CHARACTERIZATION OF BLEOMYCIN-RESISTANT DNA

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ABSTRACT After reaction of DNA with high concentrations of bleomycin, approximately 80% of the DNA becomes trichloroacetic acid (TCA) soluble. The remaining 20% of the DNA remains TCA insoluble. Upon further treatment of this TCA-insoluble material with high concentrations of the drug, no further drug action can be detected. Drug action is defined as fragmentation of DNA to smaller molecular size, release of free bases, and TCA solubilization. This material which is not attacked by bleomycin has been termed bleomycin-resistant DNA. This bleomycin-resistant DNA does not compete with native DNA in the bleomycin reaction indicating that there is no binding or inactivation of the drug by the resistant DNA. The resistant DNA shows very little hyperchromicity when heated through the melting temperature for the corresponding native DNA, indicating a single-stranded structure. Results of sedimentation and equilibrium analyses yield a molecular weight of about 4,000 daltons. This value is the same regardless of the source of the native DNA. Finally, the bleomycin-resistant DNA exhibits a base composition similar to that of the native DNA from which it was derived.

INTRODUCTION

The antineoplastic antibiotic bleomycin has been found to be effective against Ehrlich sarcoma (1), transplantable tumors of mice (2), human squamous cell carcinoma, Hodgkin's disease and reticuloma (3). Since there is no evidence of bone marrow, liver, or renal toxicity in humans treated with bleomycin, it is felt that this drug might be useful in the treatment of certain human malignancies. The most serious side effect encountered in some patients is a pulmonary fibrosis (4).

It is generally considered that the primary lethal effect of bleomycin is the result of its ability to cause the fragmentation of DNA (5–8). This fragmentation reaction can be observed both intracellularly (in vivo reaction) and extracellularly on isolated DNA (in vitro reaction). Although this fragmentation reaction is most likely the primary mode of action of bleomycin, there is as yet no definitive proof for this.

In our previous communications, we demonstrated, for the in vitro reaction, that in addition to the endonucleolytic-like fragmentation reaction of DNA one could

detect the release of all four of the DNA bases (9), and that there was no detectable reaction of bleomycin with RNA (10). We have postulated that the site of specificity for bleomycin activity resides in the deoxyribose moiety of DNA (10). It was noted that approximately 80% of the DNA became trichloroacetic acid-soluble after reaction with bleomycin. The remaining 20% of the DNA was resistant to further action of the drug.

The present study details the characterization of this bleomycin-resistant DNA.

METHODS

Chemicals

Bleomycin was a generous gift of Bristol Laboratories, Syracuse, N. Y. (Lot No. 71L1263), and of Nippon Kayaku Co., Tokyo (Lot No. F7071BS). [methyl-^{3}H]thymidine (20 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. [methyl-^{3}H]thymine (20 Ci/mmol) was purchased from Schwarz BioResearch, Orangeburg, N. Y. $H_3{}^{32}PO_4$ (carrier-free) was purchased from Amersham/Searle Corp., Chicago, Ill. PEI cellulose paper was purchased from Brinkmann Instruments, Inc., Westbury, N. Y. Polydeoxyadenylate-thymidylate, poly d(A-T), and polydeoxythymidylate, poly dT, were purchased from Miles Laboratories, Inc., Elkhart, Ind. Bovine pancreatic deoxyribonuclease I (E.C. 3·1·4·5, ribonuclease-free) and venom phosphodiesterase from *Crotalus adamanteus* (E.C. 3·1·4·1) were purchased from Worthington Biochemical Corp., Freehold, N. J. Tissue culture media were purchased from Grand Island Biological Co., Grand Island, N. Y. All other chemicals were purchased from general suppliers.

Growth of Cells for Labeled DNA

Bacillus subtilis (thy trp C2) DNA labeled with [^{3}H]thymidine was obtained as previously described (5). Labeled T-2 bacteriophage DNA was obtained by the addition of 1×10^8 viable phage particles to 8×10^{10} cells of *Escherichia coli* B contained in 200 ml of Hershey's Nutrient Broth containing 1.0 μCi/ml of [^{3}H] thymidine and incubated for 3.5 h at 37°C (multiplicity of infection = 0.001). Lysozyme (100 μg/ml) was added, followed after 15 min at 37°C by the addition of DNase (5 μg/ml) and an additional 30 min incubation at 37°C. Chloroform (5 ml) was added and the mixture was stored overnight at 4°C. Cell debris was removed by centrifugation for 10 min at 10,000 *g*. Phage particles were sedimented from the supernatant fluid by centrifugation for 60 min at 20,000 *g*. Labeled Chinese hamster cell DNA (Don C line kindly supplied by Dr. T. E. Stubblefield) was obtained by growing 1×10^7 cells for 24 h at 37°C in 40 ml of McCoy's Medium supplemented with 20% fetal calf serum containing 10 μCi/ml of [^{3}H]thymidine. Labeled *E. coli* B DNA was obtained by inoculating 1×10^8 cells into 1 liter of Modified Hershey's Broth containing 12 μCi/ml of $H_3{}^{32}PO_4$ and growing for 7 h at 37°C with shaking. All DNAs were isolated according to the procedure described by Marmur (11). For DNA labeled with ^{32}P, a CsCl density gradient purification step was included.

Gradient Centrifugation

Alkaline and neutral sucrose gradient centrifugation analyses were performed as previously described (5).

Paper Chromatography

Descending paper chromatography was performed on Whatman No. 3MM filter paper developed with methanol-ethanol-concentrated HCl-water (50:25:6:19) as previously described (5).

Preparation of Resistant DNA

All of the reaction mixtures contained: bleomycin (15 mg), 2-mercaptoethanol (5 mM), and DNA (300 μg) in 0.5 ml of tris buffer (0.1 M, pH 7.0). The reaction mixtures were incubated in glass stoppered tubes at 22°C for 4 h. After incubation, the mixtures were dialyzed against 1 liter (two changes of 500 ml each) of buffer (0.01 M NaCl, 0.001 M Na orthophosphate, pH 6.9) for 24 h at 4°C. After dialysis, the mixtures were reacted anew with the bleomycin (15 mg) and 2-mercaptoethanol for 4 h at 22°C. The dialysis step was repeated with 2 liters (four changes of 500 ml each) of the orthophosphate buffer for 2 days at 4°C.

Alternatively, the resistant DNA, after reaction with high bleomycin concentrations, can be obtained directly from the top of a neutral sucrose gradient followed by the dialysis step described above.

Reaction Mixtures for Competing DNAs

These reaction mixtures contained 406 μg/ml of unlabeled *B. subtilis* DNA; 11 mM 2-mercaptoethanol and bleomycin at the indicated concentrations contained in a total volume of 0.45 ml of tris buffer (0.1 M, pH 7.0). The reaction mixtures were incubated for 1 h at 22°C. After incubation, the mixtures were dialyzed against 1 liter of tris buffer (0.05 M, pH 8.0) overnight prior to their use as competing DNA.

Thermal Denaturation Analyses

Thermal denaturation profiles were measured at 260 nm using a Beckman DK-2 Ratio Recording Spectrophotometer. The DNAs were dissolved in orthophosphate buffer (0.01 M NaCl, 0.001 M Na orthophosphate, pH 6.9); both sample and solvent were degassed by bubbling with helium prior to analysis. Stoppered cuvettes were used and were heated at a rate of 0.5–1.0° C/min. The temperature was monitored during denaturation using a Beckman Conductivity Bridge Model 16B2 with a calibrated thermister placed in the reference cuvette. Complete spectra of the samples were taken before and after heating.

Sedimentation Velocity Analyses

Sedimentation velocity analyses were performed using a Beckman Model E Analytical Ultracentrifuge equipped with a high intensity light source, scanning system, and multiplexer. The analytical AN-F titanium rotor was used at 52,640 rpm. 12-mm double sector cells were used; cell centerpieces were made of Kel-F (3M Company, St. Paul, Minn.) coated aluminum or carbon-filled Epon (Shell Chemical Co., New York). The solution column contained 0.4 ml of sample (absorbance at 265 nm = 0.7–0.8); the solvent column contained slightly more. Median sedimentation coefficients were calculated.

Sedimentation Equilibrium Analyses

Sedimentation equilibrium measurements were performed with the same rotor and cells as described above using the short column technique of Van Holde and Baldwin (12). The

solution sector contained 80 μl of sample (column height = 0.22 cm; absorbance at 265 nm = 0.4–0.5) and 10 or 15 μl of FC43 fluorocarbon fluid to serve as the base. The solvent column consisted of 105 μl of the final dialysate buffer. Measurements were recorded after 5 h at 29,500 rpm at 20°C. Apparent molecular weights were calculated from plots of ln absorbance vs. r^2. The value of the density increment used in this calculation was that of Cohen and Eisenberg (13) for native calf thymus DNA in 1.000 M NaCl at 25°C: $(\partial\rho/\partial C_2)^0_\mu$ = 0.414. Although bleomycin resistant DNA is single-stranded, no appreciable error results from using the density increment of native DNA in this calculation, since the density of denatured DNA is within 1% of that of the native molecule at neutral pH (14, 15).

Enzymatic Digestion of DNA and Chromatography of Products

Approximately 0.5 ml (50 μg) of ^{32}P-labeled *E. coli* native DNA or resistant DNA was digested by sequential treatment with pancreatic DNase I and venom phosphodiesterase according to the method of Spencer et al. (16). This treatment resulted in the digestion of approximately 99% of the native DNA and 60% of the resistant DNA to the 5'-mononucleotides. The products of the hydrolysis were separated by column chromatography using Bio-Rad AG-50 resin (Bio-Rad Laboratories, Richmond, Calif.) (17) and PEI cellulose thin-layer chromatograms (developed using 0.5 M formic acid-NH_4 formate buffer, pH 4.2) were traced after autoradiography on X-ray film; the resultant spots were cut out and counted in POPOP-PPO-toluene solution (18). The separated mononucleotides from the column chromatogram were counted by Cerenkov radiation.

RESULTS

When native DNA was reacted with high concentrations of bleomycin followed by neutral sucrose gradient centrifugation, the profile shown in Fig. 1 *A* was obtained. This bleomycin-treated DNA has lost approximately 80% of the TCA precipitable counts when compared with the control, confirming our previous results (5). The profile was the same regardless of which labeled base was used in the preparation of the DNA. This DNA, shown in part *A* of Fig. 1 (reband), when reacted anew with bleomycin showed no further release of TCA soluble material when compared with the control which was not retreated with the drug. The relative sedimentation distance for the treated DNAs of Fig. 1 *A* and *B* is consistent with that of low molecular weight single-stranded DNA. The same results as shown in Fig. 1 were obtained when T-2 phage DNA, Chinese hamster cell DNA, or poly d(A-T) were used.

Fig. 2 shows the results of competition experiments with the resistant DNA and with DNA only partially degraded by bleomycin. These experiments (Fig. 2) demonstrate that unlabeled, untreated DNA competed with labeled DNA in the bleomycin reaction. This competition is manifested by the position of the DNA in the gradient (greater competition by the unlabeled DNA resulting in less fragmentation of the labeled DNA) and by the area under the peaks (the smaller area indicating more TCA solubilization and consequently less competition). When labeled DNA was mixed with unlabeled DNA, which was partially degraded by bleomycin followed by reaction with high bleomycin concentration, there was less competition. Finally,

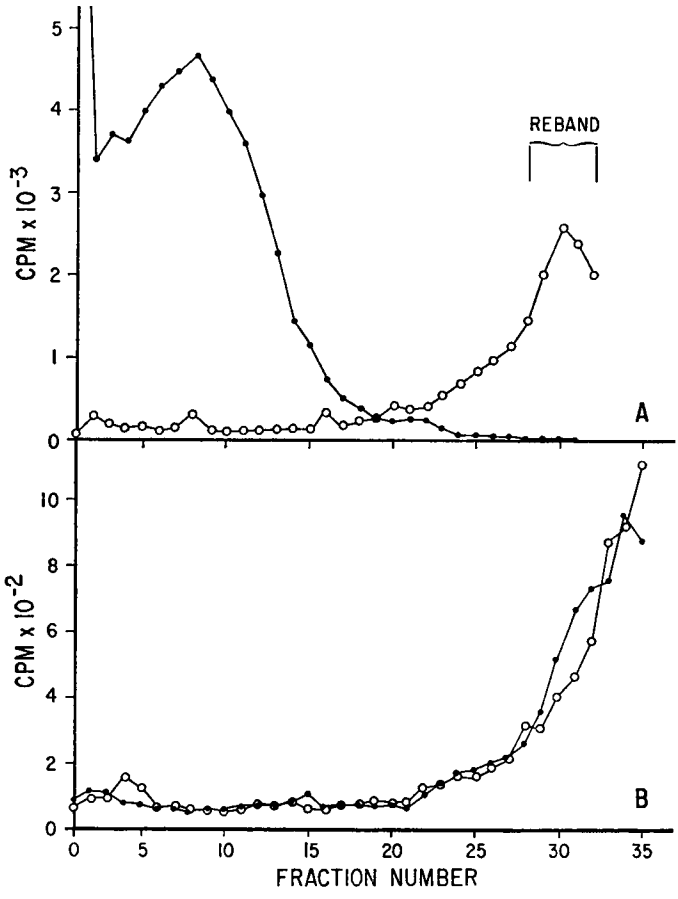

FIGURE 1 Sucrose gradients of bleomycin-resistant DNA. Sedimentation was to the left. The gradients were centrifuged at 5°C for 4 h at 149,000 *g*. (*A*) Neutral sucrose gradients. The reaction mixtures contained 75 μg/ml of [^{3}H]thymine-labeled *B. subtilis* DNA and 25 mM 2-mercaptoethanol. ●, control, no bleomycin; ○, 4.0 mg/ml of bleomycin. (*B*) Alkaline sucrose gradients. The reaction mixtures contained dialysed DNA from the top of the gradient shown above (reband) divided in half plus 10 mM 2-mercaptoethanol. ●, control, no additional bleomycin; ○, 10 mg/ml of bleomycin. The reaction mixtures were incubated for 1 h at 22°C. Background counts were not subtracted.

when the labeled DNA was mixed with bleomycin-resistant DNA followed by reaction with high bleomycin concentration, there was no competition.

When resistant [^{3}H]thymine-labeled DNA was reacted anew with bleomycin, there was no thymine released as demonstrated by paper chromatography. However, thymine was released, as expected, after perchloric acid hydrolysis of the resistant DNA. We have previously demonstrated that all four free bases are released after reaction of labeled native DNA with bleomycin (9).

Fig. 3 shows the melting characteristics of resistant DNA derived from *B. sub-*

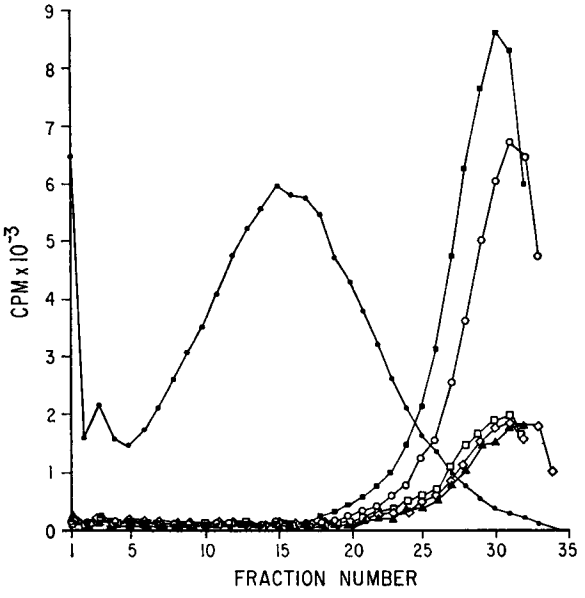

FIGURE 2 Competition of unlabeled native *B. subtilis* DNA and unlabeled end-products with [³H]thymine-labeled native DNA in the bleomycin reaction. Alkaline sucrose gradients; sedimentation was to the left. The gradients were centrifuged at 5°C for 4 h at 149,000 *g*. The reaction mixtures contained 37.5 μg/ml of [³H]thymine-labeled DNA plus 20 mM 2-mercaptoethanol. ●, control, no bleomycin, no competing DNA; ◇, 10 mg/ml of bleomycin, no competing DNA; ■, 10 mg/ml of bleomycin, 101 μg/ml of competing DNA untreated with bleomycin (see methods); ○, 10 mg/ml of bleomycin, 101 μg/ml of competing DNA pretreated with 5.6 mg/ml of bleomycin; ▲, 10 mg/ml of bleomycin, 101 μg/ml of competing DNA pretreated with 22 mg/ml of bleomycin; □, 10 mg/ml of bleomycin, 101 μg/ml of competing DNA pretreated with 22 mg/ml of bleomycin (competing DNA was from the top of a neutral sucrose gradient). Background counts were not subtracted.

tilis. This resistant DNA shows very little hyperchromic shift when heated through the melting temperature for the corresponding native DNA. The spectrum of resistant DNA at high temperature is not appreciably different from its spectrum at room temperature.

Table I shows the results of sedimentation velocity analyses on resistant DNAs from various sources including the synthetic deoxyribopolymers poly d(A-T) and poly dT. The Svedberg values are quite similar regardless of the source of DNA and they are fairly constant among the experiments. Very similar results are seen in Table II which shows the apparent molecular weights as determined by short-column sedimentation equilibrium. Again the molecular weight is independent of the source of the DNA. Data from a typical experiment are shown in Fig. 4.

Table III shows the nucleotide composition of native *E. coli* DNA, following enzymatic hydrolysis and column chromatography. Although the value for 5′-AMP does not equal that of 5′-TMP as shown in the table, this experimental error was

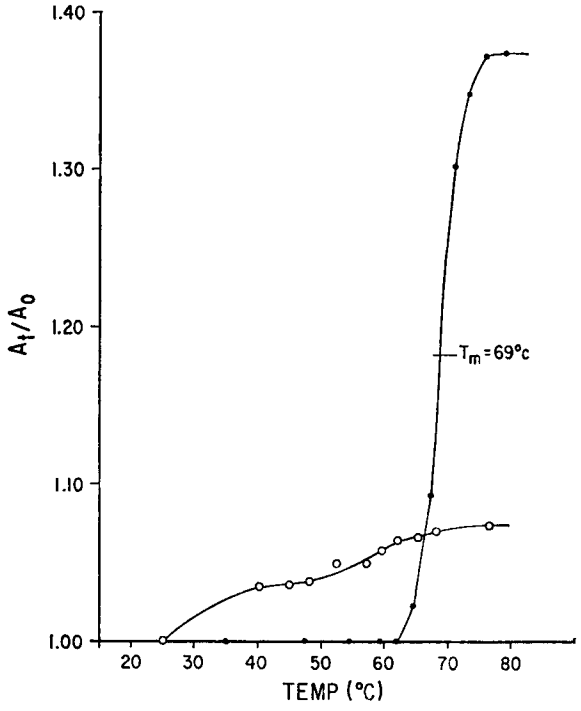

FIGURE 3 Melting profile of resistant DNA. ●, Native *B. subtilis* DNA; ○, resistant DNA derived from *B. subtilis*.

TABLE I

MEDIAN SEDIMENTATION COEFFICIENTS OF BLEOMYCIN-RESISTANT DNA*

Source	Experiment number							
	773	704	691	700	752	834	839	884
B. subtilis	1.11	1.19	1.03	0.96	0.95			
E. coli	1.18	1.53						
Calf thymus	1.13							
Poly d(A-T)						1.37	1.02	
Poly dT								1.32

* S_{20} at 1.00 M NaCl, 0.001 M Na orthophosphate, pH = 6.9.

consistent and probably reflects a low estimation for 5′-AMP caused by incomplete elution from the column or conversely a consistently high value for 5′-TMP as a result of some inorganic phosphate which elutes with 5′-TMP. Table IV shows the nucleotide composition of the resistant DNA from *E. coli*. Since this resistant DNA was incompletely hydrolyzed enzymatically, some oligonucleotides were found to elute with the 5′-TMP using the column chromatography system. This results in the high values shown in the first row of Table IV. However, the 5′-TMP can be

TABLE II

APPARENT MOLECULAR WEIGHT OF BLEOMYCIN-RESISTANT DNA*

Source	Experiment number				
	749 *a*	749 *b*	754	765	790
B. subtilis	3,500	4,300	3,200	3,100 3,800	4,600
E. coli	4,000	4,500			4,500
Calf thymus	3,800	4,500			4,300

* Assume $(\partial\rho/\partial C_2)^0_\mu = 0.414$ in 1.00 M NaCl, 0.001 M Na orthophosphate, pH = 7.0. Apparent weight average molecular weights calculated from the relative concentrations at the meniscus and at the bottom of the cell (12) were consistently about 4% higher than the molecular weights recorded here, which were estimated by a linear fit of ln A vs. r^2.

TABLE III

E. COLI DNA—NUCLEOTIDE COMPOSITION

Nucleotides	Experiment number				
	816-1	816-2	816-3*	808	Average
5′-TMP	25	25	51	25	25.0 ± 0.2
5′-CMP	28	27	51	28	27.1 ± 0.4
5′-GMP	26	26	26	26	26.2 ± 0.3
5′-AMP	21	22	22	22	22.0 ± 0.4

AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; TMP, thymidine monophosphate.

* Thin-layer chromatography.

TABLE IV

E. COLI RESISTANT DNA—NUCLEOTIDE COMPOSITION

Nucleotides	Experiment number				
	823	818	811	822	Average
5′-TMP and oligonucleotides	59	59	57	59	58.4 ± 0.8
5′-CMP	14	14	15	14	14.2 ± 0.3
5′-GMP	14	14	15	14	14.1 ± 0.3
5′-AMP	13	13	14	13	13.3 ± 0.3

separated from the contaminating oligonucleotides using thin-layer chromatography. When this was done, it was found that the percentage of 5′-TMP was closely similar to that of the other 5′-mononucleotides.

Spectrophotometric measurements indicated an unusual absorbance at 290 nm

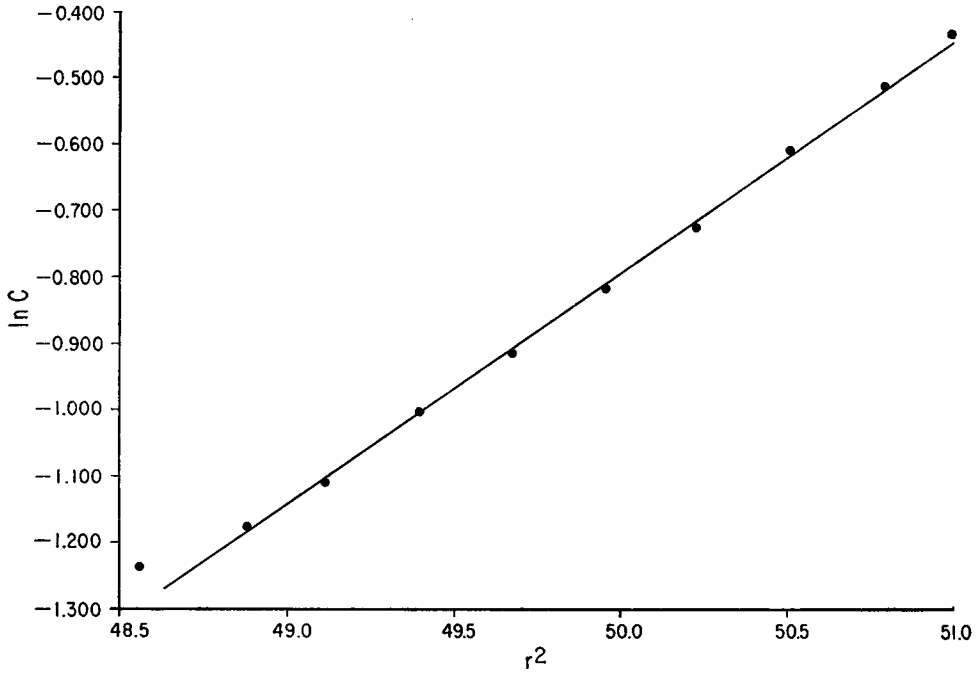

FIGURE 4 Plot of ln concentration (absorbance) versus distance (r^2 in square centimeters) from the axis of rotation in a typical sedimentation equilibrium experiment. $\bar{M}_w$ (apparent) is 4,300 daltons from a linear fit of ln A vs. r^2, 4,500 daltons from a quadratic curve fitted by least squares. The apparent Z-average molecular weight for this experiment is 6,100 daltons.

for the dialyzed, resistant DNA. When this resistant DNA was further purified using phenol followed by dialyses, this unusual absorbance disappeared and the spectrum for the resistant DNA resembled that of denatured, untreated DNA.

DISCUSSION

The results clearly demonstrate the presence of a fraction of natural or synthetic deoxyribopolymer which is resistant to further action of bleomycin. This resistance is manifested by the failure of the DNA, previously reacted with high drug concentration, to undergo further fragmentation, to release free bases or to be further solubilized by TCA when reacted anew with additional high drug concentrations. The failure to undergo further fragmentation and solubilization is seen both in Fig. 1 and in Table I which shows the constancy of Svedberg values after repeated drug treatment. The failure to cause the release of additional free base is described in the text. Thus none of the three parameters of bleomycin activity (fragmentation, TCA solubilization, and release of free base) can be demonstrated for the resistant DNA. This is further strengthened by the results of the competition experiments

presented in Fig. 2 which show that the resistant DNA has absolutely no effect upon the activity of bleomycin toward native DNA.

That this resistance to the action of bleomycin is a function of some specific sequence of bases is highly unlikely since the base composition of the resistant DNA from *E. coli* is similar to that of the parental DNA. In addition, since the alternating copolymer poly d(A-T) and the homopolymer poly dT both yield a resistant fraction of similar size to the resistant fraction from native DNAs, the base sequence must not be the factor which confers the resistance to bleomycin action.

Our results indicate that the resistant DNA is a single-stranded molecule containing approximately 12 nucleotides. We have previously shown that both single- and double-stranded DNA is degraded by the drug (5), therefore the single-strandedness of the resistant DNA per se should not confer resistance. We are presently investigating the possibility that single-stranded regions may indeed be necessary to provide a point of attack for bleomycin. Presently, we have no information concerning the possibility of missing bases along the backbone of the resistant DNA, which could conceivably have an effect on the action of the drug.

All of the DNAs and synthetic polymers studied yield a molecule with a molecular weight of approximately 4,000 daltons. The most likely explanation for the lack of reactivity of bleomycin is the small molecular size. A simple hypothetical model to account for such a size requirement would be that bleomycin has at least two different binding sites for DNA, both of which must be operable for drug action. If these sites on the drug molecule were situated further apart than the distance occupied by 12 or 13 nucleotides (the length of the resistant DNA) then the drug could not attach to such a molecule and consequently would be inactive.

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REFERENCES

1. UMEZAWA, H., M. ISHIZUKA, K. KIMURA, J. IWANAGA, and T. TAKEUCHI. 1968. *J. Antibiot. (Tokyo)*. **21**:592.
2. TAKEUCHI, M., and T. YAMAMOTO. 1968. *J. Antibiot. (Tokyo)*. **21**:631.
3. ICHIKAWA, T. 1970. *Prog. Antimicrobial Anticancer Chemother.* **2**:288.
4. SHASTRI, S., R. E. SLAYTON, J. WOLTER, C. P. PERLIA, and S. G. TAYLOR. 1971. *Cancer.* **28**:1142.
5. HAIDLE, C. W. 1971. *Mol. Pharmacol.* **7**:645.
6. SUZUKI, H., K. NAGAI, H. YAMAKI, N. TANAKA, and H. UMEZAWA. 1969. *J. Antibiot. (Tokyo)*. **22**:446.
7. NAGAI, K., H. SUZUKI, N. TANAKA, and H. UMEZAWA. 1969. *J. Antibiot. (Tokyo)*. **22**:569.
8. NAGAI, K., H. SUZUKI, N. TANAKA, and H. UMEZAWA. 1969. *J. Antibiot. (Tokyo)*. **22**:624.
9. HAIDLE, C. W., K. K. WEISS, and M. T. KUO. 1972. *Mol. Pharmacol.* **8**:531.
10. HAIDLE, C. W., M. T. KUO, and K. K. WEISS. 1972. *Biochem. Pharmacol.* **21**:3308.
11. MARMUR, J. 1961. *J. Mol. Biol.* **3**:208.

12. VAN HOLDE, K. E., and R. L. BALDWIN. 1958. *J. Phys. Chem.* **62**:734.
13. COHEN, G., and H. EISENBERG. 1968. *Biopolymers.* **6**:1077.
14. MESELSON, M., and F. W. STAHL. 1958. *Proc. Natl. Acad. Sci. U. S. A.* **44**:671.
15. CHAPMAN, R. E., JR., and J. M. STURTEVANT. 1969. *Biopolymers.* **7**:527.
16. SPENCER, J. H., R. ČERNÝ, E. ČERNÁ, and A. D. DELANEY. 1972. *J. Virol.* **10**:134.
17. BLATTNER, F. R., and H. P. ERICKSON. 1967. *Anal. Biochem.* **18**:220.
18. RANDERATH, K., and E. RANDERATH. 1964. *J. Chromatogr.* **16**:126.