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This paper is dedicated to Professor David Shugar on the occasion of his 80th birthday

"Small is beautiful": major modifications in DNA structure or dynamics by small substituents or ligands*

Wilhelm Guschlbauer

CEA/Saclay, Service de Biochimie et Génétique Moléculaire, Bât. 142, F-91191 Gif-sur-Yvette Cedex, France

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This short review assembles the contributions of the author's laboratory to the structural aspects of DNA. DNA was modified by small ligands and/or substituents. There are three aspects to this work:

a) Protonation of guanosine and DNA and the formation of triple- and quadruple-strands of guanosine, its nucleotides, their polymers and DNA.

b) Substitution of the 2'-position of deoxyribose by the most polar atom, fluorine: studies on 2'-deoxy-2'-fluoro-nucleosides, -nucleotides and their polymers, studied both by structural and biological methods.

c) The effect of introducing the methyl group in the large groove of DNA: NMR studies of oligonucleotides containing N6-methylated adenine residues, and enzymatic and molecular biology work on Dam methylase are reported.

Anniversaries are festive occasions which offer the opportunity for reflection and retrospection. The approach I shall summarize here was started in 1965 when I had the great chance as a young postdoc to co-author a review with A.M. Michelson. It turned my interest to the study of the conformational capacities of nucleic acids and the perturbations caused in their structure by the interaction with ligands or substitution by atoms or molecules with the smallest van der Waals radii. These small groups give frequently rise to very large effects on the structure or the dynamics of nucleic acids. Here I shall describe briefly the principal steps of work done and the results obtained. The utilisation and the study of small perturbants has been characterized by three principal phases:

- I) The proton, the smallest and most ubiquitous cation and ligand (r_{vdW} = 1.20 Å): protonation of guanosine and DNA and formation of triple- and quadruple-strands of guanosine, the nucleotides, their polymers and DNA.
- II) Substitution of the 2' position of deoxyribose by a fluorine atom (r_{vdW} = 1.35 Å), barely larger than hydrogen, but the most polar atom: studies on 2'-deoxy-2'-fluoro-nucleosides, -nucleotides and their polymers. These studies covered a broad range of topics from chemical synthesis to pharmacology, encompassing most physico-chemical and enzymological techniques.
- III) More recently, the study of the effect of introducing another small substituent, the methyl group (r_{vdW} = 2.00 Å), in the large groove of DNA: this started with an NMR study of oligonucleotides containing N6-methylated adenine residues and is pursued now in the work on Dam methylase.

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PROTONATION OF DNA OR "WHY IS GUANOSINE DIFFERENT?"

After the proposal of the double helix by Watson & Crick [1] and the complementary pairing of A with T and G with C, many researchers set out to verify this hypothesis along two lines: on one hand, in their second paper, Watson & Crick [2] had envisaged that the change of *one* base to its alternative tautomeric form could induce mispairing during the replication mechanism [2]. Much work has been devoted to the role of tautomers in bases in DNA structure [3–6]. The existence of such tautomers in a DNA structure has been demonstrated unambiguously only recently [7]. On the other hand, X-ray structures of co-crystals between the complementary bases (or nucleosides) gave some surprising results: co-crystals of A and U (or T) *never* formed the Watson-Crick base pair, but gave rise to the Hoogsteen pair [8] or the reversed Hoogsteen pair [9]. Only co-crystals of stoichiometry A:U = 1:2 showed both Watson-Crick and Hoogsteen pairing [10, 11]. On the other hand, deoxyguanosine and 5-bromodeoxycytidine co-crystallized as the Watson-Crick pair [12], but with another conformation around the glycosidic linkage, which was *syn* [13], implying a parallel helicity. Attempts to obtain a G·C Hoogsteen pair in crystals have consistently failed, since an additional proton would be necessary to form it.

On the polynucleotide level it had been demonstrated early that poly(A)·poly(U) could disproportionate to form a triple stranded complex poly(U)·poly(A)·poly(U) [14]. A detailed study of the experimental conditions allowed to trace a "phase diagramme" of this system [15–17]. This work was extensively reviewed a long time ago [18, 19].

How to approach the problem of the G·C Hoogsteen pair? Can G·C pairs assume the Hoogsteen configuration in a polynucleotide structure or DNA? We have addressed the problem from several points of view.

We started by looking at the CD spectra of all nucleosides at neutral and acid pH. All nucleosides, *except guanosine*, maintained their characteristic Cotton effects. Only guanosine *inverted* its sign. In analogy with model compounds (Fig. 1) which are either fixed in the *syn* conformation, like 8-bromo-guanosine, or which assume easily this conformation, like 7-methyl-guanosine or formycin B, it was proposed that guanosine changed its conformation from *anti* at neutral to *syn* at acid pH [19–21]. Later NMR and NOE work plainly confirmed this interpretation of our early CD data [22].

With the studies on guanosine we were confronted with a unique phenomenon in nucleic acid chemistry: the gel formation by guanosine and its derivative [23, 24]. This phenomenon was strongly concentration dependent and proceeded in three steps: tetramerisation, dimerisation of two tetramers and polymerisation of the octamers [23]. Recently, it has been found that the telomeric ends of chromosomes form similar tetrameric structures (for reviews see ref. [24, 25]).

When DNAs were titrated to low pH, characteristic changes in ORD and CD spectra (Fig. 2) were observed [26]. These changes were proportional to the G·C content (Fig. 3) and took place in the spectral region where the inversion of CD spectra of guanosine occurred. Simultaneously, the Jena group [27] made very similar observations. They interpreted these results by the protonation of the cytidine residue in the center of the helix. Our proposal was more radical: protonation took place on the easily accessible N7 of guanosine in the major groove and a change to the *syn* conformation was pro-

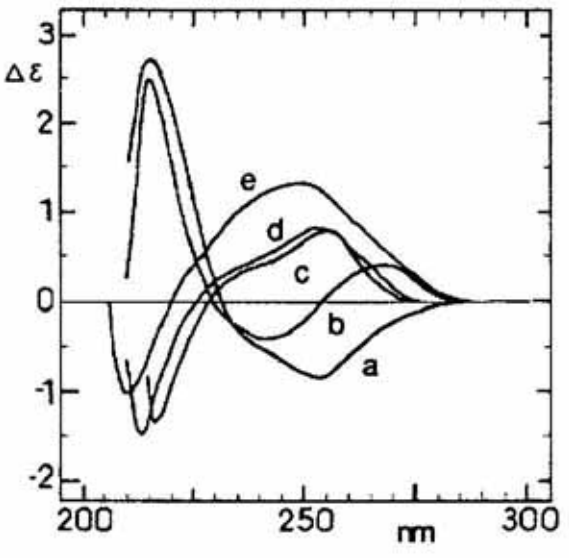

Fig. 1. CD spectra of various purine nucleosides. a, Guanosine, pH 7; b, 8-bromo-guanosine; c, 7-methyl-guanosine; d, guanosine, pH 1; e, formycin B. Adapted from ref. [21].

posed to form a Hoogsteen pair with the cytidine residue, thus satisfying the protonation sites of both bases.

To bolster this proposal, we began a series of studies on synthetic polynucleotides. The first was to re-investigate a curious result of Giannoni & Rich [28] who had observed a two-step transition during the acid titration of poly(I)·poly(C) [$(rI)_n \cdot (rC)_n$] and proposed the formation of protonated $(rC^+)_n \cdot (rI)_n$ Hoogsteen pairs. It had been postulated by Michelson *et al.* [18] that an intermediate protonated triple-stranded structure could be formed. A detailed study of the system $(rI)_n \cdot (rC)_n$ at various ionic strength and pH conditions promptly demonstrated (Fig. 4) the existence of a protonated triple stranded intermediate $(rC^+)_n \cdot (rI)_n \cdot (rC)_n$ [29] before rearrangement to the Hoogsteen pair $(rC^+)_n \cdot (rI)_n$ [30]. Similarly, the system $(rG)_n \cdot (rC)_n$ gave rise to the triple stranded structure $(rC^+)_n \cdot (rG)_n \cdot (rC)_n$ [31], but precipitated before the double stranded Hoogsteen pair could be formed. For such structures a conformational change of the purine nucleosides was not necessary, nor evident, although this possibility had been suggested [30].

Further studies on various poly-ribonucleotide and deoxyribonucleotide duplexes revealed that this kind of two-step disproportionation reaction was fairly general [32–36].

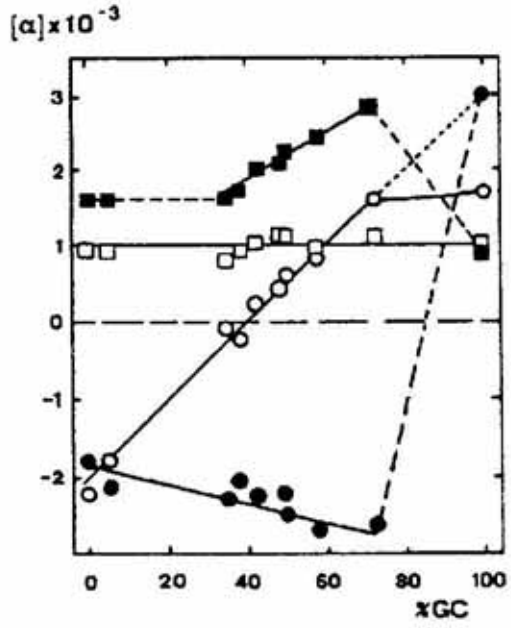

Fig. 2. Optical rotatory dispersion of various DNAs as a function of G·C content in 0.15 M NaCl at pH 7 (filled symbols) and pH 3.1 (open symbols). Circles: α_{290}, squares: α_{260}. Replotted from ref. [26].

Duplex-triplex disproportionation reactions took place upon acid titration of poly(dG)·poly(dC) [$(dG)_n \cdot (dC)_n$] [32–33]. A detailed study of this very complex system, however, revealed some interesting details. Commercial $(dG)_n \cdot (dC)_n$ samples were found to contain a mixture of several species [34] of which the duplex was not always the predominant one. In particular, a triplex $(dG)_n \cdot (dG)_n \cdot (dC)_n$ was found to be easily formed (Fig. 5a) and ubiquitous at neutral pH in equilibrium with double stranded $(dG)_n \cdot (dC)_n$, free $(dC)_n$ and associated (probably four-stranded) $(dG)_n$ [34]. The different complexes are characterized by very different CD spectra (Fig. 5b). Only alkaline treatment (pH > 11.5) and slow titration to neutral pH [32, 33] re-established the double stranded $(dG)_n \cdot (dC)_n$. At pH < 3, a new stable form was found [33] which was probably the Hoogsteen paired duplex $(dC^+)_n \cdot (dG)_n$ (Fig.

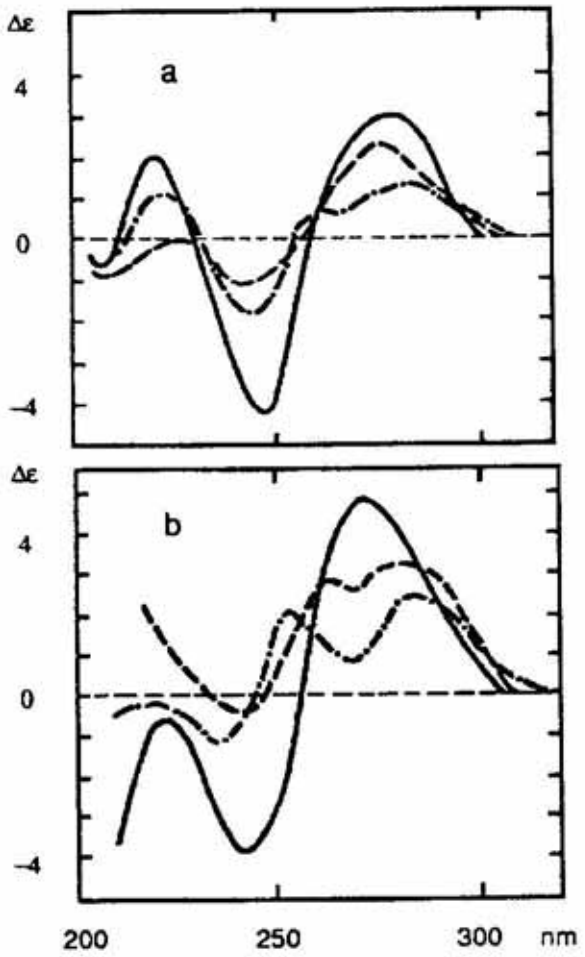

Fig. 3. CD spectra of DNAs at pH 7 (---), pH. 3.2 (- -) and pH 2.5 (- • -): a) Cytophaga johnsonii (32% G·C) and b) Micrococcus lysodeiktikus (72% G·C). Redrawn from ref. [26].

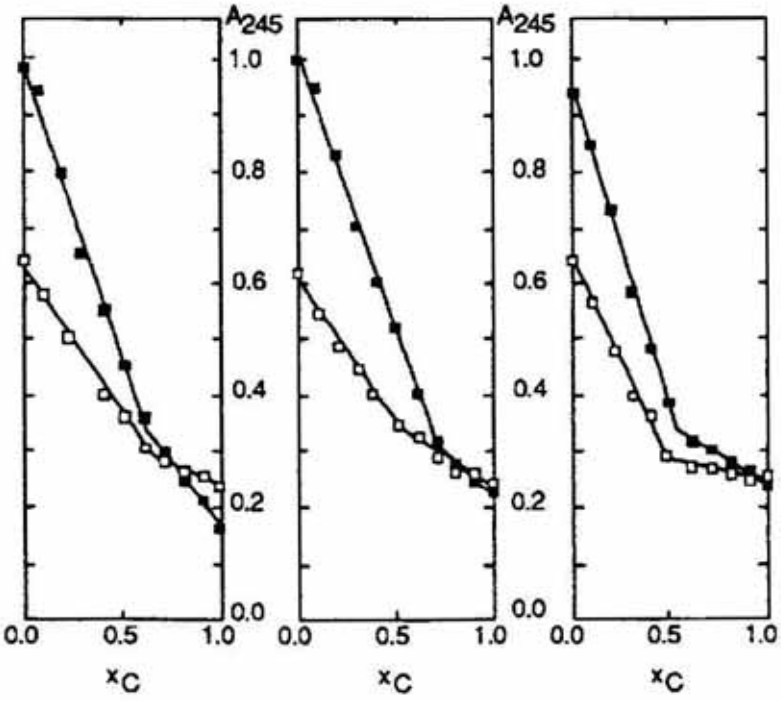

Fig. 4. Mixing curves between poly(I) and poly(C) under different experimental conditions.
Left panel, ■ pH 3, 0.03 M NaCl; ❑ pH 3.9, 0.6 M NaCl; central panel, ■ pH 4.7, 0.03 M NaCl; ❑ pH 5.2, 0.6 M NaCl; right panel, ■ pH 7.25, 0.03 M NaCl; ❑ pH 7.25, 0.6 M NaCl.

6). Note the similarity of this spectrum with that of natural DNAs at pH < 3 (see Fig. 3).

At pH < 6 triple stranded hybrid complexes formed between $(dG)_n \cdot (dC)_n$ and $(rC)_n$ [34]. Triplex formation was even observed with the corresponding ribo-oligonucleotides of different chain length [35]. While $(rC)_n$ could compete for the $(dC)_n$ strand in the duplex $(dG)_n \cdot (dC)_n$ at neutral pH, the oligo(rC) could not [36]. The formation of triplexes below pH 6 encouraged us to try to interact oligo(rC)s of various chain length with natural DNA. We chose T7 DNA which is known to contain oligo(dC) clusters [37]. Spectroscopic and t_m data showed that interaction took place between T7 DNA and $(rC)_n$ with n = 4, 5, 7, 9, 12

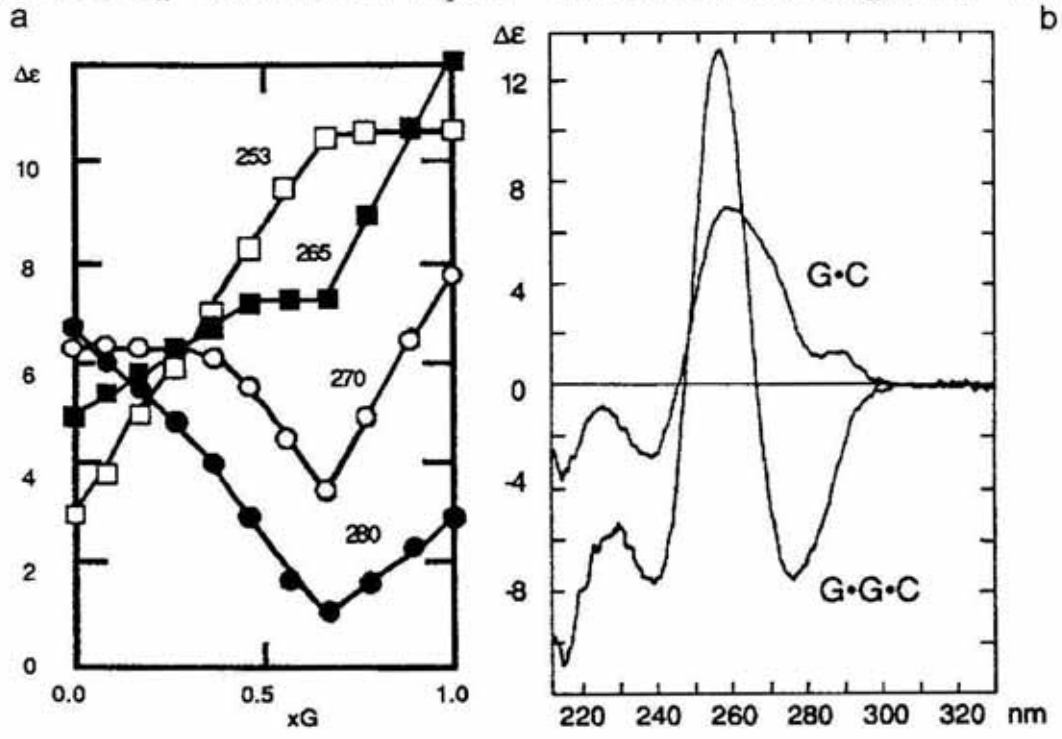

Fig. 5. a, CD mixing curve of poly(dC) with poly(dG) at pH 8, 48 h after heating the mixtures; b, Computed CD spectra of $(dG)_n \cdot (dC)_n$ and triplex $(dG)_n \cdot (dG)_n \cdot (dC)n$. Adapted from ref. [34].

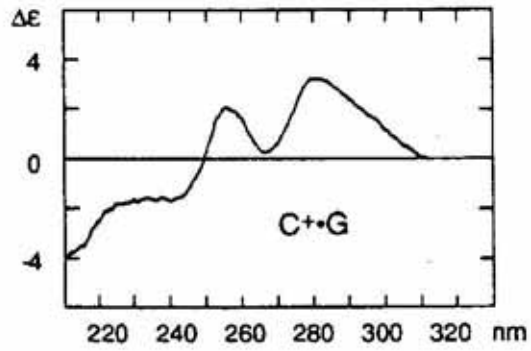

Fig. 6. Computed CD spectrum of the Hoogsteen paired duplex $(dC^+)_n \cdot (dG)_n$ [33].

[38]. What was most interesting, however, was the demonstration that *E. coli* RNA polymerase (with σ factor) was strongly inhibited by $(rC)_n$ with n = 7, 9 or 12 (Fig. 7). This inhibition did not take place on the promoter sites, but was due to either triplex formation or a local replacement of the dC clusters by oligo(rC) [38].

A different approach to study triplexes was used by Morgan & Wells [39] who transcribed $d(G\text{-}A)_n \cdot (d(C\text{-}T)_n$ at pH 8 and obtained $r(C\text{-}U)_n$ or $r(G\text{-}A)_n$, depending on the nucleoside triphosphates used. Below pH 6.5, $r(C\text{-}U)_n$ interacted with the parent duplex to form a triple stranded complex $r(C^+\text{-}U)_n \cdot d(G\text{-}A)_n \cdot (d(C\text{-}T)_n$. In the presence of $r(C\text{-}U)_n$ transcription was inhibited. The latter observation was confirmed for other polydeoxynucleotide duplexes [40, 41].

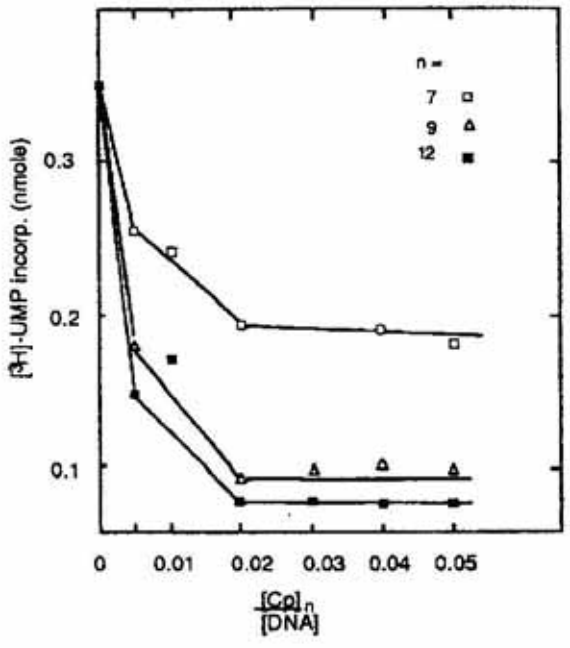

Fig. 7. Inhibition of transcription of T7 DNA by E. coli RNA polymerase (δ) as a function of increasing amounts of oligo(rC)s of chain length indicated [38].

Further development in triple strand helices was halted because funding was withheld with the unbeatable argument: "Everybody knows that nucleic acids are single or double stranded, but do not have three or four strands!". Several years later, numerous laboratories have begun intensively to work on triplex structures to control gene expression or to interfere with the binding of proteins on specific DNA targets. This has been accomplished either by selective triplex formation (see e.g. ref. [42–47]) or in conjunction with additionally stabilizing intercalating drugs (see e.g. ref. [48–50]).

FLUORINATION OF THE 2'-POSITION OF THE RIBOSE MOIETY OR "WHY IS DNA DIFFERENT FROM RNA?"

The essential difference between RNA and DNA, both from the structural and the biological view point, resides in the difference of the sugar puckering of the polynucleotide chain. While in the free nucleoside the two canonical sugar puckers, *N* (3'-*endo*) and *S* (2'-*endo*), are in rapid equilibrium, the sugar conformers of the polynucleotide chain are essentially fixed: RNA in the *N*-form, DNA in the *S*-form.

We had started our work by postulating that the differences between RNA and DNA were not determined by the size or the hydrogen bonding capacity of the 2'-substituent, but by its polarity [51]. In line with these findings were early observations [52–54] which indicated that the physico-chemical and biological properties of poly-2'-*O*-methyl- or -2'-*O*-ethyl-nucleotides were very similar to those of polyribonucleotides.

On the other hand, Cushley *et al.* [55] and our laboratory [56] found that the electronegativity of the 2'-substituent had a profound influence on the conformational equilibrium of the nucleosides: 2'-deoxy-2'-fluoronucleosides showed a pronounced preference for the *N* conformer, indicated by the very small coupling constant $J_{1'2'}$ and the large $J_{3'4'}$ coupling in the ^{1}HNMR spectra (Fig. 8). More detailed studies with adenine- [57] and uracil- [58] nucleosides clearly confirmed the early hypothesis: the more polar the substituent, the larger shall be the displacement below the plane of the sugar (Fig.

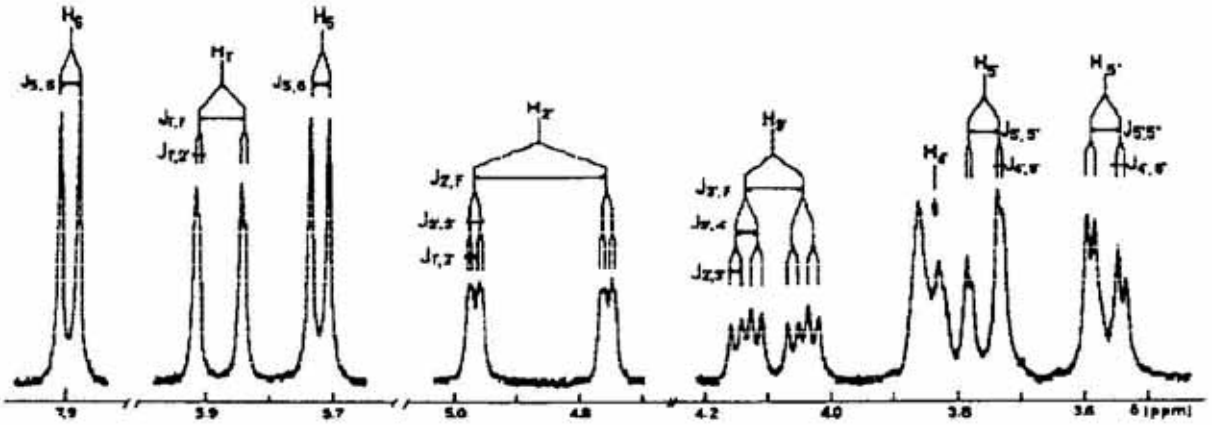

Fig. 8. ^{1}*HNMR spectrum of dCfl in DMSO [56].*

9b). X-Ray diffraction studies (Fig. 9a) presented further evidence for the preferential 3'-*endo* conformation of the 2'-deoxy-2'-fluoronucleosides [59]. Olson [60] gave a theoretical explanation to these observations: if the most polar groups (O4' and substituents on C2' and C3') are in the *gauche* (±60°) position to each other, the conformation shall have the lowest energy ("*gauche*-effect"). This is not only true for the 2'-substituent, but also for the 3'-replacements [55, 61]. For 2'-deoxy-2'-fluororibose this is the 3'-*endo* conformation. In other words these nucleosides behave like "super-ribosides".

On the polynucleotide level, $(dUfl)_n$ was shown to be similar to $(rU)_n$, but not to $(dU)_n$ [62]. Similarly, $(dCfl)_n$ showed great similarities with $(rC)_n$, but not with $(dC)_n$ [63]. Extensive work by Ikehara's group on 2'-deoxy-2'-fluoropurine-polynucleotides [64, 65] confirmed the ribose-like character of these polymers. We therefore embarked on the preparation of the six polymers of the I·C series, containing deoxyribose, ribose or 2'-deoxy-2'-fluororibose. Figure 10 shows the t_m values as a function of ionic strength [66]. In all three series the t_m values decrease in the order $(dCfl)_n > (rC)_n > (dC)_n$ and $(dIfl)_n > (rI)_n > (dI)_n$. The CD spectra (Fig. 11) show the "evolution" of the nine duplexes on the substitution in the purine or pyrimidine strand. The four duplexes in the upper left show the typical A-form CD spectra of $(rI)_n \cdot (rC)_n$ which are the same within the experimental error. Only these four duplexes interacted with $(rI)_n \cdot (rC)_n$ antibodies and also induced interferon, with the exception of $(dIfl)_n \cdot (dCfl)_n$ [66].

The similarity of the CD spectra of the $(dC)_n$ containing duplexes (right column of Fig. 11) suggested possible intermediate structures be-

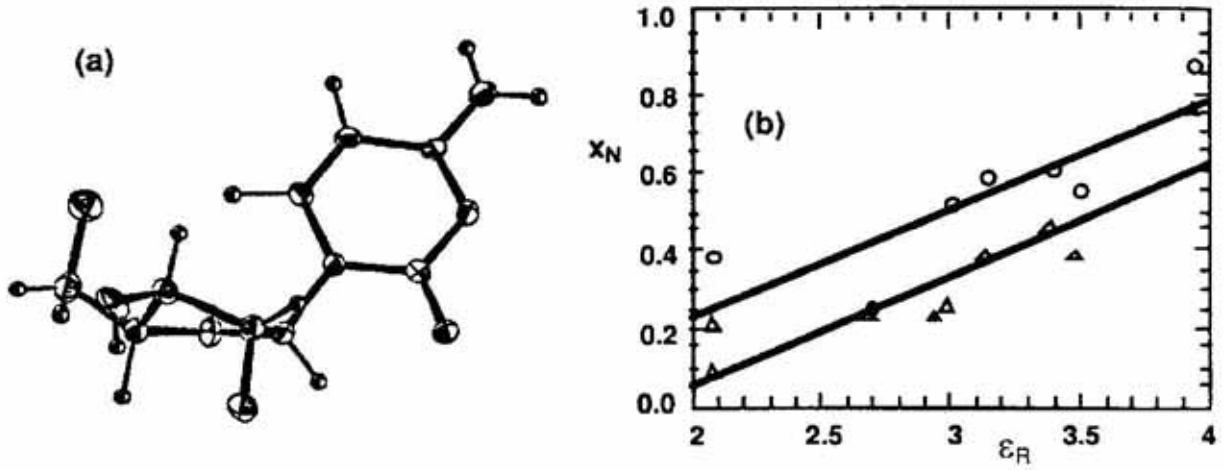

Fig. 9. (a) Crystal structure of dCfl [59]; (b) Dependence of the mole fraction in 3'-endo conformation (x_N) on the electronegativity of the 2'-substituent: (Δ) 2'-substituted adenosines [57]. (o) 2'-substituted uridines [58].

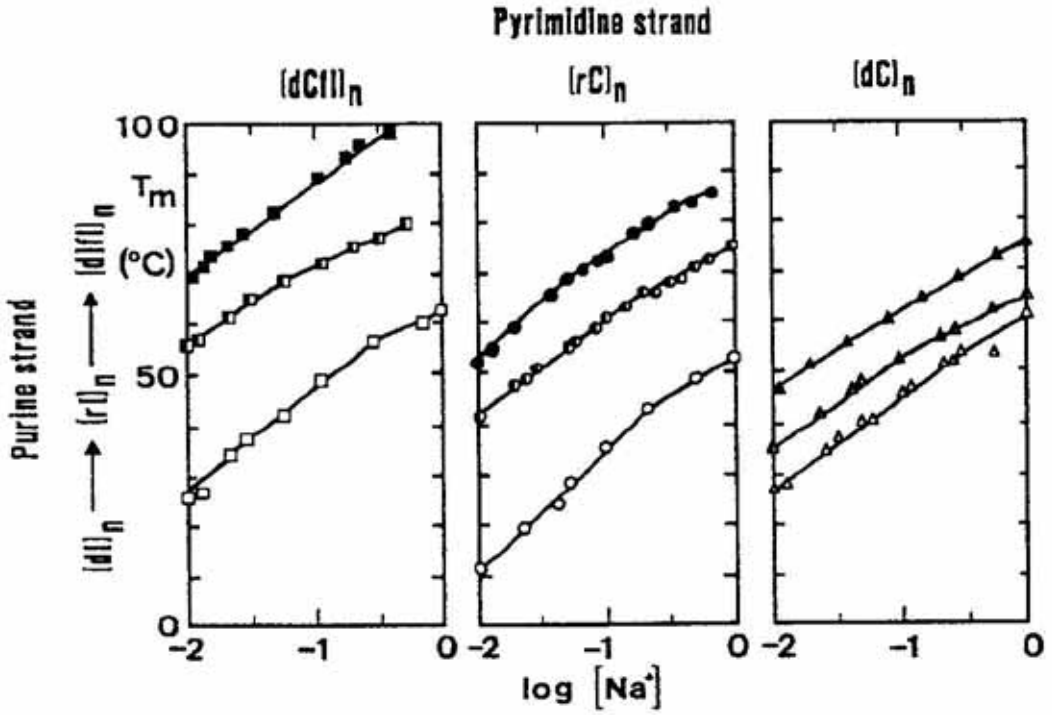

Fig. 10. t_m Values of the nine 2'-substituted homopolymer duplexes investigated (1 mM Tris, varying amounts of NaCl, pH 7.8). Adapted from ref. [66].

tween the B-form of $(dI)_n \cdot (dC)_n$ and the A-form duplexes of the $(rI)_n \cdot (rC)_n$ type. This was tested by the use of the groove binding antibiotics netropsin and distamycin which specifically require the B-form. Both antibiotics interacted not only with $(dI)_n \cdot (dC)_n$ and $(dA)_n \cdot (dT)_n$, but also with $(dIfl)_n \cdot (dC)_n$, $(dAfl)_n \cdot (dU)_n$ and $(dAfl)_n \cdot (dT)_n$ [67, 68], but did not interact at all with the $(rI)_n \cdot (dC)_n$ and $(rA)_n \cdot (dT)_n$ duplexes. This suggested that the 2'-fluoropolynucleo-

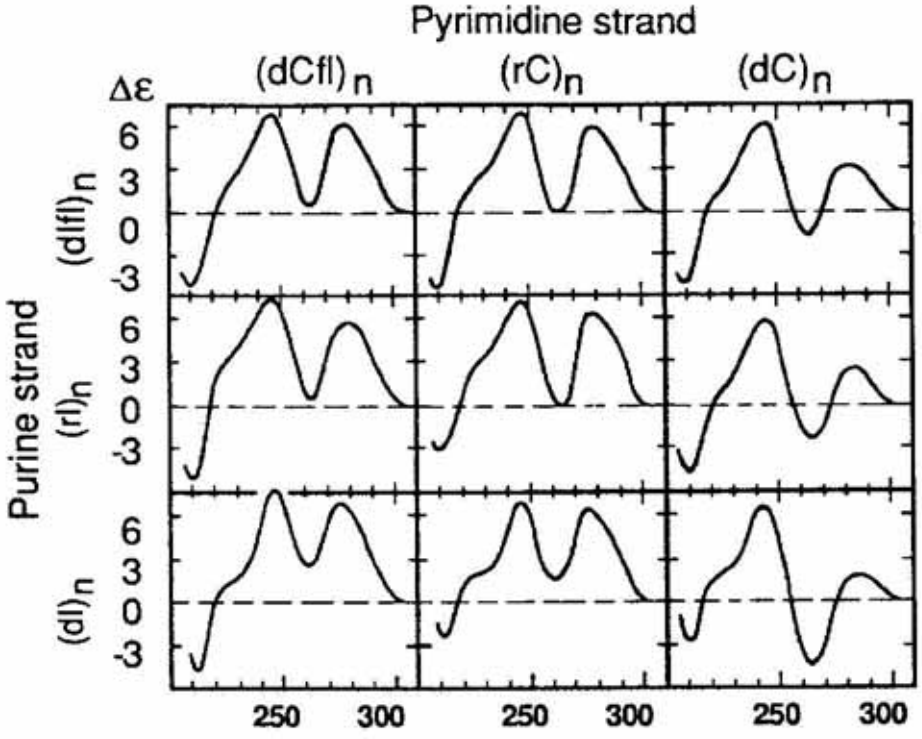

Fig. 11. CD spectra of the nine 2'-substituted homopolymer duplexes investigated (0.1 M NaCl, 1 mM Tris, pH 7.8). Adapted from ref. [66].

tides, particularly those containing purines, but not the ribo-polymers, may change their conformation from the *N* to the *S* form under external influences. Such change in conformation implies, however, a passage through the energetically unfavourable O4'-*endo* conformation (P = 90°) with a concomitant large energy barrier of about 2 to 4 kcal/mole [60, 69]. The energy barrier may, however, be smaller and crystal packing forces are sufficient to overcome this barrier. Thus the crystal structure of dUfl·2 H_2O shows the unusual puckering 4'-*exo* -O'-*endo* (P = 71°) [59] and that of dIfl·H_2O the 3'-*endo*-4'-*exo* pucker (P = 42°) [70]. This lowering of the energy barrier of 2'-deoxy-2'-fluoronucleosides also explains the acceptance of dUflMP by thymidylate synthetase, an enzyme which methylates dUMP to dTMP [71], while all other sugar substituted dUMP analogues were inhibitors. Similarly, dCflTP was also found to be a substrate both for RNA and DNA polymerases [72–75].

Recently, 2'-fluoronucleotides were incorporated into 17-mer oligonucleotides and the corresponding phosphorothioates [76, 77]. The CD spectra confirmed that the duplexes adopted a fully A-form conformation [76] and that t_m values were much higher than those of other duplexes. The duplexes were also used for antisense hybridization. While these compounds were resistant to RNase A, they were degraded by S1 nuclease or snake venom phosphodiesterase [77].

METHYLATION OF ADENINE IN THE LARGE GROOVE OF DNA OR "HOW TO MARK DNA FOR REPAIR?"

Another small substituent, natural this one, is the methyl group. The virtually universal methyl donor is *S*-adenosylmethionine (AdoMet). It intervenes in a very large number of enzymatic reactions. In particular, methylation of DNA [78] has been found to be a complex reaction with considerable repercussions in the cellular machinery. In bacteria, methylation of cytosine or adenine in DNA is a primitive form of immune response. The role of all the restriction-modification systems [79] found in bacteria is to protect the bacterial DNA from outside invasion, but also to protect its own DNA from self-destruction.

The *Escherichia coli* mismatch repair system consists of a number of gene products, all of which have specific enzyme functions. Two of these gene products, M·EcoDam and MutH recognize a specific nucleotide sequence, hemimethylated GATC. M·EcoDam plays a role in strand discrimination during mismatch repair [80]. It appears to function as a final "approval" system by N6-methylating the symmetrically disposed adenine residue, while MutH cuts at the hemimethylated GATC site to signal the strand to be repaired [81–83].

With these considerations in mind, we have addressed several questions:

- a) Does adenine methylation induce structural or other changes in hemi- or bi-methylated GATC sites?
- b) How does M·Dam methylase find its substrate and how does it act on the DNA site? How are the DNA and AdoMet, the methyl donor, bound and recognized?

NMR studies on N^6-methyladenine containing oligodeoxynucleotides

We have studied by high-resolution NMR a series of unmethylated, hemi-methylated and bi-methylated oligonucleotides duplexes containing the M·Dam recognition site GATC (Table 1).

One and two-dimensional NOE measurements showed that in all cases the overall helical structure is a canonical B-form and is unaffected by methylation [84–87]. Figure 12 shows partial NOESY spectra of the aromatic-anomeric region of complexes IIIb and IIIc. The similarities between the spectra indicate that the structure and distances in both helices are very similar and no major distortions have occurred.

On raising the temperature the chemical shifts of the non-exchangeable protons of the unmethylated oligodeoxynucleotides showed typical sigmoid curves. For the bi-methylated [84–86] and hemimethylated duplexes [87], upon an increase in temperature the resonances of the duplex state decreased in intensity and new resonances appeared corresponding to the single stranded species (Fig. 13), indicating that the two species were in slow exchange on the NMR time scale. The melting process could be followed up to the t_m on the duplex resonances by integrating them. Above the t_m, however, line broadening did not allow accurate integra-

Table 1
DNA sequences studied by ^{1}HNMR (mA = N6-methyladenine)

```
     5'G G A T C C
Ia     • • • • • •
       C C T A G G 5'

     5'G G A T A T C C
IIa    • • • • • • • •
       C C T A T A G G5'
       1                10
     5'G C G A T C A T G G
IIIa   • • • • • • • • • •
       C G C T A G T A C C5'
      20                11
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```
     5'G GmA T C C
Ib     • • • • • •
       C C TmA G G5'

     5'G GmA T A T C C
IIb    • • • • • • • •
       C C T A TmA G G5'

     5'G C G A T CmA T G G
IIIb   • • • • • • • • • •
       C G C T A G T A C C5'

     5'G C GmA T C A T G G
IIIc   • • • • • • • • • •
       C G C T A G T A C C5'

     5'C G C G TmA C G C G
IVb    • • • • • • • • • •
       G C G CmA T G C G C5'
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tions. The t_m values are decreased in hemi- or bi-methylated duplexes [88].

Since in duplex Ib, d(GGmATCC), the two N-methylamino groups are in close contact, which could be the cause of the described unusual behaviour of the complexes IIIb and IIIc, another pair of oligonucleotides was studied, duplex IIa and IIb, where the mA·T pairs are separated by two alternating A·T pairs [85, 86]. In duplex IIb a slow exchange between the duplex and single strand forms was again observed in the bi-methylated complex, indicating that this is an intrinsic result of adenine methylation and not of steric interference.

The *dynamics* of the opening and closing (the "breathing") of the double helix is therefore slowed down by adenine methylation. Lifetimes in the closed and open states are increased in bi- and hemi-methylated duplexes. We can estimate for the opening and closing rates a decrease of at least two orders of magnitude for the bi-methylated [84–86] and one order of magnitude for the hemi-methylated duplexes [87]. The slowing down of the closing

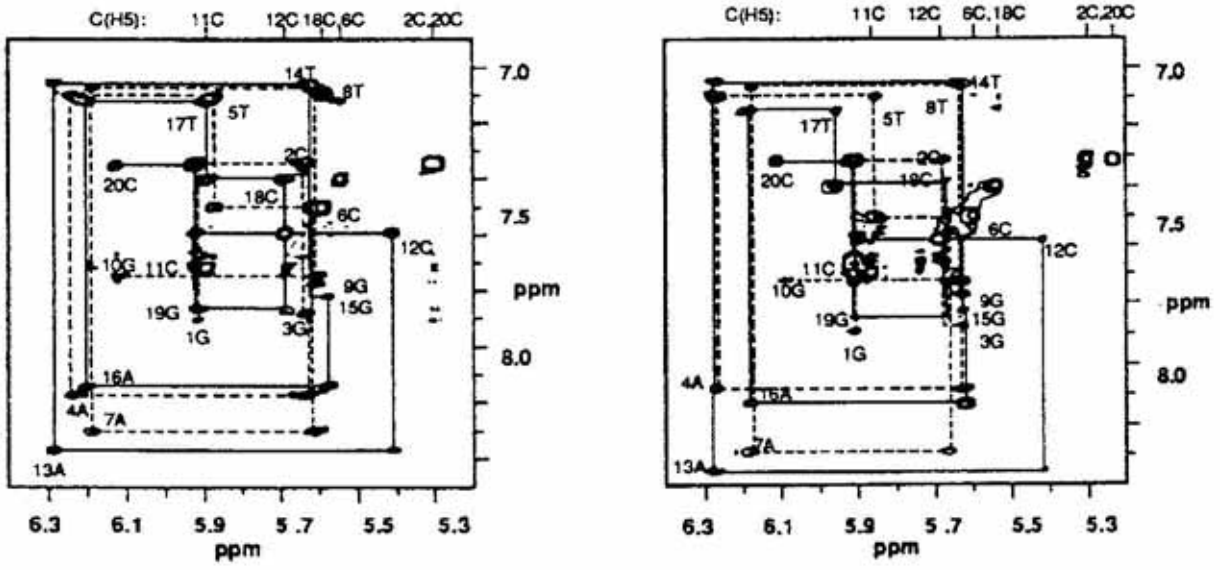


Fig. 12. Expanded NOESY contour maps of the aromatic-anomeric region of complexes IIIb and IIIc (from ref. [8]). NOE connectivities are represented for the top (- - -) and bottom (-------) strand, respectively. Adapted from ref. [87].

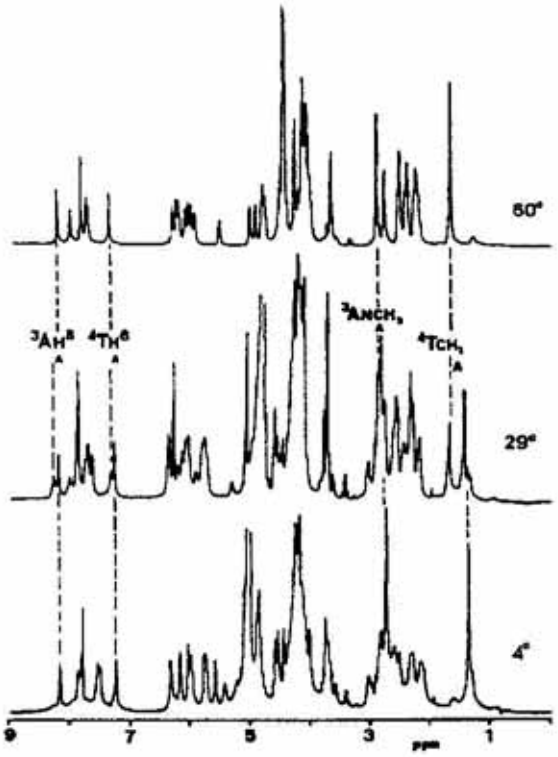


Fig. 13. 500 MHz NMR spectra of complex Ia (d(GGmATCC) in D_2O as a function of temperature).
Note the connectivities between resonances of double and single strand (dashed lines) species at 29°C (only given for $^3AH^8$, $^4TH^6$, $^3ANCH_3$ and $^4TCH_3$). Adapted from ref. [84].

rates can be explained by the isomerization of the methyl-amino group (Fig. 14), which prefers the *cis* isomer (*K*(*cis*)/(*trans*) = 20), unfavourable for Watson-Crick base pairing [89]. This, in turn, decreases the overall thermal stability of the duplex [88]. Interestingly, the same phenomenon is observed in oligonucleotides containing the N4-methylated cytidine residue [90].

The increased basicity of the methyl-amino group, however, increases the strength of base pairing in the bi- and hemi-methylated duplexes: thus, for instance, the imino resonances of d(GGATCC) disappear above 23°C, while those of d(GGmATCC) were still observed at 32°C (Fig. 15) [84]. This increase of the basicity of the methyl-amino hydrogen is responsible for the reduction of opening rates.

A significant sequence effect has been observed: lifetimes of the hemimethylated fragment CmATG of duplex IIIb were longer than those of GmATC (duplex IIIc) [87]. It is noteworthy that CGCGTmACGCG (duplex IVb), where mA is 3′ (and not 5′) to the T residue, a doubly methylated sequence apparently unknown in natural DNAs, possesses a greatly perturbed structure which is overwound and bent [91].

These results lead to the proposal that the distinctly different dynamic properties of GATC in the three different states of methyla-

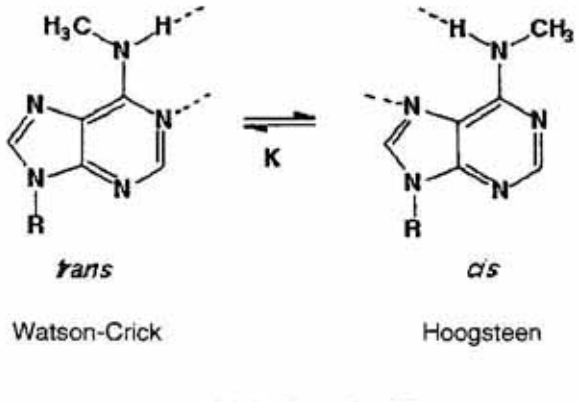


Fig. 14. Isomerization of the methylamino group in N6-methyladenine.

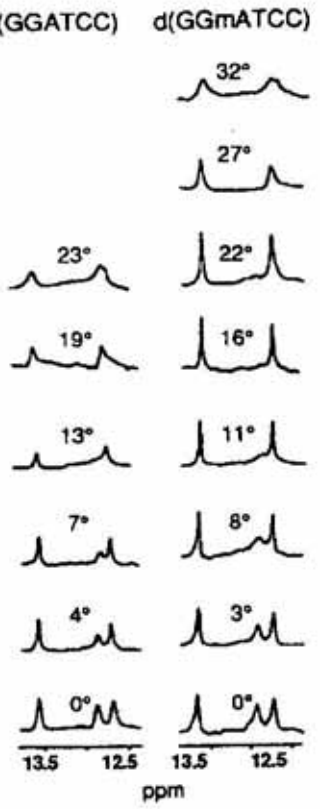


Fig. 15. NMR spectra of imino protons in 90% H_2O/10% D_2O of d(GGATCC) (duplex Ia) and d(GGmATCC) (duplex Ib) as a function of temperature (from ref. [84]).

tion are the basis of a *kinetic* recognition mechanism by adenine methyltransferases, like M·Dam, and possibly other proteins involved in mismatch repair of DNA, like MutH.

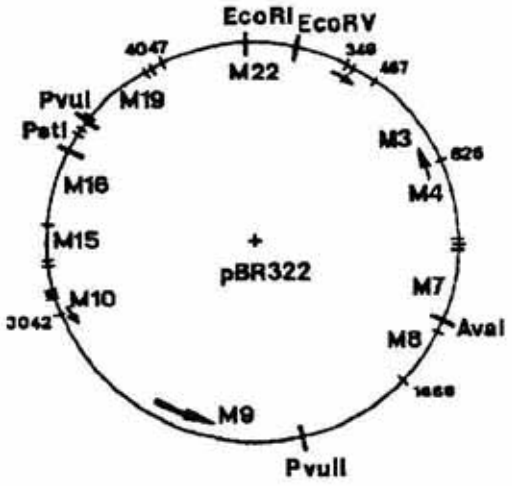
Enzymatic studies on M·Dam methylase from *E. coli*

The methylation of the 22 GATC sites of DNA from pBR322 (*dam*⁻) by M·Dam methylase from *E. coli* have been studied [92]. The rates of methylation of specific sites were the same in closed circular and linear DNA [92]. We have localized the first sites methylated on pBR322 DNA (Fig. 16a). Two complementary restriction enzymes were used to follow methylation of GATC sites. R·MboI cleaves only unmethylated GATC sites, while R·DpnI cuts exclusively on bi-methylated GmATC sites. Therefore, digestion patterns will appear with the latter enzyme only when bi-methylation has occurred. If either hemi- or bi-methylation occurred, R·MboI digests would give rise to larger fragments, due to the "fusion" of the two fragments surrounding the methylated GATC site. Combination of the two enzymes can thus distinguish between hemi- and bi-methylation events. Since no R·DpnI digestion patterns appeared at less than 10 methyl groups in average per DNA molecule, only one methyl group is transferred per binding event [93].

At very low degree of methylation (1 to 2 methyl groups per plasmid DNA) fragments M9+M10 (1374+75) and, to a lesser degree, M22+M1 (665+27) appear (Fig. 16b), indicating methylation of sites 3043 and 350. They are followed (at 2 to 3 methyl groups/DNA molecule) by fragments M8+M9+M10 (75+1374+207) and M7+M8+M9+M10 (75+1374+207+317), as well as M22+M1+M2 (665+27+91). This shows that sites 1669 and 1462, on the one hand, and site 372, on the other, are strongly methylated suggesting a processive mechanism for M·Dam methylation. pBR322 DNA linearized at position 185 by R·EcoRV did not affect the processive methylation on sites 3043, 1668 and 1461. On the other hand, in DNA linearized at position 2066 by R·PvuII the preferential methylation at position 3043 was conserved, but the processive methylation on positions 1669 and 1462 was interrupted. On the other hand this process was not interrupted when DNA was linearized by R·EcoRV (Fig. 16b).

Preferential hemimethylation thus took place primarily at positions 3043 and 350. The rapid methylation of the second and third GATC site suggests a processive mechanism. In both cases the flanking regions contain three G·C pairs on one side and two A·T pairs and a G·C pair on the other side, although the orientation is different in the two sites (Table 2). The next sites methylated were in both cases the first GATC on the A·T rich side.

The mechanism of methylation of GATC sequences was further investigated [94] using some of the synthetic oligonucleotides of Table 1. Gel retardation experiments with Dam methylase showed, however, that the oligonucleotides were too short. On the other hand, assays performed with a 321 bp DNA fragment from pBR322 with one GATC site gave rise to a



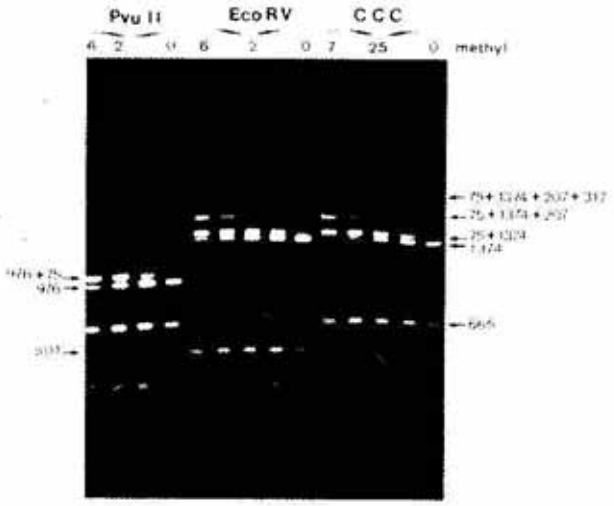


Fig. 16. a, pBR322 (dam⁻) map indicating major single restriction sites and the 22 GATC sites; b, gel electrophoresis of in vitro methylated pBR322 DNA (dam⁻), closed circular (right) and linearized by R·PvuII (left) or R·EcoRV (center).

Table 2

Positions and environment of GATC sequences in pBR322 (only the top strand is shown) and distances (Δ) between them (length of R·MboI fragments).

Polarity of the adjacent sequences is indicated by arrows.

		position	Δ
M1	TACGC **GATC** ATGGC	350	
	→		27
M2	CTGTG **GATC** CTCTA	377	
			91
M3	GGGAA **GATC** GGGCT	468	
	←		359
M4	ACGAT **GATC** GGCCT	827	
	←		272
M5	TCAAG **GATC** GCTCG	1099	
			31
M6	ACTTC **GATC** ACTGG	1130	
			15
M7	CCGCT **GATC** GTCAC	1145	
			317
M8	CGCAT **GATC** GTGCT	1462	
			207
M9	TTCCG **GATC** TGCAT	1669	
			1374
M10	CTCTT **GATC** CGGCA	3043	
	←		75
M11	AAAAG **GATC** TCAAG	3118	
			11
M12	AAGAA **GATC** CTTTG	3129	
			8
M13	CCTTT **GATC** TTTTC	3137	
			78
M14	AAAAG **GATC** TTCAC	3215	
			12
M15	ACCTA **GATC** CTTTT	3227	
			105
M16	TCAGC **GATC** TGTCT	3332	
			341
M17	CCAAC **GATC** AAGGC	3673	
			18
M18	TACAT **GATC** CCCCA	3691	
	←		46
M19	CCTCC **GATC** GTTGT	3737	
			258
M20	TCAAG **GATC** TTACC	3995	
			17
M21	GTTGA **GATC** CAGTT	4012	
			36
M22	CAACT **GATC** TTCAG	4048	
			655

shift pattern with a number of bands increasing with the quantity of enzyme added, corresponding to the probe shifted by one, two or more molecules of Dam methylase. Since there was no accumulation of the first complex, Dam methylase apparently exhibited affinity also for unspecific DNA, which was not greatly different for specific and unspecifc DNA. We finally settled for a tetradecamer

5′GCGC**GATC**ATGGCG3′

3′CCCG**CTAG**TACCGC5′

with the asymmetry characteristics of the preferred target sequences in Table 2.

In the presence of AdoMet, the association of Dam methylase to its specific site was increased by a factor of about five: K_D of the enzyme for the 14-mer fragment in the presence of 100 μM AdoMet was about 60–70 nM, but about 400 nM in the absence of AdoMet. The complex, 14-mer duplex/Dam methylase/AdoMet migrated slightly faster than complexes formed with specific DNA in the absence of AdoMet or with non-specific DNA. Apparently, the simultaneous binding of AdoMet and the GATC site induced a conformational change in the enzyme.

Adenosylhomocysteine (AdoHcy) and sinefungin are competitive inhibitors for AdoMet of Dam methylase with K_i values very similar to the K_m of AdoMet (about 5 μM) [94]. The presence of either of these two analogues did not change the shift pattern of binding of Dam methylase on the 14-mer duplex (Fig. 17, panel A). Complexes formed in the presence of these analogues still migrated like those formed in the absence of AdoMet, unable to induce the conformational change that increased the binding affinity of the enzyme for GATC. These two

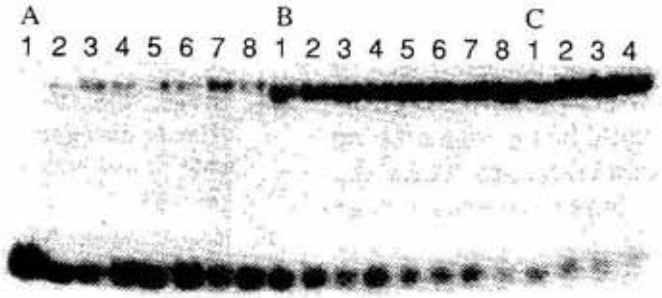


Fig. 17. Effect of AdoMet and its analogues on the binding of the Dam methylase on the tetramer duplex. 10 nM of duplex was incubated at 0°C with 100 nM of Dam methylase with various concentrations of AdoMet or/and of analogues (from ref. [94]).

Panel A: lane 1: no enzyme; lanes 2-4: AdoHcy 1, 10, 100 μM; lanes 5-7: sinefungin 1, 10, 100 μM; lane 8: no AdoMet, no analogues. Panel B: lanes 1-8: AdoMet 100, 200, 400, 600, 800 nM, 2, 4, 10 μM. Panel C: 400 nM AdoMet; lane 1: + 400 nM AdoHcy; lane 2: + 4 μM AdoHcy; lane 3: + 400 nM; lane 4: + 4 μM sinefungin.

analogues did not, however, even in an excess over AdoMet concentrations, suppress the increase of affinity of AdoMet for its specific site on Dam methylase (Fig. 17, panel C). This suggests that Dam methylase contains two binding sites for AdoMet: first, an allosteric one, to reinforce the affinity of the enzyme for the GATC site with a strong K_D, and secondly, the catalytic site, which binds the methyl donor for transferring the methyl group. Sinefungin and AdoHcy could compete for the catalytic, but not for the allosteric site.

To further investigate the details of this ternary complex formation, ^{3}HNMR was used [95]. Experiments were done with a fivefold excess of sinefungin over AdoMet, since it did not induce the conformational change, to avoid the rapid methylation of the oligonucleotide substrate. The added oligonucleotide induced a fast conformational change resulting in a downfield shift, followed by a slower upfield shift of the [^{3}H]CH_3-AdoMet resonance. This indicates that the environment of the methyl group changes before the methylation reaction takes place.

Finally, recently we have studied the kinetics of methylation of 14-meric hemi-methylated oligonucleotides containing base substitutions [96]. Interestingly enough the enzyme is rather tolerant of many modifications. The relative free energy of dissociation (related to K_m values) appears to be inversely related to the thermodynamic free energy ($\Delta\Delta G^o$) which indicates that lower thermodynamic stability favours the binding of the enzyme in the ternary complex. On the other hand, the transition state free energy $\Delta\Delta G_t$ (related to k_{cat}/K_m) shows a reasonable correlation with $\Delta\Delta G^o$ which suggests that the specificity of the interaction is related to the thermodynamic stability of the duplexes.

CONCLUSION

In this short mini-review it is attempted to summarize the importance of the influence of small perturbants on DNA structure.

The ubiquitous presence of H^+ ions, which can also be supplied or withdrawn by ionizable groups in proteins, gives protonation of DNA a particular importance. Like methylation which, however, only takes place on specific and highly defined sequences, protonation could and can take place on a small region of the DNA sequence, e.g. dG·dC-clusters. Such minute changes are difficult to localize (see ref. [97]), but can have important repercussions on the function of the genome. The use of triple-strand forming oligomers to control gene expression or transcription [38–50] is an example.

The case of fluorine substitution is different, primarily because it is not a natural substituent. Here we have demonstrated that the *reduction* of the size of the prosthetic 2′-OH group did not change the intrinsic conformational properties of the thus modified compound. It also conferred some of the properties of the "small" brother, the deoxynucleosides, insofar as under certain outside effects, like interactions with enzymes, DNA-binding drugs etc., the 2′-deoxy-2′-fluoro-nucleosides can assume the 2′-*endo* (*S*) conformation, both in the monomeric and in the polymeric state.

In conclusion, small substituents can induce large effects!

The work reported is the fruits of the collaboration with many students and postdocs, as well as many colleagues to which the author is greatly indebted. The list would be too long and the danger of leaving somebody out would be great. To all of them I wish to express my deep gratitude.

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