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The Mode of Interaction of Mitomycin C with Deoxyribonucleic Acid and Other Polynucleotides *in Vitro* †

Maria Tomasz,* Carmen M. Mercado,‡ Janet Olson, and Nithiananda Chatterjie§

ABSTRACT: Reductive activation of mitomycin to a partially reduced form rather than to the fully reduced hydroquinone greatly increases the binding affinity of the drug to native and denatured DNA: complexes of up to a few nucleotides per mitomycin molecule are readily formed in a few minutes at room temperature. The partially reduced form of mitomycin is believed to be the semiquinone radical. The complexes exhibit the following properties: same ultraviolet spectrum as apomitomycin derivatives, increased T_m, resistance to enzymatic hydrolysis. Bound mitomycin is not released under a variety of strong conditions known to dissociate noncovalent complexes. Depurination releases the drug and normal amounts of free guanine and adenine. Upon acid hydrolysis of the complex to bases normal base ratios are obtained. Monomeric nucleotides and GpC do not react with activated mitomycin. Poly(dG) binds mitomycin well while binding to poly(dA), -(dT), or -(dC) is negligible. Binding to poly(dG·dC) or poly(dI·dC) is relatively inhibited. Cross-linking is not observed with ds-RNA. The cross-links in DNA are much more stable than bifunctional mustard-induced cross-links. The hydrolysis product of mitomycin lacking the aziridine ring binds well to DNA but exhibits no cross-linking activity. A model is proposed to explain the results. Covalent bond formation is preceded by a noncovalent association, presumably of the intercalative type, between the mitomycin semiquinone and the polynucleotide, which then facilitates covalent bond formation between appropriately situated functional groups of DNA and the antibiotic. The site of covalent bonds is possibly the O^6 position of guanine.

A large variety of antibiotics are known to interact with the nucleic acids of the cell. As these drugs combine with DNA and/or RNA they modify the natural structure of the nucleic acid, resulting in an inhibition of its template function. Frequently this effect is one or even the only fundamental cause of the biological effect of the antibiotic, as, for example, in the case of the most extensively studied nucleic acid modifying antibiotic, actinomycin. Since most antibiotics are complex molecules the structure of the drug–DNA complex is not known with certainty in a single case. In most instances the affinity in such complexes is noncovalent. Covalent association has been recognized, however, in one unique case: the mitomycin antibiotics. (For a recent review of this field and specific references see Goldberg and Friedman, 1971.)

The mitomycins, objects of the present study, are a class of potent antibiotics. They also exhibit high antitumor activity and act as lysogenic inducers, mutagens, and carcinogens. (For specific references, see Szybalski and Iyer, 1967.) The most studied member of the group is mitomycin C (I) although the others, *e.g.*, porfiromycin (II), are very similar, both chemically and biologically. They are small

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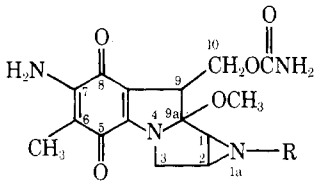
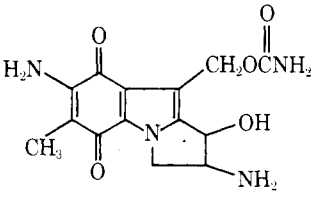
‡ Present address: John Jay College, The City University of New York, New York, N. Y. 10019.

§ Present address: Cornell University Medical College, New York, N. Y. 10021.



I, mitomycin C (R = H)
II, porfiromycin (R = CH_3)



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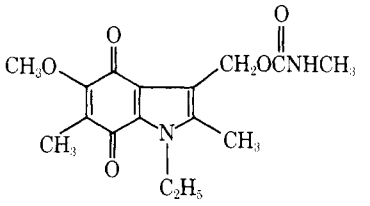

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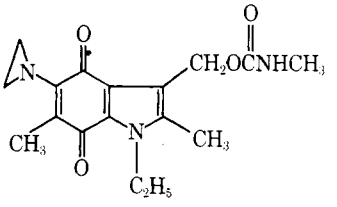

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but complex molecules with several potential biologically active groups, such as the C-1, C-2 aziridine ring; the C-10 carbamate group; and the dihydroindoloquinone moiety. These antibiotics preferentially inhibit DNA synthesis in bacteria and DNA appears to be the direct target for their lethality (see the review quoted above). The DNA of MC[1]-treated bacteria is cross-linked (Iyer and Szybalski, 1963; Matsumoto and Lark, 1963). No such or any other effect of MC on DNA could be observed *in vitro*. However, when a chemical reducing agent or a NADPH-dependent cell extract was added to the DNA-mitomycin mixture cross-link formation was reproduced *in vitro* (Iyer and Szybalski, 1964). In addition to the cross-links, attachment of the drug in a monofunctional (non-cross-linking) manner to DNA was also observed, in a ratio 10:1 to the cross-links, approximately (Szybalski and Iyer, 1964; Weissbach and Lisio, 1965). A similar ratio was found in DNA-MC complexes formed *in vivo* (Szybalski and Iyer, 1964). Based on these findings and a theoretical analysis of the functional groups of the molecule Iyer and Szybalski (1964) postulated that the reduced (hydroquinone) form of the mitomycins functions as a bifunctional alkylating agent, in a conceptual analogy to synthetic cross-linking agents such as HN2 (Geiduschek, 1961). Thus, the aziridine ring and the C-10 carbamate are the two "leaving groups," whose displacement by nucleophilic groups in opposite strands of DNA will result in a cross-link. The predominant monofunctional alkylation was postulated to arise by only one of the leaving groups reacting with the nucleic acid. Since the cross-linking efficiency *in vitro* increased with increasing (Gua + Cyt) of the DNA, guanine or guanine and cytosine were suggested as possible sites of interaction.

This model of the reactive groups of the mitomycins while attractive has remained hypothetical. Reactive sites of DNA are also unknown. Contrary to a previous suggestion that in analogy to bifunctional mustards, guanine-7 positions are involved (Lipsett and Weissbach, 1965) we showed (Tomasz, 1970) by a sensitive radioactive assay developed for this purpose that this is not the case. The suggestion has been also disputed on other grounds (Szybalski and Iyer, 1967).

Along another line of developments structure–activity relationship studies reported from three laboratories (Weiss *et al.*, 1968; Kinoshita *et al.*, 1971a,b; Mercado and Tomasz, 1972) revealed that analogs lacking the C-1,C-2 aziridine rings (for instance III, IV, V, and many others) are also potent antibiotics and mimic other characteristic effects of the parent mitomycins, most notably a selective inhibition of DNA synthesis. These findings suggest that factors other than aziridine-mediated cross-link formation may be important in the overall modification of DNA by the mitomycins and their close analogs.

The reason for lack of evidence regarding the chemistry of the DNA–mitomycin complex is probably that in contrast to other antibiotics the *in vitro* system of interaction (Iyer and Szybalski, 1964) has not been a simple and convenient one for study. The problem is associated with a requirement, unique among DNA-modifying antibiotics, for activation of the drug. The activated form is short-lived, never has been isolated, its structure is uncertain, and what is recovered after the reduction is inactive and an ill-defined mixture (Patrick *et al.*, 1964; Iyer and Szybalski, 1964). Another problem is that only marginal amounts of mitomycin have been reported to incorporate into DNA *in vitro* (maximally one per several hundred nucleotides; Weissbach and Lisio, 1965). At such low levels of substitution no physicochemical characteristics[2] (spectral changes, binding isotherms, changes in T_m, etc.) or chemical properties (base ratio changes, formation of modified bases) would be easily detected.

In view of these problems we undertook exploration of the chemical effects of mitomycin on DNA and other polynucleotides. As a primary goal we hoped to increase the efficiency of the *in vitro* interaction system so that the chemical effects will be amplified and detectable by the usual methods.

Experimental Section

Materials

The following substances were obtained commercially: mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan), *Escherichia coli* K_{12}DNA (General Biochemicals, Chagrin Falls, Ohio), rRNA, 16S + 23S, and poly(I·C) (Miles Laboratories, Elkhart, Ind.), all other synthetic polynucleotides (P-L Biochemicals, Milwaukee, Wis.).

Commercial *E. coli* K_{12}DNA was further purified by digestion with ribonuclease T-1 (ca. 10^2 units/ml in a 1 mg/ml of DNA solution in $1/10$ SSC, 1 hr, 37°), followed by precipitation and repeated deproteinization by $CHCl_3$ according to the Marmur procedure (Marmur, 1961). Analysis indicated less than 0.5% protein.

Denatured DNA was prepared by adding 3 N NaOH to DNA solution to pH 12.7, stirring for 10 min, followed by neutralization with 1 N HCl, then dialysis in 0.017 M sodi-

[1] Abbreviations used are: MC, mitomycin C; PM, porfiromycin; SSC, standard saline citrate (0.15 M NaCl–0.02 M sodium citrate (pH 7.4)); $1/10$ SSC, SSC diluted tenfold with distilled water; HN2, di(2-chloroethyl)methylamine.

[2] With the exception of cross-link formation for which the detection methods are extremely sensitive (Iyer and Szybalski, 1963).

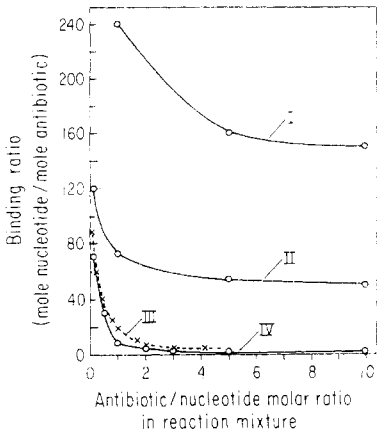


FIGURE 1: DNA-mitomycin binding curves. (I, II) Conventional activation (method A): (I) [^{3}H]porfiromycin-native DNA complex; (II) [^{3}H]porfiromycin-denatured DNA complex. (III, IV) New activation (method B): (III) mitomycin C-native DNA complex; (IV) mitomycin C-denatured DNA complex. All complexes were isolated by Sephadex G-100 chromatography.

um phosphate buffer (pH 7.5). The stock solution was stored frozen.

Mitomycin C stock solution was prepared in 30% methanol (Spectrograde) and kept at −15° in the dark.

[1a-^{3}H]Porfiromycin (8.3 Ci/μmol) was prepared by methylation of mitomycin C with [^{3}H]methyl iodide by a published procedure for nonradioactive synthesis (Stevens *et al.*, 1965), followed by purification by paper chromatography in 1-propanol–1% NH_4OH (2:1, v/v) on Whatman 40 sheets from which it was eluted in the same solvent.

The hydrolysis product of mitomycin C (III) was prepared by a published procedure (Stevens *et al.*, 1965).

Methods

DNA melting and cooling curves were taken on a Gilford instrument. Ultraviolet spectra were obtained using a Cary 14 spectrophotometer.

Paper Chromatography. The descending method was used. Solvents: I, 1-propanol–concentrated NH_4OH–H_2O, 55:10:35 (v/v); II, 90% methanol–H_2O; III, isobutyric acid–0.5 M NH_4OH, 10:6 (v/v); IV, NH_4OAc–ethanol, 7:3 (v/v); V, isopropyl alcohol–concentrated HCl–H_2O, 170:41:39 (v/v).

Mitomycin containing solutions and paper chromatography equipment were routinely protected from prolonged exposure to light by covering with aluminum foil.

Activation of Mitomycin or Porfiromycin in Situ and Complex Formation with Polynucleotides. METHOD A. This is the conventional procedure as introduced in its general form by Iyer and Szybalski (1964). The polynucleotide and MC or PM were mixed in 0.017 M sodium phosphate buffer (pH 7.5) (0.7–1 μmol of polynucleotide/ml) and deaerated by bubbling He gas (Matheson "research grade," >99.9999% purity) through the solution for 20 min. The reducing agent (0.1 M $Na_2S_2O_4$, freshly prepared by adding the solid to similarly deaerated water) was then added at once, using a syringe, in 1.5 molar excess to mitomycin.[3] The bubbling continued for 10 min, then the mixture was exposed to air and the polynucleotide–antibiotic complex was isolated by one of several separation methods.

[3] The stoichiometry of the quinone to hydroquinone reduction requires 1 mol of $Na_2S_2O_4$/mol of mitomycin. We "standardized" our solid $Na_2S_2O_4$ from time to time by titrating mitomycin with fresh $Na_2S_2O_4$ solution. Sudden decolorization of the MC solution indicated the end point.

TABLE 1: Effect of Excess Nonreduced MC on the Binding of Reduced MC to DNA.

	μmol of MC Added Overall[a]	μmol of MC Reduced[b]	Binding Ratio (mol of Nucleotide/mol of MC)[c]
Control	1	1	72
Sample	10	1	3

[a] To 1 μmol of DNA (denatured) in 1.5 ml of 0.017 M sodium phosphate buffer (pH 7.4) deaerated as in Methods. [b] In one step, see Methods. [c] Assayed by the spectroscopic method.

METHOD B. Same as method A, except the reducing agent was *not added at once* but rather in five equal portions at 5-min intervals.

Separation of the Polynucleotide–MC Complex from Unbound Antibiotic. The most convenient method was Sephadex G-100 chromatography (2.5 × 30 cm column, for 1–4 μmol of polynucleotide, in max. 6-ml volume), using SSC as eluent. Alternatively, for DNA–MC complexes, hydroxylapatite (Bio-Gel HTP, DNA grade) chromatography was occasionally employed (Bernardi, 1969) using a very short column (1 × 1 cm, 200 ml of a linear gradient of 10^{-3} to 0.5 M sodium phosphate buffer (pH 6.8)) upon which the unbound antibiotic separated widely from the later emerging DNA–MC complex (native or denatured).

Assay of the Binding Ratio in Polynucleotide–MC (or PM) Complexes. The binding ratio is defined here as molar ratio of mononucleotide unit to antibiotic. Two different assays were used, depending on the magnitude of the binding ratio of the complex, as follows.

(a) RADIOACTIVE METHOD FOR HIGH BINDING RATIOS (>50). [1a-^{3}H] Porfiromycin was employed in forming these complexes. Mononucleotide was assayed by measuring ultraviolet absorbance in SSC. The following extinction coefficients were used at 260 mμ: DNA (native), 7000; DNA (denatured), 7910; RNA (ribosomal), 7200. These values were determined by ourselves by phosphate analysis (Ames and Dubin, 1960) of stock solutions. For the synthetic polynucleotides ϵ values at λ_{max} as given by the manufacturer were used. Bound [^{3}H]PM was determined by the following counting procedure, adapted after Hattori *et al.* (1965): 0.5 ml of the [^{3}H]PM–polynucleotide complex solution was hydrolyzed by incubation with 40 μl of concentrated HCl for 24 hr at 50°. Then 0.1 ml of hyamine hydroxide and 10 ml of Aquasol was added and the mix was counted in a liquid scintillation counter. For control for quenching an aliquot of the stock [^{3}H]PM solution of known specific activity, see above, was mixed with the same amount of stock polynucleotide as that in the complex sample and this mixture was similarly hydrolyzed and counted. A 45–50% quenching was observed regularly. Tritium counting efficiency of the counter was 25%.

(b) SPECTRAL METHOD FOR LOW BINDING RATIOS (<50). Mononucleotide was assayed by the phosphate analysis method of Ames and Dubin (1960), since the ultraviolet absorbance at 260 mμ as in (a) may not be accurate for

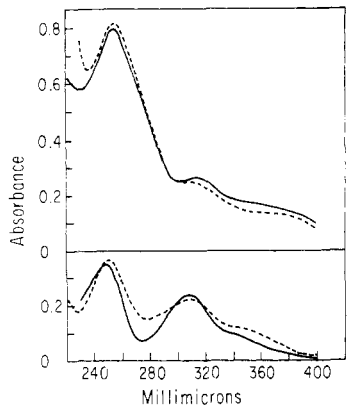


FIGURE 2: Ultraviolet spectra of DNA-mitomycin C complexes. All spectra were taken in SSC, pH 7.5. Upper section: (—) native DNA-MC complex; (- - -) denatured DNA-MC complex. Lower section: (- - -) computed spectrum of MC bound to denatured DNA, obtained by subtracting the spectrum of DNA alone at the same concentration from the spectrum of the complex (binding ratio *ca.* 3); (—) spectrum of III at the same molar concentration.

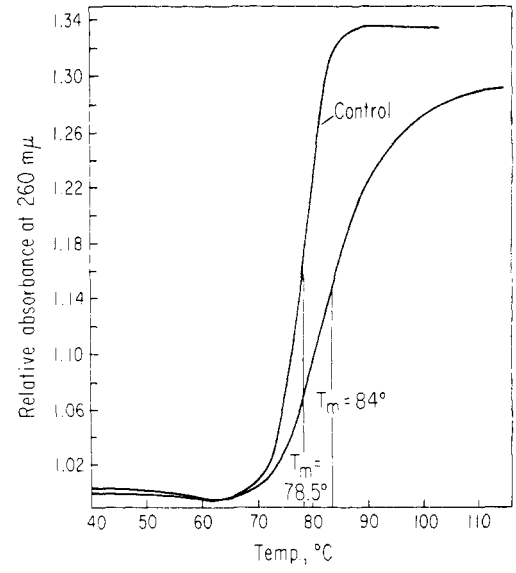


FIGURE 3: Melting curve of a DNA-mitomycin C complex (binding ratio 26) and control DNA. Buffer, DSC.

this purpose as the A_{260} of the drug itself becomes appreciable at lower binding ratios. Bound mitomycin was determined by ultraviolet absorbance of the complex at 310 mμ (see Figure 2), using ϵ_{310} 11,500 (SSC) for denatured DNA and 11,000 for native DNA (see below).

In the intermediate binding ratio range (30–70) either methods a or b may be used with reasonable accuracy.

Determination of the Molar Extinction Coefficient of Bound Mitomycin. Complexes of [1a-^{3}H]porfiromycin with poly(dG) and native or denatured DNA were prepared and their radioactivity and 310 mμ absorbance were correlated. This extinction coefficient is strictly valid for porfiromycin only but it was applied for mitomycin C complexes as well since the spectral properties of these compounds and their derivatives are generally very similar (Stevens *et al.*, 1965).

Depurination of the Complex. This was accomplished by heating at 90° in 5% Cl_3CCOOH for 10 min in the absence of salts. Apurinic acid (a 2-μmol batch) was isolated by chromatography over a Sephadex G-25 (fine), 2.5 × 25 cm column with 0.017 M sodium phosphate buffer (pH 7.4) as eluent. The apurinic acid was mostly excluded although broadened somewhat. Adenine and guanine were eluted in a later band together.

Apurinic Acid Preparation. DNA was heated in 5% Cl_3CCOOH at 90° for 10 min, then passed through a Sephadex G-10 (fine) column from which the apurinic acid was completely excluded.

Assay of Cross-Linked DNA. The melting curve reversibility method was applied as described for MC by Iyer and Szybalski (1963), except that 1/10 SSC was used in our work as buffer.

Results

Extent of Binding of Mitomycin C and Porfiromycin to Native and Denatured DNA using Different Methods of Activation (*Figure 1*). (a) Using the conventional activation method (A) binding to native DNA leveled off at *ca.* 150 nucleotide/antibiotic binding ratio. Denatured DNA reacted with more antibiotic under the same conditions, leveling off at a ratio of *ca.* 50.

(b) Using method B, *i.e.*, adding the required amount of reducing agent in small portions at a time, resulted in a 10–20-fold increase in antibiotic binding by both DNAs, leveling off at *ca.* 5 and 2–3 nucleotide/antibiotic binding ratios in native and denatured DNA, respectively.

(c) In yet another modification of the conventional procedure, activation was carried out in the presence of a large excess of nonreduced mitomycin C (Table I): at tenfold molar excess of mitomycin to denatured DNA in the reaction mixture only one-tenth of the stoichiometric amount of reducing agent was added altogether, in one step, leaving nine-tenths of the mitomycin in its inactive, quinone form. The resulting complex was saturated with mitomycin (binding ratio 3–4), representing a 24-fold increase in bound mitomycin over that in the control experiment where no excess quinone form was present. Table I further illustrates these results.

Properties of the Mitomycin–DNA Complex. The purple color of the complex solutions is apparent at 20 and lower binding ratios and 10^{-4} M DNA concentrations. Complexes of native DNA have a great tendency to precipitate at the saturation binding ratios (4–6) and cannot be redissolved. Denatured DNA complexes are more soluble. Alcohol precipitation results in a precipitate with a crystalline appearance (shiny plates).

Uv Spectra (*Figure 2*). The denatured DNA complexes display a characteristic band at 310 mμ while in native DNA complexes this is red-shifted to 313–315 mμ. The extinction coefficient at λ_{max} is 11,500 in denatured DNA and 11,000 in native DNA complexes. The computed spectrum of the bound mitomycin alone resembles closely the spectrum of "mitosene" derivatives of mitomycin C (or of porfiromycin), as seen on comparison with the spectrum of the hydrolysis derivative of mitomycin C (III, ϵ_{310}(SSC) 12,500; Stevens *et al.*, 1965).

Melting Behavior (*Figure 3*). Native DNA-mitomycin complexes of high MC content exhibit an increase of T_m and broadened transition profiles, as seen in Figure 3.

Stability of the Binding. The complexes of both native and denatured DNA possessing high or low binding ratios were shown to be stable upon prolonged dialysis (in SSC, DSC, or 0.017 M sodium phosphate (pH 7.5)), upon repeated chromatography over Sephadex G-100 or hydroxylapatite columns, and upon incubation at room temperature

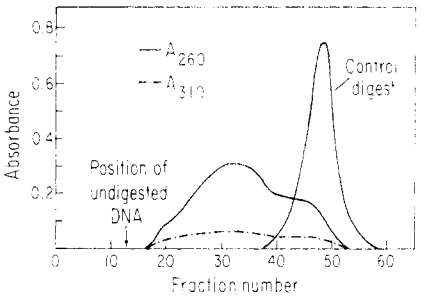


FIGURE 4: Pattern of the enzymatic digest of mitomycin-denatured DNA complex on Sephadex G-100 chromatography. The complex (binding ratio, 8; 2–3 μmol/ml) was incubated at 37° with 15 μg of DNase/ml in 0.01 $Mg(OAc)_2/Mg(OH)_2$ (pH 7.1) for 3 hr, then the pH was raised to 8.6 by addition of NaOH, snake venom diesterase was added to 100 μg/ml in 2 equal portions at 0 and 3 hr and the mixture was incubated overnight at 37°, then chromatographed over a Sephadex G-100 column. Control DNA was similarly digested and chromatographed.

with the following (all solutions contained SSC in addition): (a) 5% sodium dodecyl sulfate, 4 hr; (b) pH 12 (by adding NaOH solution), 15 min; (c) 7 M urea, 40 min; (d) 20% formamide, 1 hr; (e) 1 M NaCl, 3 hr; (f) 0.1 M $MgCl_2$, 3 hr. Stability to these agents was evaluated by passing the mixture through Sephadex after the incubation, then analyzing the uv spectra of the complex-containing fraction for any loss of bound mitomycin.

Upon paper chromatography in system III a small fraction (10–20%) of bound [^{3}H]porfiromycin was eluted from a denatured DNA complex, but after drying the chromatogram and resubmitting to fresh elution no more loss of antibiotic occurred from the complex remaining at the origin.

Heat Stability at 100°. Two different denatured DNA–[^{3}H]profiromycin complexes were tested: one made by method A, binding ratio 86, and one by method B, binding ratio 6. Each was heated in SSC at 100° for 30 min then passed through Sephadex and the counts per unit DNA were assayed. Both complexes lost a small amount of antibiotic, 16 and 23%, respectively. A native DNA–MC complex (method B, binding ratio 17) lost 10% MC under similar conditions.

Partial Resistance to Enzymatic Degradation (Figure 4). The complex was only partially degraded by the combination of DNase and snake venom diesterase digestions as seen from the Sephadex G-100 pattern. Absorbance at 310 mμ paralleled the A_{260} of the fractions indicating that bound mitomycin was not released from fragmented DNA. Another aliquot of the digest was submitted to paper chromatography in system III. Only traces of the four 5-mononucleotides could be detected while the bulk of the uv absorbing material remained at and near the origin.

Depurination. The depurinated complex lost all its bound mitomycin as could be seen from its uv spectrum which was identical with that of control depurinated DNA. The released adenine/guanine fracton (see Methods) was separated into its components on rechromatography over a Sephadex G-19 (fine) column (7.5 × 40 cm, same buffer as above). The molar ratio Gua/Ade was found to be 0.9 ± 0.1, same as that from the control depurinated DNA. Overall recovery of (Gua Ade) was 89% (control, 86%).

Depurination of [^{3}H]PM–DNA complexes prepared by method A was also carried out. Complete loss of bound antibiotic was indicated by loss of radioactivity from the depurinated complex.

Apurinic Acid as Substrate for Mitomycin Binding. Apurinic acid was submitted to reaction with mitomycin C in a 1:1 molar ratio, using activation method B, then the mixture was passed through a 2.5 × 25 cm Sephadex G-25 (fine) column in 0.017 M sodium phosphate (pH 7.4) to separate the apurinic acid from unbound mitomycin. The apurinic acid contained no bound mitomycin as seen from its ultraviolet spectrum indicating that activated mitomycin has no affinity toward apurinic acid.

Base Ratios. A complex (denatured DNA–mitomycin C, binding ratio 3–4) was hydrolyzed in 7.5 N $HClO_4$ and analyzed for base ratios, in the usual manner (Wyatt, 1955), using solvent system V for paper chromatographic separation of the bases. The base ratios were normal and identical with those of control DNA determined simultaneously.

Attempts at Alkylation of Guanine, Guanosine, and 5′-GMP by Mitomycin. These experiments were aimed primarily at reproducing previous work (Lipsett and Weissbach, 1965) and clarifying some uncertainties. They were carried out exactly as described there, featuring activation of mitomycin by KBH_4 in aerobic solution, adding the nucleotide substrate only after the reduction was complete and excess KBH_4 was destroyed. The mixtures were analyzed by paper chromatography. In addition, we ran the following controls on paper: (a) reaction mixture but nucleotide substrate omitted, (b) nucleotide substrate (guanine, guanosine, or 5′-GMP) as standard, (c) mixed spot of (a) and (b). Combinations of nonradioactive substrate and antibiotic or ^{14}C substrate and cold antibiotic or ^{14}C substrate and [^{3}H]porfiromycin were all tested.

In nonradioactive runs we invariably obtained only unreacted guanine, guanosine, or 5′-GMP, and three spots from reduced mitomycin (system I). The R_F of the strongest one corresponded to that reported for "mono-guanyl mitomycin" (R_F 0.8) and another spot, at the origin, corresponded to "diguanyl mitomycin" (R_F 0). *These same three spots* occurred in the controls with no guanine substrates. In system II similar results were obtained. Using [^{14}C]guanine substrates providing a much more sensitive method of detection than uv absorption we noted the appearance of false spots and streaks, as a small percentage of the total counts, mainly at the origin. These on elution and rechromatography traveled at the R_F of the standard guanine compounds indicating that their previous different positions were artificial. Guanine and its derivatives are known for such behavior, due to poor solubility. In the reaction mixtures overlap of ^{14}C counts with one or more of the above mentioned three spots of reduced mitomycin or [^{3}H]porfiromycin inevitably occurred but, contrary to the above authors, we could not consider this as evidence for the formation of guanine–antibiotic products, particularly since the mixed spot (c) control showed exactly the same pattern. Experiments in which tenfold amount of mitomycin was used gave similarly negative results.

In another series the more effective anaerob activation method B was applied to mixtures of either 5′-dAMP, dCMP, dTMP, or dGMP, and fivefold molar excess of mitomycin C in unbuffered aqueous solution. No spots other than those present in the controls were apparent on paper chromatography in systems I or III. Similarly negative results were obtained using ^{14}C-labeled guanine, guanosine, or 5′-GMP. Also, no products were obtained with GpC as substrate using either activation methods A or B, and five- to tenfold molar excess of antibiotic. GpC was recovered quantitatively after the reaction upon elution from chromatogram (system I or IV).

Specificity of Binding to Synthetic Polynucleotides (Table II). These experiments were carried out under a standard set of conditions for comparison. The results show unequivocal binding specificity for guanine, both in the single- and the double-stranded series. The data for the guanine analog poly(dI · dC) suggest that the drug can bind to I residues as well. Secondary structure appears to inhibit the overall binding (compare binding to poly(dG) with that to poly(dG · dC)). While poly(dI · dC) binds the antibiotic, its ribo analog is completely inert. The binding ratios of DNAs and RNA under the same conditions are higher than that of poly(dG), demonstrating the unique preference of the drug for poly(dG) at this binding level.

Binding Capacity of Analogs Lacking the C-1,C-2 Aziridine Ring. A complex was readily formed between III and either native or denatured DNA, under the conditions of Figure 1, at 1:1 molar ratio of DNA to III in the activation mixture. The obtained binding ratios were 24 and 11, respectively. (The comparative values for mitomycin C itself are 18 and 8, as seen in Figure 1, from curves III and IV, respectively.) Analogs IV and V also formed complexes under these conditions, as seen from ultraviolet spectra, but quantitative binding ratios were not determined.

Lack of Cross-Link Formation by the Aziridine-Lacking Derivatives. No cross-linking effect by III, IV, and V could be detected, using regular amounts or large excess of these drugs to native DNA (1:1 or 1:10 DNA–drug molar ratio) in the reaction mixture (activation method A).

Cross-Link Studies with Mitomycin C. In contrast to DNA, reovirus RNA was not cross-linked by MC under the conditions specified in the preceding experiment.

The stability of cross-links in DNA induced by MC and by HN2 were compared directly, under heating or alkaline conditions. The results (Table III) show that MC-induced cross-links are stable while HN2-induced ones are unstable under these conditions.[4]

TABLE II: Specificity of Binding of [^{3}H]Porfiromycin to Polynucleotides.[a]

Substrate	Binding Ratio (mol of Nucleotide/mol of porfiromycin)[b]
Synthetic polynucleotides	
Poly(dAT·dAT)	2064
Poly(dA·dT)	2000–3400
Poly(dG·dC)	162
Poly(dI·dC)	374
Poly(rI·rC)	5040
Poly(dG)	38
Poly(dC)	2112
Poly(dT)	5321
Poly(dA)	3170
Natural polynucleotides	
DNA (native)	244
DNA (denatured)	72
RNA (ribosomal)	95

[a] Reaction conditions: 1 μmol of substrate and 1 μmol of [^{3}H]porfiromycin in 1.0 ml of 0.017 M phosphate buffer; activation method A. The complex was isolated by gel filtration chromatography followed by dialysis. [b] Assayed by the radioactive method.

Discussion

In our early efforts to prepare extensively substituted DNA–mitomycin complexes we were puzzled by the fact that the binding leveled off at high nucleotide/drug binding ratios, about 150 with native and 50 with denatured DNA (Figure 1, curves I and II). This behavior is in contrast to other better known covalent modifiers of DNA, for example, the common methylating agents (*e.g.*, Uhlenhopp and Krasna, 1971) or the carcinogens *N*-acetylacetoxyaminofluorene (Miller *et al.*, 1966) or 7-bromomethylbenzanthracene (Pochon and Michelson, 1971) where substitution of DNA to near stoichiometric binding ratios at one or more bases can be readily achieved *in vitro*. We hypothesized that the "sluggishness" of the *in vitro* system contrasting so sharply the enormous biological potency and rapidity of action of these antibiotics (*e.g.*, Iyer and Szybalski, 1963) may be due to some imperfection of the activation method. This method was originally developed by Iyer and Szybalski (1964) and used in all subsequent work (*e.g.*, Weissbach and Lisio, 1965; Cohen and Crothers, 1970). Pertinent to this was the curious fact, noted previously (Iyer and Szybalski, 1964), that after the addition of the reducing agent reduced mitomycin lost its cross-linking ability (as well as biological activity; Schwartz *et al.*, 1963). One might interpret this to indicate that not the fully reduced drug but rather a transient intermediate of the reduction process, namely the half-reduced, semiquinone radical form, is the active agent. In the usual activation method, however, *excess* reducing agent is added at once, thus the semiquinone intermediate would be rapidly quenched by further reduction (Chart I). In order to test whether this transient form plays a role in the reaction with nucleic acids we introduced one simple modification of the activation: the

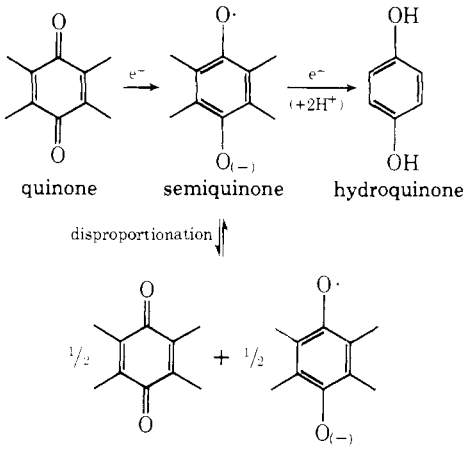
TABLE III: Comparative Stabilities of Cross-Links Induced by MC and HN_2, a Bifunctional Mustard.

Cross-Linking Agent	% Loss of Cross-Linked DNA[a,b] 60°, 2 hr, pH 7.2, 1/10 SSC	60°, 2 hr, pH 10, 1/10 SSC
MC	0	0
HN_2	76	64

[a] DNA was 65–70% cross-linked initially in each case. The degree of cross-linking was assayed by melting the sample at 70° for 5 min in 51% methanol–DSC (pH 7.2) then quenching in ice, reequilibrating at room temperature, and comparing the hyperchromicity to that of non-cross-linked control DNA (Geiduschek, 1971). HN_2–DNA showed a certain (10–15%) loss of cross-linking just by this assay alone as shown when the heating and quenching cycles were repeated on the same sample. This loss was subtracted from the overall figure. [b] Preparation of 65–70% cross-linked DNA: (a) DNA (1.27 μmol) and MC (0.6 μmol) in 1.8 ml of SSC; activation method A; (b) DNA (3.1 μmol) and HN_2 (4.9 μmol) were incubated in 4 ml of 0.5 M NaOAc for 1 hr, 37°. The cross-linked DNAs were precipitated by ethanol and redissolved in 1/10 SSC.

[4] These results confirm the "unpublished experiment" of Lawley and Szybalski, quoted by Szybalski and Iyer (1967).

CHART I



required (stoichiometric) amount[3] of $Na_2S_2O_4$ was not added at once but rather in five portions, at 5-min intervals, so as to allow temporary buildup of the semiquinone before the reduction was completed. This resulted in greatly increased binding and no saturation effect until binding ratios of a few nucleotides per mitomycin molecule were reached (Figure 1, curves III and IV.) By all criteria tested the nature of the binding in these highly substituted complexes is qualitatively the same as in those prepared conventionally. The new complexes are completely cross-linked. In addition, they show the same remarkable heat stability, resistance to enzymatic degradation (Weissbach and Lisio, 1965) and lability to acid. These properties will be discussed below in greater detail.

More substantial evidence that the binding effect is dependent on the semiquinone rather than the fully reduced, hydroquinone concentration was provided by the experiment in which, in addition to the usual components, a large excess of *nonreduced* mitomycin in constant amount was present during the activation (Table I). Given the well-known scheme of quinone reductions (Chart I; Walling, 1957) it follows that under these conditions the concentration of the semiquinone is *maximized* at the expense of the hydroquinone concentration (which is therefore *minimized*), both because the disproportionation equilibrium is reversed by excess quinone and because the excess quinone competes effectively for the available reducing agent with the semiquinone. The resulting great (24-fold) increase of the extent of binding to DNA under these conditions leads us to believe that the primary reactive species is the half-reduced, semiquinone radical form rather than the fully reduced hydroquinone, as previously postulated. This explains the limited binding obtained with the conventional activation procedure where the excess reducing agent prevents any appreciable buildup of the semiquinone intermediate.

Direct evidence by esr spectroscopy for the semiquinone intermediate in the course of reduction of the mitomycins has been reported earlier and this radical has been stated to be "quite stable" (Patrick *et al.*, 1964). A theoretical argument for the observed stability, based on resonance theory, was presented by Murakami (1965). Furthermore, he postulated the importance of the mitomycin semiquinone radical form in the mode of action of the antibiotic. In spite of these indications, it should be pointed out that the presence of the semiquinone radical in our particular system has not been proven by direct esr detection. Therefore an alternative, more complicated explanation of our results, namely a mixed quinone/hydroquinone dimer or mixed polymer as the binding species, cannot be entirely excluded. It is clearly desirable to have a conclusive, esr clarification on this point.

The affinity of mitomycin activated by our new procedure toward DNA is rather striking. From Table I, for instance, it is seen that a maximum of 2 mol of semiquinone[5]/mol of DNA-nucleotide in the reaction mixture was sufficient to saturate the binding sites of denatured DNA (binding ratio 3:4) at room temperature in 10 min (shorter periods were not tested).

In view of these findings *in vitro* it is of great interest that the occurrence of the semiquinone form of several quinone type antibiotics and some simple quinones in biological systems has been reported. Rubiflavine, streptonigrin, and phenazine methosulfate were shown to be reduced to semiquinone radicals by bacterial suspensions provided with an electron source, as observed directly in the esr cell (White and Dearman, 1965). The radicals displayed considerable stability. Similar results could be obtained by chemical reduction. The substituted quinone ring system of streptonigrin is very similar to that of the mitomycins. This is pointed out by the authors, along with the similarity in bactericidal action, and in the response to redox type synergizing and antagonizing agents, although their efforts to detect the MC semiquinone radical itself in their system were tentatively negative (Ishizu *et al.*, 1968). All considered, it is certainly plausible to assume the occurrence of the semiquinone form of mitomycin during its cellular reduction, and we believe that characterization of the rapid reaction of this form with DNA *in vitro* is fundamental to the understanding of the action *in vivo*. Before formulating any ideas on the basic nature of this interaction, however, more of its properties will be discussed.

Base Specificity. The fact that the binding of activated porfiromycin shows virtually absolute requirement for guanine[6] in the synthetic polydeoxynucleotide series (Table II) suggests that the binding to DNA should also be at guanine residues. Consistent with this is that depurinating conditions remove all bound MC from DNA and from poly(dG) and that, conversely, apurinic acid does not bind MC. Iyer and Szybalski (1964) noted that the cross-linking efficiency of the antibiotic *in vitro* as well as *in vivo* increases with increasing (Gua + Cyt) content of DNA. While all this points toward guanine as the crucial base in the interaction we were not able to obtain direct proof by the usual methods. Thus, perchloric acid hydrolysis of the complex yielded normal base ratios, and upon depurination normal amounts of purines were recovered. These experiments suggest the possibility that the MC–DNA linkage is acid sensitive and dissociates under acidic degradation conditions of DNA. Alkyl groups in O^6-alkylated guanine residues are known to exhibit such behavior (Lawley and Thatcher, 1970). Interestingly, Szybalski and Iyer (1967) suggested the O^6 position of guanine as the only possible point of attachment of MC in forming a cross-link without distortion of the double helix. An additional argument (although based on negative evidence) for the O^6 position is that the 7 and 8 positions of guanine have been ruled out previously by a new alkylation assay method (Tomasz, 1970). This assay was repeated with the new, highly substituted complexes, yielding the same results (unpublished experiment). Noninvolvement of the Gua-7 position is now confirmed by the observed great

[5] Assuming that the semiquinone → hydroquinone step does not occur to any extent.

[6] Or the nonnatural analog, hypoxanthine.

heat stability of the binding since alkyl groups at the Gua-7 position should be unstable to heat (Lawley, 1966). Analogous stability behavior is observed for the cross-links, in direct contrast to the heat lability of HN2 cross-links (Table III) which are known to be at 7 positions of guanine. It is unlikely, therefore, that the MC-induced cross-links are at the same position.[4]

Further efforts to prove directly a covalent attachment of mitomycin to guanine were unsuccessful. The DNA–MC complex remained resistant to enzymatic hydrolysis, even under strong conditions, and to heat as well as to alkali treatments. Consequently, no fragments could be obtained for analysis. Tests for reaction of activated mitomycin with monomeric guanine derivatives, other bases, and GpC were unequivocally negative. Lack of interaction on the monomer level with guanine derivatives confirms the original report of Iyer and Szybalski (1964) but contradicts the work of Lipsett and Weissbach (1965) who described the formation of "guanyl mitomycin" and "diguanyl mitomycin" as products of such reactions. Despite extensive efforts their results could not be substantiated (see Results for further details).

In view of these negative results reinvestigation of the very concept of covalent linkage between DNA and the mitomycins seemed to be in order. Although Iyer and Szybalski (1964) have shown that the cross-links are stable to heat, alkali, formamide, and in CsCl gradients, the stability of the monofunctional binding which represents the "bulk" (90% or more) of the overall binding (Szybalski and Iyer, 1964; M. Tomasz, unpublished) has not been tested. Therefore, a comprehensive probing of the stability of the mitomycin–DNA complex was carried out under conditions known to dissociate noncovalent complexes of DNA with various antibiotics. Many such complexes are characteristically stable to dialysis, chromatography, or even to cesium salt gradients and only certain specific conditions can induce their dissociation. For instance, sodium dodecyl sulfate dissociates actinomycin (Müller and Crothers, 1968) and chromomycin complexes (Behr *et al.*, 1969), high pH (12.5) breaks up hedamycin and rubiflavine complexes (White and White, 1969), urea dissociates actinomycin (Hartmann and Coy, 1962), Mg^{2+} and formamide dissociate daunomycin (Calendi *et al.*, 1965), while high ionic strength releases rubiflavine and hedamycin (White and White, 1969; Joel and Goldberg, 1970). Heating above T_m has been reported to cause dissociation for most of these drugs, although a remarkably high temperature (93°) was required in the case of daunomycin (Doskočil and Frič, 1973). Since the mitomycin–DNA complex remained stable under *all* of these conditions we conclude that its overall mode of binding is covalent.

These conclusions present an apparent paradox: why does mitomycin fail to react with mononucleotides? Such discrimination against monomers is characteristic of *noncovalently* binding drugs, such as, for example, the classical intercalating agents proflavine (Peacocke and Skerrett, 1956) and ethidium bromide (Waring, 1966a), and the antibiotics listed above. Covalent DNA modifiers, on the other hand, readily yield monomer reaction products as has been shown with synthetic alkylating agents (Lawley, 1966), the carcinogens acetylacetoxyaminofluorene (Miller *et al.*, 1966) and 7-bromomomethylbenzanthracene (Pochon and Michelson, 1971; Rayman and Dipple, 1973), among others. This paradox can be resolved when we consider the possible mode of involvement of the semiquinone form.

Role of the Semiquinone Form. The ultraviolet spectra of DNA-bound mitomycin (Figure 2) show that the only change in the indoloquinone chromophore is aromatization by the elimination of CH_3OH from the 9 and 9a positions, known to occur in the reduced state of the mitomycins spontaneously, even in the absence of DNA (Iyer and Szybalski, 1964; Patrick *et al.*, 1964). This indicates that the semiquinone radical function itself had not entered into any covalent reaction with DNA; otherwise, reoxidation to the intact indoloquinone chromophore could not have occurred. This conclusion is in line with the general notion that semiquinone radicals, because of their relative stability, "rarely partake in radical chain processes" (Walling, 1957). Thus, while evidence was presented above that the semiquinone radical form of mitomycin is rate determining in the binding to nucleic acids, it is also evident that the radical is not involved covalently. This, in effect, means that this form combines with DNA in a noncovalent manner, as a prerequisite for subsequent formation of the covalent links observed. Noncovalent affinity of free radicals to polynucleotides has been observed before: Ohnishi and McConnell (1965) described the oriented, noncovalent binding of chlorpromazine free radicals to DNA, interpreted it as intercalation, and noted that the radical was markedly stabilized in its DNA-bound state. Binding of the 5-methylphenazinium cation radical to DNA displayed similar characteristics, as reported in detail by Ishizu *et al.* (1969). In further analogy to these systems, certain characteristics of the mitomycin binding resemble intercalation. Thus, binding data (Figure 3, Table II) indicate that denatured DNA and RNA bind MC as well, or, at higher drug concentration, better, than native DNA. (Similar findings were first reported by Szybalski and Iyer (1964) and Weissbach and Lisio (1965). The intercalating drugs acridines and ethidium display similar behavior, along with daunomycin (Doskočil and Frič, 1973), recently shown to belong to the intercalating class (Ward *et al.*, 1965; Pigram *et al.*, 1972). An increase in T_m' also characteristic of this class (Waring, 1966b) is apparent in the MC–DNA complex (Figure 4), but this would be expected also from the presence of multiple cross-links, according to the theory of Crothers *et al.* (1965), and it is uncertain what portion of the effect if any is caused by intercalation. Judging from the chemical structure of mitomycin, however, stacking of its indole ring system between bases is feasible,[7] especially in the light of recent work showing that the indole ring of tryptophan derivatives binds to polynucleotides, apparently by intercalation between stacked bases (Gabbay *et al.*, 1972; Dimicoli and Helène, 1974).

The proposed model assigns for the first time a specific role for the indoloquinone ring system of the mitomycins, shown to be indispensable for biological activity (Kinoshita *et al.*, 1971b). It also explains the conspicuous lack of reaction with mononucleotides. The primary, noncovalent binding by the semiquinone is specific to the polynucleotide structure and since it is prerequisite for subsequent formation of covalent bonds the monomers do not react at all.

An interplay between noncovalent and covalent forces in the interaction of a drug with DNA, as proposed here, has been assumed previously in other cases. Thus, aromatic carcinogens were postulated to intercalate in DNA followed by

[7] Especially in the aromatized form, resulting by elimination of CH_3OH in the reduced state, as discussed above.

covalent alkylation of DNA groups by the metabolically activated side chains of the carcinogen (Ames *et al.*, 1973). As another example, the light-induced covalent cross-linking of DNA by psoralene is evidently preceded by noncovalent intercalation (Dall'Acqua *et al.*, 1969). A common property of these drugs and mitomycin is that they are activated into short-lived reactive forms. It appears almost a necessity that any efficient interaction of such forms with DNA would involve facilitation by fast, specific noncovalent binding since otherwise they would react indiscriminately with more abundant cell components.

Role of the Aziridine Ring. The fact that the three biologically active aziridine-lacking analogs (III, IV, V) bind to but do not cross-link DNA in our system strongly suggests the aziridine ring as one of the covalent points of attachment of the drug to nucleic acids. This is corroborated by similar lack of cross-linking ability of some other aziridine-lacking analogs *in vivo* (Otsuji and Murayama, 1972). Thus one of the two hypothetical groups in the Iyer–Szybalski theory now appears to be substantiated experimentally, while the other (C-10 carbamate) remains still hypothetical. Our results may also explain the observed antibiotic activities of aziridine-lacking analogs: since such analogs are now shown to possess the monofunctional binding capacity of the mitomycins it is very likely that this is the basic factor in their activity. In the case of the natural mitomycins the cross-links represent additional damage to the DNA template functions in the cell.

In conclusion, the mitomycin-activating agent–DNA system is chemically more complex than previously believed. The suggested pattern of interplay of specific noncovalent and covalent interaction may prove to be a more general principle in the field of metabolically activated drug–DNA interactions. The unexpected affinity of the semiquinone radical of mitomycin toward polynucleotides may be relevant to the action of other quinone-type drugs and further work could lead to recognition of factors involved in the stabilization of certain radicals by association with polynucleotide structure. Experiments can be visualized to probe into this and other details of the presented hypothesis and some are in progress in our laboratory.

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Synthesis of Oligodeoxyribonucleotide Ethyl Phosphotriesters and Their Specific Complex Formation with Transfer Ribonucleic Acid†

Paul S. Miller, J. C. Barrett,‡ and Paul O. P. Ts'o*

ABSTRACT: Oligodeoxyribonucleotide ethyl phosphotriesters, d-Tp(Et)Gp(Et)G and d-Tp(Et)Tp(Et)Cp(Et)A, were synthesized by a stepwise, chemical procedure. These triesters are complementary, respectively, to 3′-CpCpA-OH (the 3′-amino acid accepting terminus) and to -UpGpApA- (the anticodon region) of phenylalanine tRNA from yeast and *Escherichia coli.* Tritium-labeled triesters were prepared by exchange of the H-8 protons of adenine and guanine in the oligomers in tritiated water. The association constants for binding of the triesters to their complementary regions on tRNA were measured by equilibrium dialysis and were compared with those of oligodeoxyribonucleotides and oligoribonucleotides of the same sequences. In 1 M NaCl–10 mM $MgCl_2$ at 0°, the association constants of the oligomers with both $tRNA^{Phe}_{yeast}$ and $tRNA^{Phe}_{coli}$ are very similar. The association constants of the ribooligonucleotides are 8 to 20 times larger than those of the corresponding deoxyribooligonucleotides, while the deoxyribooligonucleotide triesters exhibit binding constants slightly higher than those of the deoxyribooligonucleotides. These differences are discussed in terms of the differences in conformations of the various oligomers. At low salt concentration (0.1 M NaCl, 1 mM EDTA), the oligonucleotide triesters have the same binding constants as at high salt concentration, whereas the corresponding deoxyribo- and ribooligonucleotides show a four- to sixfold decrease in their binding constants. This reflects the removal of the charge repulsion between the neutral triesters and the tRNA. The binding of oligomers to modified $tRNA^{Phe}_{yeast}$ was also examined. Removal of the Y base decreased the binding of anticodon-complementary oligomers sixfold while removal of the 3′-CpA residues decreased the binding of the 3′-CpCpA-OH complementary oligomers 6- to 20-fold. This study provides the chemical and physicochemical basis for the investigation of the biochemical effects of these triesters on the aminoacylation of tRNA which is reported in the following paper (Barrett, J. C., Miller, P. S., and Ts'o, P. O. P. (1974), *Biochemistry 13,* 4897).

Oligodeoxyribonucleotide alkyl phosphotriesters are oligodeoxyribonucleotide analogs containing an alkylated 3′-5′ internucleotide phosphate linkage. In the preceding papers of this series (Miller *et al.,* 1971; DeBoer *et al.,* 1973; Kan *et al.,* 1973) we have shown that these oligodeoxyribonucleotide analogs have the following novel characteristics. (1) The triesters are uncharged at neutral pH. (2) Triesters form base-paired complexes with complementary polynucleotides. The complexes have a higher stability than similar complexes formed by the parent diester, presumably due to the removal of charge repulsion between the phosphate of polymer and the alkyl phosphotriester of the oligomer. (3) Trityl-containing derivatives of these compounds are very soluble in organic solvents, a feature which allowed us to investigate hydrogen-bonded, base-pairing interactions of these compounds in chloroform. (4) The methyl or ethyl groups of the dimeric phosphotriesters serve as reporters in proton magnetic resonance (pmr) studies on the conformation of the dimer, especially at the backbone region.

† From the Division of Biophysics, Department of Biochemical and Biophysical Sciences, The Johns Hopkins University, Baltimore, Maryland 21205. *Received June 24, 1974.* This work was supported in part by a grant from the National Institutes of Health (GM-16066-06) and a grant from the National Science Foundation (GB-30725X). This is paper No. 4 in a series entitled: Alkyl Phosphotriesters of Dinucleotides and Oligonucleotides. Paper No. 3 in this series is Kan *et al.* (1973).

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