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SPECTROPHOTOMETRIC STUDIES OF THE INTERACTION OF ANTHRAMYCIN WITH DEOXYRIBONUCLEIC ACID

VLADIMIR STEFANOVIĆ*

Hoffmann-LaRoche, Inc., Nutley, N.J., U.S.A.

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Abstract—Qualitative and quantitative aspects of the interaction between anthramycin methyl ether (AME) and DNA have been investigated spectrophotometrically by utilizing alterations in the anthramycin methyl ether spectrum that result from complex formation. Certain nucleic acid polymers, purine and pyrimidine derivatives, and various agents have been employed in examining the molecular nature of the interaction.

These experiments suggest that binding involves both electrostatic attraction between the AME and the anionic phosphate groups of DNA, and a more specific interaction apparently involving seven-membered ring portions of AME and DNA. The secondary structure of DNA also affects its binding to AME.

The ability of the compounds of the anthramycin series to complex with DNA was correlated with their biological activity. The possibility of predicting biological activity of a new compound from this series by using only spectrophotometric assay is therefore indicated.

ANTHRAMYCIN (A) (Fig. 1, formula 1) is a new antitumour antibiotic, which is produced by *Streptomyces refuineus, var. thermotolerans* (*NRRL* 3143) and is isolated[1] in the form of its crystalline methyl ether (AME), 5, 10, 11, 1a-tetrahydro-9-hydroxy-11-methoxy-8-methyl-5-oxo-1 H-pyrrolo (2, 1-c) (1,4) benzodiazepine-2-acrylamide hydrate (Fig. 1, formula 2). The structure was elucidated by Leimgruber *et al.*[2] and chemotherapeutic properties were reported by Grunberg *et al.*[3]

AME inhibits the synthesis of nucleic acids by intact organisms and by enzyme preparations. These effects are of interest partly because they can be useful in the study of gene action and related problems and also because of the selectivity and specificity of this antibiotic, which possesses a new and interesting chemical structure. Low concentrations of AME selectively suppressed cellular and enzymatic DNA-directed RNA synthesis; DNA synthesis was affected only at mugh higher concentrations.† This fact has suggested that the mode of action of AME is similar to that of actinomycin D.

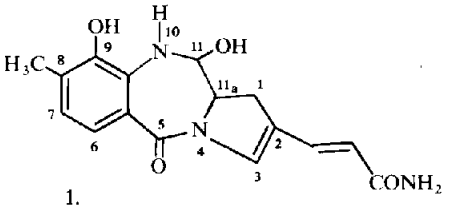
It has been established that the inhibition of RNA synthesis by actinomycin D[4] requires the binding of actinomycin with DNA; this same requirement was recently established for other anticancer antibiotics: kanamycin, cinerubin, chromomycin, mithramycin, olivomycin, and nogalamycin.[5] The nature and consequences of the intraction of these compounds with DNA have been the subject of many investigations.[6]

Our finding that a new antibiotic, AME, also reacts with DNA further indicates a more general aspect of the mode of action of these structurally different antibiotics.

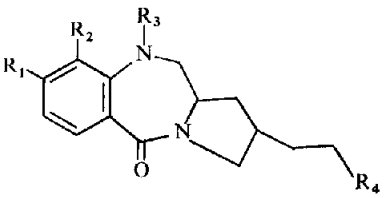
* Present address: Boston University Medical Center, Boston, Mass., U.S.A.

† H. Bates, personal communication.

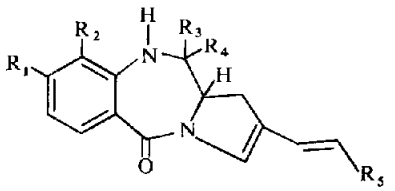
We have further investigated a series of analogs of AME (Fig. 1), and we have studied the relationship between the structural features of the compounds and their microbiological and antitumor activity; we have correlated this biological activity with the ability of these compounds to interact with DNA.

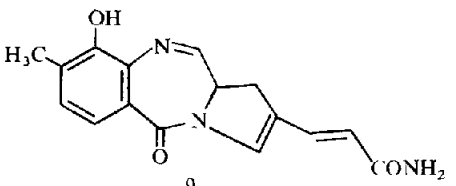


1.



7. $R_1 = R_2 = R_3 = H$, $R_4 = CONH_2$
8. $R_1 = R_2 = R_3 = H$, $R_4 = COOCH_3$





9.

2. $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = CONH_2$
3. $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$, $R_4 = OC_2H_5$, $R_5 = CONH_2$
4. $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$, $R_4 = OCH_2C_6H_5$, $R_5 = CONH_2$
5. $R_1 = CH_3$, $R_2 = OCH_3$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = CONH_2$
6. $R_1 = CH_3$, $R_2 = OH$, $R_3 = OCH_3$, $R_4 = H$, $R_5 = CONH_2$

FIG. 1. Structures of anthramycin and related compounds.

We feel, therefore, that further elucidation of the reaction between AME and DNA may provide valuable information concerning the nature of polymerization reactions, which synthesize RNA and DNA, and that it may lead to models for physiological regulation of template activity. The investigation concerns the specific molecular nature of the AME–DNA interaction.

MATERIALS AND METHODS

AME* and other compounds from this series were crystalline, chromatographically pure compounds, prepared in the chemical research laboratories of Hoffmann-LaRoche, Inc., Nutley, N.J.

The various purines, pyrimidines, and their derivatives were obtained either from Schwartz Laboratories, Nutritional Biochemical Corp., or from Calbiochem. RNA was obtained from Nutritional Biochemical Corp., DNA (calf thymus and salmon sperm) from Calbiochem, and DNA (*Escherichia coli* K_{12}) from General Biochemicals.

Test systems. Systematic measurements were accomplished in the following manner: A "stock" solution of DNA (calf thymus) was prepared in 0·001 M phosphate buffer,

* Because of the water insolubility of AME and other compounds in this series, methanol or dimethylsulfoxide (DMSO) was used for primary solubilization of these compounds, giving final stock solutions containing less than 2 per cent of the above solvents.

pH 7·0, at a concentration of 1·200 mg/ml. This solution was then mixed with equal volumes of the stock solutions of the compounds listed in Table 1 and dissolved in the same buffer. Thus, solutions (Solution A) containing 0·600 mg/ml of DNA and the indicated concentrations of the examined compounds were obtained. To serve as controls, the stock solution of each material was diluted with an equal volume of buffer to give the same concentration of examined compounds (Solution B) as in previous solutions; the stock solution of DNA was also diluted with an equal volume of buffer to serve as a background control (Solution C).

Diluted solutions were stored at room temperature for not more than a few hours. Ultraviolet absorption of these solutions was measured in a Cary model 14 M recording spectrophotometer with 0·2 cm cells. Absorbance, is therefore presented as Abs/0·2. Difference in λ_{max} (in millimicrons) between Solution A and Solution B is reported under $\Delta\lambda_{max}$ in Table 1 and difference in $\varepsilon\lambda_{max}$ under $\Delta\varepsilon\lambda_{max}$ in the same table.

The various purines, pyrimidines and their derivatives were used in concentrations of 2 μmole/ml, with the exception of thymidylic acid, which was used at a concentration of 1·5 μmole. Concentrations of RNA and DNA of various origins were 0·600 mg/ml, or as indicated.

In order to determine the influence of various agents on the bathochromic shift and hypochromic effect observed in solutions of AME after addition of DNA, samples containing DNA and AME were treated with methanol at concentrations of 10, 20 30, and 45 per cent; with $MgCl_2$ at concentrations of 0·01 and 0·1 M; with urea at a concentration of 0·2 M; and with formaldehyde at 0·5 M. For heating studies, a stock solution of DNA in a closed vial, was placed in an oven, at 90° for 15 min where the temperature of the DNA solution did not exceed 60° (for this time period), and at 105° for 75 min.

It was also established that the spectroscopic alterations that occurred after the addition of DNA were complete within several seconds, and showed no further change with time or additional mixing. Within the concentration ranges of polymer and ligand which were used in these experiments, no precipitation of the complex occurred.

The hypochromic effect could be measured with a reproductibility of 97·5 per cent. The experimental error of 2·5 per cent can be attributed to the uncertainties of measurements of wavelengths, absorbance, and volume.

Activity against transplanted tumors in mice (*Ehrlich carcinoma*-solid form and *Sarcoma*-180) was tested as described by Grunberg *et al.*[3] The animals were sacrificed 8 days after implantation of the tumor and the average weight of the tumors from the untreated control group (C) was compared with the average weight of the tumors from the treated group (T); if the C/T ratio was 2·0 or greater (50 per cent or more inhibition), positive antitumor effect was considered to have occurred. For determination of microbiological activity of AME *in vitro*, plate-cup assay against *Bacillus sp.* "*TA*" (NRCB-3167) was employed. To the zone size (30 mm) obtained by the crystalline antibiotic (0·1 mg/ml of pH 6·7 phosphate buffer), a value of 16,000 units/mg* was arbitrarily assigned.

* Details of the microbiological procedure for the determination of AME *in vitro* will be reported by J. Berger *et al.*, elsewhere.

TABLE 1. INTERACTION OF DNA WITH AME AND RELATED COMPOUNDS*

No.	Compound	Solution A (without DNA)					Solution B (with DNA)				
		Conc. (μg/ml)	λ_{max} (mμ)	Abs/0·2§	Mol.wt.	$\Sigma\lambda_{max}$	λ_{max} (mμ)	Abs/0·2	$\varepsilon\lambda_{max}$	$\Delta\lambda_{max}$† (mμ)	$\Delta\Sigma\lambda_{max}$‡ (%)
1	Anthramycin	35·9	334	4·185	315·3	36,758	342	3·410	29,951	8	18·5
2	AME	34	334	3·710	347·4	37,903	342	3·000	30,649	8–9	−19·1
3		33·4	333	3·500	343·3	35,981	342	2·800	38,786	9	19·9
4		35·7	333	3·180	405·4	36,109	343	2·500	28,955	10	−19·8
5		35	334	3·190	343·7	31,328	334	3·100	30,049	0‖	2·9‖
6	AME epimer	37	334	4·410	347·4	28,870	343–4	3·395	31,840	9–10	18·0
7		26	333	2·495	273·3	2622	333	2·515	2643	0‖	0·8‖
8		30	333	2·879	292·3	28,033	333	2·912	28,324	0‖	+1·1‖
9	Anhydroanthramycin	35·6	333	4·465	297·3	37,287	343	3·620	30,231	10	−18·9
10	Product of oxidation of AME	65	437–8	1·450			438			1	
11	Actinomycin D (Merck)	40	439–40	2·535	1247·5	22,588	459	1·835	16,350	19–20	−27·6
12	Actinomycin D (Hoffmann-LaRoche)	40	439–40	2·395	1247·5	21,340	459	1·745	15,548	19–20	−27·1

* Values are means of five determinations. Compounds numbered 1–9 correspond to compounds of the same numbers in Fig. 1.

† = Difference in λ_{max} (in mμ) between solution containing mixture of AME and DNA and a solution containing only AME.

‡ = Difference in the molar absorbtivity at λ_{max} (expressed in per cent) between a solution containing AME and a solution containing mixture of AME and DNA.

§ Absorbance obtained using 0·2 cm cell.

‖ $P < 0{\cdot}001$.

RESULTS

Screening of compounds of the anthramycin series. Compounds of the anthramycin series (Table 1, Fig. 1) were examined for the ability to produce a complex with calf thymus DNA and were subjected to the conditions described under "Test system". Results of the measurements of absorbance and wavelength of maximum absorbance are reported in Table 1. Structures of the examined compounds are presented in Fig. 1 and typical spectral changes of AME in Fig. 2.

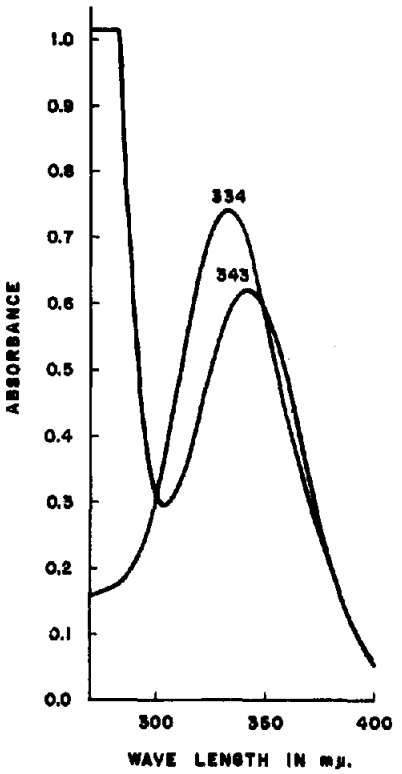


FIG. 2. Spectral change of AME in solution with DNA. Conditions are described in Table 2, No. 5.

It is evident from these results that only AME and five other compounds of this series have demonstrated a bathochromic shift accompanied by a hypochromic effect in absorbance. Both phenomena are of the same order of magnitude for all six compounds.

Four other compounds did not exhibit a significant change in absorbance or λ_{max} when measured in solution with DNA. At the same time, only those six compounds capable of forming complexes with DNA have shown microbiological activity *in vitro* against *Bacillus sp. "TA"* (15,000–16,000 units/mg) and activity *in vivo* against *Sarcoma*-180 and *Ehrlich carcinoma* solid tumor in mice (0·2 mg AME/kg).

It can be concluded, therefore, that the microbiological activity *in vitro* and antitumor activity *in vivo* of compounds belonging to the anthramycin series are correlated with their ability to complex with DNA. The capacity to form a complex with DNA

should be considered a primary cause of activity of AME and other antitumor compounds in this series.

Compound No. 10, which has not been completely characterized, contains a phenoxazone nucleus and was obtained by enzymatic oxidation of AME.

Actinomycin D behaved as has been described in the literature and served as an overall internal standard for the procedure used.

Effect of altering ratio of DNA *and* AME. Stock solutions of DNA were prepared in concentrations of 2·400 mg, 1·200 mg, 0·600 mg and 0·300 mg/ml of 0·001 M phosphate buffer, pH 7·0 Equal volumes of the AME (0·070 mg/ml) in the same buffer were mixed with the various solutions of DNA for spectrophotometric analysis.

AME was present in a concentration equal to 0.035 mg/ml in all cases, whereas the DNA concentrations varied from 1·200 to 0·150 mg/ml. The samples were treated as described in "Test system". The results are presented in Table 2.

TABLE 2. THE EFFECTS OF VARIOUS CONDITIONS AND AGENTS ON THE INTERACTION BETWEEN AME AND DNA*

No.	AME conc. (mg/ml)	DNA conc. (mg/ml)	Condition	$\Delta\lambda_{max}$† (mμ)	$\Delta\varepsilon\lambda_{max}$‡ (%)
1	0·035	0·150		4§	− 6·0§
2	0·035	0·300		3§	−12·7§
3	0·035	0·600		8	−18·4
4	0·035	1·200		9–10	−18·7
5	0·035	0·600	0·001 M buffer	10	−18·1
6	0·035	0·600	0·01 M buffer	9	−17·9
7	0·035	0·600	0·1 M buffer	8	−18·0
8	0·035	0·600	DNA heated for 10 min at 90°	8	−17·4
9	0·035	0·600	DNA heat for 75 min at 105°	0§	−0·5§
10	0·035	0·600	0·5% MeOH	8	−18·1
11	0·035	0·600	10% MeOH	7§	−17·8
12	0·035	0·600	20% MeOH	5§	−17·6
13	0·035	0·600	30% MeOH	3§	−12·6§
14	0·035	0·600	45% MeOH	1§	−2·0§
15	0·035	0·600	0·1 M $MgCl_2$	2§	−8·0§
16	0·035	0·600	0·01 M $MgCl_2$	4§	−12·6§
17	0·035	0·600	0·5 M HCHO	3§	−7·9§
18	0·035	0·600	0·2 M urea	9	−18·6
19	0·035	0·600	Calf thymus DNA	9	−19·4
20	0·035	0·600	*E. coli* K_{12} DNA	7§	−18·3
21	0·035	0·600	Salmon sperm DNA	8–9	−21·1‖

* Values are means of five determinations.

† Difference in λ_{max} (in mμ) between solution containing mixture of AME and DNA and a solution containing only AME.

‡ Difference in the molar absorbtivity at λ_{max} (expressed in %) between a solution containing AME and a solution containing AME and DNA.

§ $P < 0{\cdot}001$.

‖ $P < 0{\cdot}01$.

The results demonstrate, that the greatest $\Delta\varepsilon_{max}$ and $\Delta\varepsilon\lambda_{max}$ occurred at a ratio of either 1·200 or 0·600 mg/ml. At ratios where AME was 0·035 mg/ml and DNA was less than 0·600 mg/ml, the $\Delta\lambda_{max}$ still had not reached a maximum, indicating that insufficient DNA was present to completely complex the antibiotic.

Effect of varying ionic strength of buffer. The concentrations of DNA and AME were kept at 0·600 mg/ml and 0·035 mg/ml, respectively. In a manner similar to the previous experiments, the buffer strengths of the solutions were prepared to give final buffer concentrations of 0·1–0·01 and 0·001 M.

It is obvious from Table 2 that, at the concentrations employed here, $\Delta\varepsilon\lambda_{max}$ remains constant regardless of the ionic strength of buffer. However, there does seem to be a greater $\Delta\lambda_{max}$ when 0·001 M buffer is used, as opposed to 0·1 M. The differences between 0.01 and 0·001 M are insignificant.

The previously described procedure was repeated, substituting 0.001 M tris buffer, containing 0·001 M NaCl (pH 7·0) in place of 0·001 M (pH 7·0) phosphate buffer. The same qualitative and quantitative changes in the spectra of AME in the presence of DNA were produced.

Effect of denaturation of DNA *with heat.* The experiment was performed in a manner described under Materials and Methods. The results are presented in Table 2.

It appears that DNA, which has been heated for 10 min at 90°, is almost fully active in complexing with AME. Upon prolonged heating of DNA for 75 min at 105°, its ability to complex with AME is completely inhibited.

Effect of methanol. Solutions were prepared as usual except that varying amounts of methanol were incorporate in the buffer. The solution containing 0·5% methanol was used as the control. As the methanol concentration is increased, there is a gradual decrease in $\Delta\lambda_{max}$, until at 45% methanol there is practically no $\Delta\lambda_{max}$. On the other hand, the $\Delta\varepsilon\lambda_{max}$ is not noticeably decreased until about 30% of methanol is present, and when we reach a 45% methanol content, there is an abrupt drop in the $\Delta\varepsilon\lambda_{max}$. This suggests that methanol concentrations above 30 per cent are necessary to prevent formation of a complex between DNA and the antibiotic.

Effect of magnesium chloride, formaldehyde and urea. Solutions were prepared as described under Materials and Methods and results are presented in Table 2. It is obvious from these results that magnesium chloride inhibits DNA–AME complex formation. Formaldehyde at a concentration of 0·5 M prevented complex formation between DNA and AME. Urea, at a concentration of 0·2 M was without effect, as evidenced by the usual $\Delta\lambda_{max}$ of 9 mμ and $\Delta\varepsilon\lambda_{max} = -18{\cdot}6$ per cent.

Effect of origin of DNA. Three DNA's of different origins—salmon sperm DNA, *Escherichia coli* K_{12} DNA, and calf thymus DNA—were compared on the basis of their ability to complex with AME. The procedure was exactly as described in Materials and Methods and the results are presented in Table 2.

These results show that salmon sperm DNA causes a 1·7 per cent greater $\Delta\varepsilon\lambda_{max}$ and *Escherichia coli* K_{12} DNA a 1·1 per cent lower $\Delta\varepsilon\lambda_{max}$ than that caused by calf thymus DNA when in solution with AME. In respect to $\Delta\lambda_{max}$, the only significant change is produced by *Escherichia coli* K_{12} DNA, which exhibits a $\Delta\lambda_{max}$ of 7 mμ.

AME and nucleic acid derivatives. Because of the bathochromic shift and decrease in absorbance demonstrated by AME in the presence of DNA in pH 7·0 (0·001 M) phosphate buffer, a number of nucleic acid derivatives were examined for their ability to complex with AME. The concentration of the nucleic acid derivatives was 2 μmole/ml. In experiments with RNA its concentration was 0·600 mg/ml and thymidylic acid was used at a concentration of 1·5 μmole/ml.

None of the compounds listed below caused any change in λ_{max} and $\varepsilon\lambda_{max}$ of AME: RNA, deoxyguanosine, uracil, thymine, cytidine, 2,6-diaminopurine, adenine,

ADP (Na salt), ATP (di-Na salt), adenylic acid, guanylic acid, thymidine, thymidine-diphosphate (Ba salt), thymidylic acid (Ca, 2 H_2O) and deoxycytidine·HCl.

DISCUSSION

The ability of AME to affect the functioning of DNA in biological systems suggests that this compound might be useful in studying the mechanisms involved in certain DNA-dependent cellular processes. Our investigation concerns the specific molecular nature of AME–DNA interaction, and examines the structural requirements for the participating molecules by the use of various nucleic acid polymers and different anthramycin analogs.

The spectrophotometric method used in those experiments to determine complex formation utilizes a change in the absorbtion spectrum of the ligand (AME or analogs) on being bound by DNA.

Of the ten compounds related to AME, only six compounds have shown both *in vitro* microbiological activity, e.g. against *Bacillus sp. "TA"* (approximately 16,000 arbitrary units/mg), and *in vivo* activity against *Sarcoma*-180 and *Ehrlich carcinoma* solid form tumor in mice (0·2 mg AME/kg). All other compounds related to anthramycin that were incapable of forming a complex with DNA were also devoid of biological activity. It might be considered, therefore, that complexing with DNA could be a primary cause of *in vitro* and particularly of *in vivo* antitumor activity of AME. The use of u.v. spectrophotometry for investigating the ability of compounds of the anthramycin series to complex with DNA also represent a fast and practical method for screening new compounds of this series for possible antitumour activity.

Concerning structural features of anthramycins for interaction with DNA and subsequent biological activity, free positions 9 and 10 in the molecule appear to be necessary. The epimeric AME (Fig. 1, No. 6), which could be obtained by treatment of anhydroanthramycin with methanol at room temperature, showed identical behavior when in solution with DNA and the same biological properties.

Compound No. 9 (anhydroanthramycin) can be considered as identical with compound No. 1 (anthramycin) in aqueous solution. Position II can be a hydroxyl or an ether group. Availability of more diversified anthramycin analogs would afford the possibility of more significant structure activity correlation in this series of antitumor compounds.

Concerning the mode of complexing of AME with DNA, it can be mentioned that neither free purine, nor pyrimidine, nor their derivatives were able to complex with AME. It seems, therefore, reasonable to assume that the three dimensional structure of DNA furnishes a specifically oriented array of functional groups which together account for the interaction with AME. Since the spectroscopic changes of the chromophore groups caused by complex formation with DNA are only slightly affected by ionic strength, we can assumed that electrostatic forces are only partially operating in the binding.

$MgCl_2$ affected the binding of AME to DNA as seen in Table 2. In those experiments, $MgCl_2$ was added to a solution containing AME and DNA in the concentration indicated. Earlier experiments have shown that the order of addition was inconsequential and that incubation of either AME or the metal ions with DNA did not affect the results. Mg^{2+} reduced the AME–DNA interaction to a much greater extent than might be expected on the basis of ionic strength considerations alone, which

suggests that these ions may compete more strongly with AME for binding sites on DNA. In this respect, it has been shown previously[7] that Mg^{2+} ions bind to the negatively charged phosphate groups of DNA and that these cations can prevent the interaction of other substances at these sites.

Alkaline metal cations in the same concentrations as Mg^{2+} do not have effect on the binding of AME to DNA, as is evident from the experiment with phosphate buffers of various concentrations.

Concerning the influence of heat on AME–DNA interaction, it is known that a short period of heating DNA (as in our experiment) in an oven for 10 min at 90° (temperature of the DNA solution did not exceed 60°) does not irreversibly separate two DNA strands.[8] Prolonged heating, however (e.g. 75 min at 105°), does cause irreversible separation. In our experiment, with the latter condition, AME did not complex with DNA, as was evident from the unchanged spectrum of AME. It appears, therefore, that the ability to build a complex with DNA is related to the extent of the double-stranded part of the DNA molecule. Urea at a concentration of 0·2 M was, however, without effect. Formaldehyde at concentrations of 0·5 M prevents complexing.

It appears, however, from the work of Sarker and Dounce[9] that calf thymus DNA does not react with 1·3% formaldehyde at room temperature. The conclusion, therefore, could be that the interaction of DNA with AME disrupts interstrand hydrogen bonding, exposing in this manner at least some of the amino groups in the DNA to the action of formaldehyde. Subsequent reactions of DNA with formaldehyde will then disrupt the DNA–AME complex, and will produce corresponding spectral change.

The necessity of water molecules for the formation of the complex can be deduced from the action of alcohol. The examination of the effects of pH over a broad range on the absorption spectrum of AME bound with DNA was not feasible because of the instability of antibiotics at extremes of pH. Further examination of the interaction of AME with DNA might contribute to the study of the effect of this antitumor agent and of nucleic acid function.

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