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Biochemical Pharmacology, Vol. 21, pp. 3308–3312. Pergamon Press, 1972. Printed in Great Britain.

Nucleic acid—Specificity of bleomycin*

(*Received* 14 *April* 1972; *accepted* 8 *August* 1972)

BLEOMYCIN causes a decrease in the melting temperature of DNA and, in addition, produces a scission of DNA strands both *in vitro* and *in vivo*, the former reaction requiring 2-mercaptoethanol or hydrogen peroxide.[1–3] *In vitro*, high concentrations of the drug render about 80 per cent of the DNA trichloroacetic acid (TCA) soluble[4] and also cause the release of free bases from DNA.[5]

In addition to the scission of native DNA strands, Nagai *et al.*[3] showed that bleomycin also caused the fragmentation of the synthetic deoxyribopolymers, polydeoxycytidylate and polydeoxyguanidylate. However, Suzuki *et al.*[6] observed no fragmentation of ribosomal or transfer RNA in the presence of bleomycin.

The present study was initiated to determine the reason for the apparent specificity of bleomycin toward DNA.

The bleomycin was a gift from Bristol Laboratories, Syracuse, N.Y. (lot No. 701233), and from Nippon Kayaku Company, Tokyo, Japan, (lot No. F7071BS). This bleomycin has an absorption maximum at 292 nm. Uridine-2-^{14}C (50·0 mCi/m-mole) was purchased from International Chemical and Nuclear Corp., Irvine, Calif. Polyuridylate-^{3}H (78·1 mCi/m-mole phosphorus) and the copolymer, polydeoxyandenylate-thymidylate-^{3}H (25·1 mCi/m-mole phosphorus) were purchased from Miles Laboratories, Inc., Elkhart, Ind. Uracil-2-^{14}C (56·3 mCi/m-mole) and uridine-5-^{3}H (27·8 Ci/m-mole) were purchased from New England Nuclear, Boston, Mass. Polyadenylate-^{3}H (51·0 mCi/m-mole phosphorus), thymidinemethyl-^{3}H (11·9 Ci/m-mole) and thymine-2-^{14}C (48·6 mCi/m-mole) were purchased from Schwarz BioResearch, Orangeburg, N.Y. Bovine pancreatic ribonuclease A (EC 2.7.7.16), 5 times crystallized, protease-free, was purchased from Sigma Chemical Company, St. Louis, Mo. Yeast ribonucleic acid sodium salt was purchased from Mann Research Laboratories, Inc., New York, N.Y. All other chemicals were purchased from commercial sources.

Labeled bacterial DNA was prepared from *Bacillus subtilis* 168 (*thy trp* C2) as previously described.[4] Uridine-5-^{3}H-labeled DNA, in which deoxyuridylate replaces thymidylate,[6] was prepared from confluent lysis plates of bacteriophage PBS-1 using *B. subtilis* 168 (*trp* C2) as the host bacterium. The plating procedure of Takahashi was used with minor modifications.[7–9]

Labeled RNA was prepared from *B. subtilis* (*thy trp* C2) according to the method of Okamoto *et al.*[10] The bacterial cells were grown as previously described for the isolation of DNA, except that 0·2 μCi/ml of uridine-2 ^{14}C was added to the culture medium.

DNA concentration was assayed by the diphenylamine method of Burton.[11] RNA concentration was assayed by the orcinol method of Hurlbert *et al.*[12]

* This research was supported in part by Grant CA 13246 from the United States Public Health Service and Grant G-441 from the Robert A. Welch Foundation.

Neutral and alkaline sucrose gradient centrifugation analyses were performed as previously described.[4]

Descending paper chromatography was performed on Whatman No. 3 MM chromatography paper developed with methanol–ethanol–concentrated HCl–water (50:25:6:19) for 16 hr, as previously described.[4]

All reaction mixtures were incubated in tightly stoppered tubes in Tris–EDTA buffer [0·05 M Tris, 0·1 M (ethylenedinitrilo) tetraacetic acid, pH 8·0].

There are, in general, three major structural differences between RNA and DNA. These differences are in base composition (uracil vs. thymine), in sugar content (ribose vs. deoxyribose), and in secondary structure. Any of these differences, either alone or in combination, could be responsible for the reaction of bleomycin with DNA or, conversely, responsible for its failure to react with RNA. Reaction, in this case, is defined as the ability of the antibiotic to fragment the molecule to smaller molecular size, to render at least a portion of the molecule TCA soluble and, finally, to release free bases from the molecule.

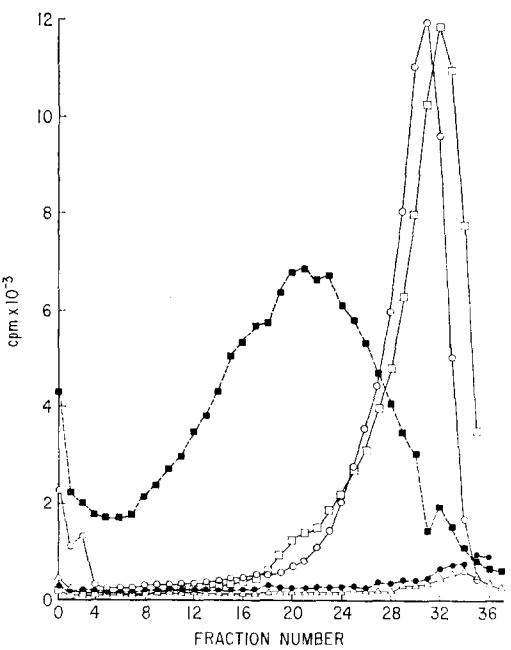

FIG. 1. Effect of bleomycin on uridine-^{14}C-labeled RNA and thymidine-^{3}H-labeled DNA. Neutral sucrose gradients. Sedimentation (4 hr) was to the left. The reaction mixtures contained: □, RNA control, 38 μg/ml of the ^{14}C-labeled RNA, 25 mM 2-mercaptoethanol; ○, 38 μg/ml of the ^{14}C-labeled RNA, 15 mg/ml of bleomycin, 25 mM 2-mercaptoethanol; △, 38 μg/ml of the ^{14}C-labeled RNA, 100 μg/ml of ribonuclease. Alkaline sucrose gradients. Sedimentation was to the left. The reaction mixtures contained: ■, DNA control, 38 μg/ml of thymidine-^{3}H-labeled DNA, 25 mM 2-mercaptoethanol; ●, 38 μg/ml of the ^{3}H-labeled DNA, 15 mg/ml of bleomycin, 25 mM 2-mercaptoethanol. The reaction mixtures were incubated for 2 hr at 22°.

The sucrose gradient represented by Fig. 1 shows that bleomycin does not act on RNA. Under the same experimental conditions and using the same solution of bleomycin, the DNA becomes fragmented, and more than 80 per cent of the DNA becomes TCA soluble. Although there is no detectable fragmentation or solubilization of RNA by bleomycin, Fig. 2 shows that yeast RNA does compete with DNA in the bleomycin reaction *in vitro*. This same result is observed using *B. subtilis* RNA which

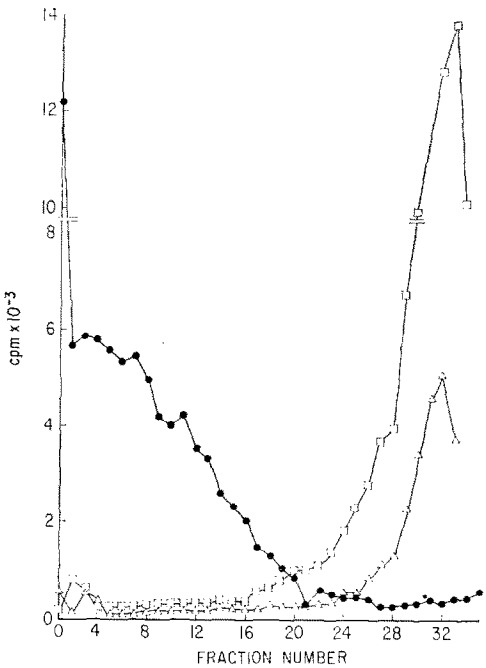

FIG. 2. Inhibition of bleomycin action on DNA by RNA. Alkaline sucrose gradients. Sedimentation (4 hr) was to the left. The reaction mixtures contained 38 μg/ml of thymidine-^{3}H-labeled DNA, 25 mM 2-mercaptoethanol, and the following additions: ●, control, no additions; △, 5 mg/ml of bleomycin; □, 5·0 mg/ml of bleomycin, 100 μg/ml of unlabeled yeast RNA. The reaction mixtures were incubated for 2 hr at 22°.

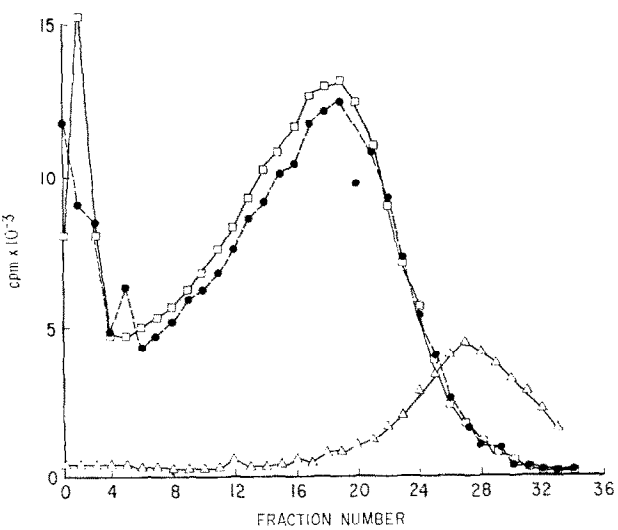

FIG. 3. Effect of bleomycin on polydeoxyadenylate-thymidylate-^{3}H. Neutral sucrose gradient. Sedimentation (16 hr) was to the left. The reaction mixtures contained 13·0 μg/ml (1·0 μCi/ml) of poly dAT-^{3}H, 20 mM 2-mercaptoethanol and the following additions: ●, control, no additions; △, 6·0 mg/ml of bleomycin; □, 40 μg/ml of ribonuclease. The reaction mixtures were incubated for 2 hr at 22°.

was treated with deoxyribonuclease I. Figure 3 shows the results of the action of bleomycin on polydeoxyadenylate-thymidylate-^{3}H (poly dAT). There is a decrease in the molecular weight (fragmentation) of the synthetic polymer, and 80 per cent of the poly-dAT becomes TCA soluble. However, when polyuridylate, which has no organized structure above 15°,[13] is reacted with bleomycin, there is no observable decrease in molecular weight, nor is there any TCA solubilization. Figure 4 demonstrates the results of paper chromatography of bleomycin-treated poly dAT and poly U. It can be seen that thymine is released from poly dAT (labeled only in the thymine moiety), but no detectable amounts of uracil are released from poly U. In addition, there is no reaction of bleomycin with the synthetic ribopolymer, polyadenylate.

When polyadenylate is mixed with polyuridylate under suitable conditions,[13] they react spontaneously to form a double-helical structure very similar to DNA in which uracil replaces thymine and is paired with adenine. Bleomycin causes neither fragmentation nor TCA solubilization of this double-stranded complex. The same result is observed in the presence or absence of ribonuclease, which degrades the unpaired portions of the complex.

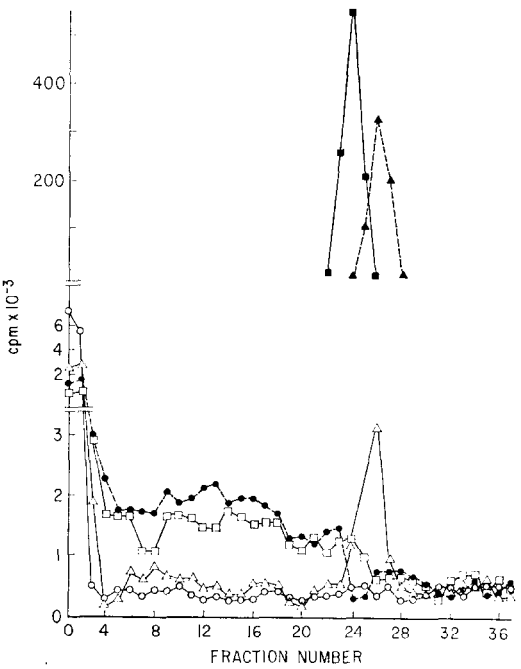

FIG. 4. Paper chromatogram of bleomycin-treated polydeoxyadenylate-thymidylate-^{3}H and polyuridylate-^{3}H. The reaction mixtures contained: ○, 13·0 μg/ml (1·0 μCi/ml) of poly dAT-^{3}H, 20 mM 2-mercaptoethanol; △, 13·0 μg/ml (1·0 μCi/ml) of poly dAT-^{3}H, 20 mM 2-mercaptoethanol, 6·0 mg/ml of bleomycin; □, 4·2 μg/ml (1·0 μCi/ml) of poly U-^{3}H, 20 mM 2-mercaptoethanol; ●, 4·2 μg/ml (1·0 μCi/ml) of poly U-^{3}H, 20 mM 2-mercaptoethanol, 6·0 mg/ml of bleomycin; 0·04 ml of each reaction mixture was spotted; ▲, 0·05 ml of authentic thymine-^{14}C was spotted as a standard; ■, 0·01 ml of authentic uracil-^{14}C was spotted as a standard. The reaction mixtures were incubated for 2 hr at 22°.

Finally, when bacteriophage PBS-1 DNA, in which deoxyuridylate replaces thymidylate, is reacted with bleomycin, the typical DNA-like fragmentation and TCA solubilization occur. Approximately 84 per cent of this DNA becomes TCA soluble after drug action. In addition, paper chromatography of PBS-1 DNA after bleomycin treatment reveals that uracil is released (Fig. 5). Thus the presence of uracil *per se* is not responsible for the lack of reactivity of bleomycin toward RNA.

From these results, it seems unlikely that the differences in secondary structure between RNA and DNA are responsible for the differences in reactivity of bleomycin. However, since all of the deoxyribopolymers are degraded and none of the ribopolymers is degraded by bleomycin, it seems most likely that the presence of deoxyribose renders DNA sensitive to reaction or, conversely, that the presence of ribose renders RNA insensitive to the reaction of bleomycin.

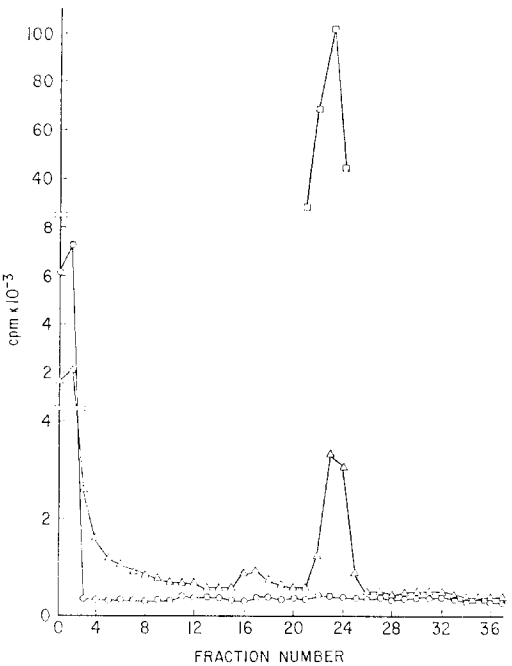

FIG. 5. Paper chromatogram of uridine-^{3}H-labeled PBS-1 DNA after reaction with bleomycin. The reaction mixtures contained: ○, 98 μg/ml of ^{3}H-PBS-1 DNA, 20 mM 2-mercaptoethanol; □, 98 μg/ml of ^{3}H-PBS-1 DNA, 20 mM 2-mercaptoethanol, 10·0 mg/ml of bleomycin; 0·06 ml of each reaction mixture was spotted; △, 0·002 ml of authentic uracil-^{14}C was spotted as a standard.

Acknowledgement—The authors wish to thank Mrs. Elsie Jackson for her excellent technical assistance.

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