemented by binding of a pyrimidine residue by Hoogsteen hydrogen bonds. Triplex structures are formed not only by homopolymers but also by repetitive sequences, for example poly(TC) with poly(GA). The ability to form triplex structures is greatly influenced by modification at the phosphate center. Thus, Miller et al. reported a hexathymidylate with an alternating methylphosphonate/phosphodiester backbone where one diastereomer forms a duplex with poly(A) whereas the other hybridizes in a triplex structure.[340] In the case of repetitive phosphorothioate oligonucleotides, the triplex formation depends on the position of the phosphorothioate residue.[298] A phosphorothioate 5′ upstream of purine promotes triplex formation, whereas one 5′ upstream of pyrimidine destabilizes a triple strand.

Double-stranded DNA can be modified or cleaved by triplex formation with derivatives having covalently bonded azidoproflavine,[190,243] EDTA/Fe,[249] chloroethylamine,[268] azidophenacyl,[243] or phenanthroline/Cu.[300] At present, however, application of this triplex formation appears to be confined to polypurine and polypyrimidine regions of DNA, which occur frequently in, for example, the control regions of eucaryotic genes. However, problems of specificity might arrive. There has merely been one report by Hogan that a 27-mer oligonucleotide with a mixed sequence inhibits the transcription of the *c-myc* gene.[372] A triplex structure was proposed, but this could not be proven experimentally.

Triplex formation is of great interest from two aspects. In the first place, inhibition at the level of transcription should be more efficient than at the level of translation, because many copies of mRNA are produced from 1 equiv of DNA. A pointer to a triple structure in Hogan's experiment is the high activity of the oligonucleotide in the 100 nM range, because unmodified oligonucleotides are usually active in the 10–100 μM range. In the second place, in the case of regulation at the DNA level, it is conceivable that there may be not only inhibition but also activation of transcription. It will certainly be of interest in the future to alter the heterocyclic base on the natural nucleosides in such a way that they preferentially form triplex structures irrespective of the particular sequence.

VI. Selection of Effective Target Sequences

A. Potential Target Sequences

The efficiency with which the function of a target sequence can be inhibited is closely connected to the mechanism of action discussed in section IV. Where the RNase H mechanism predominates, the inhibition efficiency will not depend on the binding site on the mRNA as long as this is easily accessible in the single-stranded form. As is evident from Figure 47, a large number of target sequences are suitable for inhibiting gene expression. At the level of translation, these are the 5′-non-coding region, the ribosome-binding site, the translation start region, the coding region, and the 3′-non-translated region. Potential points of attack at the level of transcription are the promoter, operator, attenuator, and terminator. Posttranscriptional targets are offered by the splice sites and the polyadenylation signals.

It is clear from most in vitro studies that antisense oligonucleotides act most efficiently when directed against the initial part of the 5′-non-coding region near the cap structure and against the region around the translation start codon.[252,290,317,420] These are better than the nontranslated region in between or the remaining coding region. The 3′-non-coding region is usually unsuitable for inhibition although it may in certain cases be a good target.[421] Thus, the best regions are those where the regulatory DNA-binding proteins are normally sited. Accordingly, it is particularly efficient to prevent binding of activators of gene expression and of translation-initiation factors. Similar conclusions regarding the target sequences have been derived from investigations on antisense RNA.[4]

When translation is inhibited by antisense oligonucleotides directed against the translation start region, there may in some circumstances be what is called the restart phenomenon. Normally, translation starts at an AUG codon. If an oligonucleotide directed against this region is used to suppress translation, it may start downstream at an internal AUG codon if this is preceded by a sequence resembling the ribosome-binding site. This may result in proteins shortened at the N terminus but still possibly functional. On inhibition of the G protein of VSV using methylphosphonate oligonucleotides directed against the start region, Engels noticed on a protein gel a concentration-dependent production of a new protein with a smaller molecular weight.[83] Similar restart problems also occur occasionally in expression of bacterial genes.[422-424]

In the case of HSV and HIV, oligonucleotides directed against particular splice sites on the RNA have proven particularly effective.[320,376] These results appear plausible on the basis of theoretical considerations, because splice sites must be readily accessible during mRNA processing. This is also true for the primer binding site of HIV, where reverse transcription of the viral genome starts.[376] Fine-tuning of the efficiency for various target sequences is not really possible because the variations in the relevant cell assay are too large.

B. Secondary Structure Considerations

Every mRNA has an individual secondary and tertiary structure that has a crucial influence on the efficiency of the target sequences. Although mRNA secondary structures can be calculated,[289,425,426] the efficiency of antisense oligonucleotides as inhibitors of protein translation has to be determined experimentally in practice.[394] Blake et al. have reported a considerable effect of the secondary structure of the binding site on inhibition efficiency.[299] One possible way of detecting adverse effects from secondary structures is to degrade the mRNA with nucleases specific for single strands.[299,341] On inhibition of globin mRNA translation by psoralen-modified methylphosphonate oligonucleotides the cross-linking achieved with sequences directed against nuclease-sensitive regions was 10–30 times that obtained with those directed against nuclease-resistant regions.[341] Psoralen-derivatized oligonucleotides are thus also suitable tools for scanning mRNA secondary structures. However, indications of the presence of double-stranded mRNA regions can also be obtained from a simple cell-free in vitro translation test: if the efficiency of an oligonucleotide is increased by a previous hybridization reaction in which a solution of the oligonucleotide is heated with the mRNA to 90 °C and then cooled, this indicates secondary structures that can be eliminated by heat.

C. The Effect of Chain Length and Tandem Targeting

In the cell-free translation test, in which the passage of the oligonucleotide through the membrane plays no part, the efficiency of inhibition correlates directly with the oligonucleotide chain length.[290,303-305] This result suggests that the longest possible oligonucleotide should also be employed in the in vivo test. Two fundamental restrictions on this should be considered. First, large amounts of oligonucleotides can be readily synthesized only for the shorter ones (≤20 bases). Second, there is evidence that penetration through membranes diminishes as the chain length increases. These factors are the justification for employing two or more oligonucleotides that bind to adjacent regions of the mRNA, i.e., a tandem system. Maher and Dolnick investigated the inhibition of cell-free DHFR mRNA translation and found an unexpected synergistic effect by a factor of 3.5 on use of two tandem oligonucleotides.[305] Gaps of one or two nucleotides between the two oligonucleotides had scarcely any effect on this, whereas no synergism was detectable with gaps of more than 16 nucleotides. Goodchild et al. reported that a tandem comprising a 23-mer and a 25-mer oligonucleotide inhibited translation of globin synthesis in a cell-free system just as well as a corresponding 48-mer antisense oligonucleotide.[290] Only in one investigation has a mixture of two tandem oligonucleotides produced only the same inhibition as with each of the oligonucleotides separately.[303] The penetration characteristic gave rise to the hope that tandem oligonucleotides will show even more pronounced effects in vivo.

VII. Assay Systems

A. Cell-Free In Vitro Translation

The advantages of cell-free test systems are the ease of manipulation and the fact that the results do not depend on secondary effects such as penetration

through membranes and stability of the oligonucleotides. Thus they are suitable for finding efficient target sequences and for examining the specificity. Prehybridization of the oligonucleotide to find and destroy mRNA secondary structures is possible in this assay. Polyacrylamide gel electrophoresis can be used to check for an alteration in the mRNA resulting from hybridization, for example an RNase H cleavage, as well as the production of short proteins. The mRNA to be translated can be labeled with [^{32}P]ATP, and the resulting protein can be detected by incorporation of [^{35}S]methionine.

Tests that are commercially available are the rabbit reticulocyte lysate (little or no RNase H) and the wheat germ extract (adequate RNase H). They are used as described, for example, by Blake et al. for inhibition of globin mRNA translation.[420] Other assays used for investigating antisense oligonucleotides are a Krebs-2 cell-free system[389] and a cell-free *E. coli* system.[301] The mRNA to be translated can be isolated from cells or prepared by using commercially available SP6 or T7 RNA polymerase in an in vitro transcription reaction on DNA as template.[221b,427-429]

B. *Xenopus* Oocytes or Eggs

In contrast to the reticulocyte lysate, the *Xenopus laevis* oocytes comprise a whole-cell system. Originally introduced by Laskey and Gurdon,[430] it is now employed in genetic engineering as a kind of in vitro translation system. The efficiency of antisense oligonucleotides in the oocyte system does not depend on penetration through membranes, because the oligonucleotides are microinjected into the oocytes. However, these contain both oligonucleotide-degrading nucleases and RNase H.[390,401] Kawasaki demonstrated the advantages of using the oocyte system for studying antisense oligonucleotides and long (490 bases) single-stranded DNA.[431] There is, moreover, the possibility of coinjecting the oligonucleotide and the heterologous mRNA. However, in order to check in vivo hybridization it is also possible to inject the oligonucleotide 1 h after the mRNA.[390] Antisense oligonucleotides can be used to inhibit the translation of both endogenous and injected exogenous mRNA. It is possible after the incubation to disrupt the oocytes and to analyze the protein products as well as the mRNA. In the oocyte system the concentration of injected mRNA decreases during the incubation with the oligonucleotide, whereas the intracellular mRNA level can be maintained by transcription in the cell assay that is described below.

C. Cell Culture Assay

Mostly used are animal cells in sera diluted with various media. Cell cultures are intrinsically closer to the in vivo situation than are the systems described above, because the stability of the oligonucleotides to nucleases and their penetration through membranes influence the results. However, the serum is often heat-incubated in order to suppress the effects of the nucleases in it. Antisense oligonucleotides have been tested with HeLa, HL-60, mouse L929, molt-3, H9, or other cells, depending on the problem. In some cases, such as with polylysine derivatives, a toxic effect of the modified oligonucleotides has been revealed by the tolerated dose levels. In the case of HIV and other viruses, the inhibition of replication is usually studied by coinjection of virus and antisense oligonucleotide. However, inhibition even occurs when the antisense oligonucleotide is added 4 days after the virus infection.[102] It must be stressed that a study comparing different antisense oligonucleotide derivatives must simultaneously be carried out in rapid sequence; otherwise the variation in the results is too great. Multiple administration of small amounts of oligonucleotide appears to have a more beneficial effect than a single high dose.[327] A suitable detection method is reaction of the suppressed protein product with the appropriate antibodies. However, systems with transient expression of a reporter gene such as CAT (chloramphenicol acetyltransferase) have also been used.[126]

A cell assay based on *E. coli*, in which the antisense oligonucleotide is directed against the *lac* operon, appears very convenient because the β-galactosidase reaction can be followed easily by the blue color produced from X-Gal.[312] However, the membrane structure of the procaryotes differs greatly from that of animal cells. In addition, the passage of oligonucleotide derivatives through the membrane depends on the *E. coli* strain used in each case.[301]

Verspieren et al. used a culture of the single-cell flagellate *Trypanosoma brucei* to investigate oligonucleotides derivatized with acridine.[236] After incubation of *T. brucei* with these oligonucleotides the morphology of the parasites was drastically altered and they ceased moving. The green fluorescence of acridine derivatives could be observed after penetration into the trypanosomes.

D. In Vivo Assay Systems

It is, of course, possible in principle to use all conventional animal models employed for testing other classes of compounds. However, animals of low weight such as the mouse will be preferred, because it is still costly to produce large amounts of oligonucleotides. The first reports to date have dealt with two in vivo tests with modified antisense oligonucleotides[247,253] and one with polyvinyl–nucleotide analogues.[171,351] In all three cases the antiviral activity of these compounds was studied on the mouse as animal model.

Vlassov investigated the in vivo activity of antisense oligonucleotides with alkylating groups against a tick-borne encephalitis virus in the mouse.[253] Repeated injection of antisense oligonucleotide (3 × 0.5 nmol per day) conferred 30–70% protection against the normally fatal infection. The injected oligonucleotides were found to reach all the tissues and organs. The oligonucleotides were eliminated relatively rapidly from the blood and were excreted with the urine. Oligonucleotides taken up by cells were stable for several hours.

A reasonably uniform distribution of oligonucleotides was also found by Miller and Ts'O,[247] who injected methylphosphonate oligonucleotides into mouse tail veins. Merely the blood–brain barrier proved impenetrable. The methylphosphonates were active against HSV-1 even on topical application to mice. Interestingly, clearance was distinctly slower with cholesterol-derivatized oligonucleotides ($t_{1/2} \approx 30$ min) than with unmodified oligonucleotides ($t_{1/2} = 3$–5 min). Similar in vivo distributions of antisense oligonucleotides have

been reported by other groups.[253]

Pitha et al. inhibited the growth of FLV (Friend's leukemia virus) in mice.[171,351] For polyvinyladenine, injection is the only reasonable way of introducing these polymers into the body. The polymers differ greatly in structure and molecular weight from the antisense oligonucleotides now customary and neither penetrate through the skin nor are they adsorbed from the food in effective amounts. They are removed relatively rapidly from the blood after injection. About one-third of the amount of polymer administered intraperitoneally is excreted with the urine in the first 2 days. The remainder is mainly recovered from the liver, spleen, thymus, and bone marrow.

VIII. Toxicity of Antisense Oligonucleotide Derivatives Compared with Their Biological Action

Sections III–VII have dealt in detail with the biological action of antisense oligonucleotides of various classes. Thus, only the results on the derivatives that are currently most promising will be summarized here in terms of their toxicity, which, in the final analysis, together with the inhibitory potency, determines their therapeutic value.

Unmodified antisense oligonucleotides are active only when nucleases are excluded, i.e., in vitro or in a cell assay in serum that has been inactivated by heat treatment. The observed effect extends over a wide concentration range from 100 nM to 100 μM, depending on the target nucleic acid, the assay system, and the length of the oligonucleotide. Because of the instability to nucleases, the unmodified oligonucleotides are suitable principally in the mechanistic investigations, but not for therapeutic use.

Methylphosphonate oligonucleotides are active in the range from 50 to 100 μM in a cell assay. Because of the poor solubility in water of the all-methylphosphonates, usually one or more negative charges are introduced in the form of a phosphate or of phosphodiester linkages. The reason for them being less effective than the phosphodiesters is that RNase H does not cut the target mRNA in the double strand. The results with methylphosphonates have been somewhat disappointing because they have been expected, owing to the lack of negative charges on the phosphorus, to pass through membranes better and to have higher activity. Methylphosphonates are active against HSV-1 in animal models, too[247] (cf. section VII.D). They have low cytotoxicity; on incubation of vero cells with 150–300 μM oligonucleotide, cell growth after 24 h is not reduced from that of the controls without methylphosphonate.[320] There is a slight reduction in the cell count after 48 h with 150–300 μM methylphosphonate although there is none at a methylphosphonate concentration of 10–25 μM. The effective concentration appears to be too high for therapeutic use, at least with regard to an antiviral application. Unfortunately, the suppression of endogenous mRNA, e.g., that of receptors, has not been investigated. It is conceivable that adequate therapeutic effects may be achieved with considerably lower doses in this case. It is noteworthy that a combination with photo-cross-linker residues allows the methylphosphonate concentration required to be reduced from 100 to 1 μM.[232]

In terms of anti-HIV activity, the phosphorothioate antisense oligonucleotides are currently the most active oligonucleotide derivatives. In a cell assay they are active against de novo infection with HIV in the approximate concentration range 0.5–5 μM. However, their lack of specificity appears to be a disadvantage, because not only specific antisense sequences but also homopolymers and sequences without homology to the viral RNA are active. Phosphorothioates inhibit directly not only HIV reverse transcriptase but also cellular DNA polymerases α and γ.[379] This is why it is surprising that a preliminary toxicity study on mice revealed no toxic effects up to a dose of 40 mg/kg of body weight.[295] This apparent contradiction would be explicable if the activity against de novo HIV infection was based not on intracellular inhibition but on inhibition of the spreading of HIV. Such a mechanism, which has in fact also been postulated for the HIV-active pentosan polysulfate,[432] is also suggested by the relatively low but specific activity of the phosphorothioates at a concentration of 25 μM against HIV in chronically infected cells.[382] This means that although the phosphorothioates are of great interest for antiviral therapy, they offer few attractions for wide use acting on the antisense oligonucleotide principle.

Phosphoramidates are somewhat less active than phosphorothioates in cell assays and, moreover, have toxic effects at a concentration of 100 μg/mL.[102]

Antisense oligonucleotides composed of α-anomeric nucleosides have, despite excellent hybridization properties, little or no activity in cell assays. It remains to be seen how far the good in vitro activity found when these oligonucleotides are coupled to photo-cross-linkers, or to other residues that modify nucleic acids, will emerge without toxicity on in vivo use.

IX. Applications

The possible applications of antisense oligonucleotides are very wide ranging because of the universality of the principle on which they are based. For example, they can be employed in fundamental research for preparing proteins shortened at the C terminus,[304] which makes structure/activity studies (deletion mapping) possible in a straightforward manner without mutagenesis of the appropriate gene. They are, furthermore, excellent tools for investigating a wide variety of mechanistic problems. Thus, they have already made crucial contributions to elucidating the functions of certain protooncogene products in cell differentiations.[314,316,433-435] The fascinating possibilities in this connection are, on the one hand, to gain insight into the development of cancer by use of the antisense oligonucleotide and, on the other hand, to employ suitably modified derivatives for cancer therapy at a later date. Since there have been, in particular, great advances recently in the characterization of oncogenes, the antisense oligonucleotides are destined to be used against cancer. Many of the recently published studies on protooncogenes deal with *c-myc*,[314,316,433,434] whose overexpression is correlated with several cancers of the lungs and colon and with neuroblastomas.[436]

Other potential endogenous points of attack are provided by inhibition of the expression of enzymes, ion channels, receptors, immunomodulators, and many other regulatory proteins. Thus, temporary inhibition

of expression of the T-cell receptor has recently been demonstrated.[437] Kawasaki has reported inhibition of the expression of interleukine-2 in oocytes using 18- to 23-mer antisense oligonucleotides in the picomolar range.[431]

Research is most advanced in the antiviral area. Even the early studies by Zamecnik and Stephenson[14,15] looked at antisense oligonucleotides directed against Rous sarcoma viruses. In addition, it has been possible to inhibit the growth of vesicular stomatitis viruses,[255,321,347] herpes simplex viruses,[58,247,320] and influenza virus[262] in cell culture. Antisense oligonucleotide research has experienced a considerable expansion in recent years due to the world-wide AIDS problem. A meeting was recently organized by the National Cancer Institute (NCI) and National Institute of Allergy and Infectious Diseases in Maryland solely devoted to the topic of antisense oligonucleotides. Many of the publications on HIV inhibition originate from the Zamecnik group or from the NCI where unmodified oligonucleotides,[252,327,376] phosphorothioates and phosphoramidates,[102,319] and methylphosphonates[295] have been successfully tested against HIV (cf. section VIII). The target sequences that could be most efficiently inhibited proved to be the splice sites[327,438] and primer-binding sites[327] of HIV.

For the sake of completeness, mention should also be made of the applications of the antisense technique in agriculture. Although synthetic oligonucleotides can be used, for example, to inhibit the translation of the mRNA of potato virus X,[389] it will probably be more economic to express antisense RNA in plants.[439,440]

X. Future Prospects

Many studies have demonstrated that antisense oligonucleotides are taken up by live cells despite their polyanionic nature. Both unmodified oligonucleotides and their derivatives with a different charge and polarity are able to pass through the cell membrane and inhibit specifically the expression of genes inside the cell. Two different groups have demonstrated the in vivo efficacy of modified antisense oligonucleotides against viral infections in mice. No toxicity problems have yet emerged. However, more extensive in vivo studies will take place only after the synthesis of oligonucleotides has been optimized and made less costly. Simply to change from deoxyribonucleotide to ribonucleotide units might reduce the costs by a factor of 10–20. The protective group technique that has been largely taken over from gene synthesis is too elaborate and costly for synthesizing antisense oligonucleotides on a large scale. If template-controlled chemical polymerization of nucleotides proves to be a reasonable alternative, it might be possible to dispense entirely with protective groups. Another future possibility is amplification of synthetic DNA similar to the polymerase chain reaction.[441] However, the large sums that still have to be found for the preparation of large amounts of antisense oligonucleotides must not be a deterrent. This problem can be solved. The consequences of abandonning the development of a therapeutic agent in the very early phase because of price considerations can clearly be seen when looking at the development of penicillin where the original price was several orders of magnitude higher than now.

However, a therapeutic use of antisense oligonucleotides also demands a lowering of the effective concentrations, which in cell assays are now about 100 nM in favorable cases but tend on average to be 10 μM. An improvement in the activity by only 2 orders of magnitude ought to make therapeutic use of antisense oligonucleotides possible. Besides increasing the ability to cross membranes, it might be of major importance to achieve the correct localization of antisense oligonucleotides in the various compartments of the cell. If these problems can be solved, antisense oligonucleotides promise to open up a new era of drug research with the possibility of rational drug design based on the nucleotide sequences of the genes causing the disease.

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