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Release of Free Bases from Deoxyribonucleic Acid after Reaction with Bleomycin

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SUMMARY

Labeled free bases could be detected chromatographically after reaction of deoxyribonucleic acid with high concentrations of the antibiotic bleomycin. Thymine, adenine, guanine, and cytosine were released from DNAs previously labeled in the base moiety with each of the four bases. No detectable amounts of nucleosides, nucleotides, deoxyribose, deoxyribose phosphate, or inorganic phosphate were released.

INTRODUCTION

The bleomycins are a group of glycopeptide antibiotics which have antineoplastic activity toward the ascitic form of Ehrlich carcinoma (1–5). This group of antibiotics has been found to be effective against certain squamous cell carcinomas in man, and lymphomas including Hodgkin's disease (6, 7). The most serious side effect encountered is pulmonary fibrosis, but there is no evidence of bone marrow, liver, or renal toxicity (8).

Bleomycin, on reaction with DNA *in vitro*, causes a decrease in the melting temperature of the DNA and also produces rapid fragmentation of DNA strands in the presence of 2-mercaptoethanol (9–12). An earlier paper from this laboratory (9) presented a confirmation and extension of the studies of the Japanese group headed by Umezawa (10–12). In the presence of high concentrations of the drug, approximately 80% of the DNA is rendered soluble in trichloracetic acid. (9).

The present study is a continuation of research on the reaction of DNA fragmentation *in vitro* in order to gain insight into the mechanism of strand breakage by bleomycin.

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MATERIALS AND METHODS

Chemicals. Bleomycin was a gift from Bristol Laboratories, Syracuse, N. Y., and from Nippon Kayaku Company, Tokyo. [*methyl*-^{3}H]Thymidine (20 Ci/mmole), [^{3}H]-adenine (8.4 Ci/mmole), $H_3{}^{32}PO_4$ (carrier-free) [8-^{14}C]guanine (41.4 mCi/mmole), and [^{3}H]uracil (2.8 Ci/mmole) were purchased from New England Nuclear Corporation. [*2-deoxyribose*-5-^{3}H]Thymidine (5.0 Ci/mmole) was purchased from Amersham/Searle. [5-^{3}H]Cytosine (11.3 Ci/mmole) was purchased from International Chemical and Nuclear Corporation, Irvine, Cal. [*methyl*-^{3}H]Thymine (20 Ci/mmole) was purchased from Schwarz BioResearch. Bovine pancreatic deoxyribonuclease I (EC 3.1.4.5, ribonuclease-free) and venom phosphodiesterase from *Crotalus adamanteus* (EC 3.1.4.1) were purchased from Worthington Biochemical Corporation. All other chemicals were purchased from commercial sources.

Labeled DNA. Bacillus subtilis 168 (*thy trp* C2), which requires thymine and tryptophan for growth, was used as the source of labeled DNA. Conditions of bacterial growth and isolation of DNA were described previously

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(9). In general, tritiated precursors were added to the growth medium at a level of 0.5 μCi/ml, ^{14}C-labeled precursors were added at a level of 0.2 μCi/ml, and $H_3{}^{32}PO_4$ was added at a level of 20 μCi/ml. Cytosine was not incorporated into *B. subtilis* DNA. Cytosine-labeled DNA was obtained by incorporating uracil, which is converted into cytosine in this organism. Guanine was not incorporated exclusively into the guanine of DNA, but some was converted into adenine. Thymine and adenine were incorporated directly with little or no interconversion.

Gradient centrifugation. Alkaline sucrose gradient centrifugation analyses were performed as previously described (9). All gradients were centrifuged in the SW 50.1 rotor of a Beckman L265B ultracentrifuge at 40,000 rpm (149,000 $\times$ *g*) at 5° for 4 hr.

Paper chromatography. Descending paper chromatography was performed on Whatman No. 3MM chromatography paper developed with methanol–ethanol–concentrated HCl–H_2O (50:25:6:19) for 16 hr, as previously described (9).

Molecular weight determination. The molecular weight of DNA was determined by sedimentation velocity in a Beckman model E analytical ultracentrifuge. After treatment of the DNA with bleomycin, the remaining trichloracetic acid-insoluble fraction was dialyzed at 4° against 2 liters of a phosphate buffer (0.01 M NaCl–0.001 M of sodium phosphate buffer, pH 6.9). The absorbance at 260 nm of this DNA solution was adjusted to 0.6 before analysis. The molecular weight was estimated according to the procedure of Eigner and Doty (13).

Bleomycin reaction mixtures. The standard reaction mixture contained bleomycin, 2-mercaptoethanol, and labeled DNA in 0.05 M Tris, pH 8.0. Details of the reaction mixtures are given in the legends to the figures. The amounts of DNA, bleomycin, and 2-mercaptoethanol were varied between experiments, but in all cases the concentrations of the drug and 2-mercaptoethanol were sufficient to cause immediate fragmentation of the DNA used. A further increase of the drug resulted in no increase of fragmentation or trichloracetic acid solubilization of the DNA (9).

Different amounts of DNA had to be used, since the specific activity of the DNA varied among the batches. All reaction mixtures were incubated for 60 min at 22° in tightly stoppered tubes to allow time for the various manipulative procedures involved in preparing gradients and chromatograms.

Acid hydrolysis of labeled DNA. Labeled DNA (0.1 ml) was evaporated to dryness, 0.1 ml of 70% perchloric acid was added, and the tube was stoppered and heated at 100° for 1 hr. After hydrolysis, 0.1 ml of water was added, and 0.1 ml of the hydrolysate was spotted for paper chromatography.

Enzymatic hydrolysis of labeled DNA. Labeled DNA (0.1 ml) was denatured at 100° for 10 min and rapidly cooled in an ice bath. The magnesium concentration was adjusted to 0.12 mM; 0.005 ml of DNase I (5 mg/ml) was added and incubated for 2 hr at 37°. After DNase I hydrolysis, 0.05 ml of venom phosphodiesterase (2.5 mg/ml) was added, and incubation was continued for an additional 2 hr at 37°. Finally, 0.1 ml of the hydrolysate was spotted for paper chromatography.

RESULTS

Figures 1 and 2 show that reaction of DNA with high concentrations of bleomycin produced not only fragmentation but also solubilization of the trichloracetic acid-precipitable radioactivity. The radioactivity shown in these gradient profiles represents only the acid-precipitable counts. The small molecular weight, trichloracetic acid-precipitable DNA represented by the tritium counts of Fig. 1 has an approximate molecular weight of 10^4 or less. This fragmentation and solubilization of DNA was observed regardless of the position of the label in the DNA, i.e., whether in the base moiety (tritium counts of Fig. 1; 80% solubilization), in the phosphorus (^{32}P counts of Fig. 1; 25% solubilization), or in the deoxyribose (Fig. 2; 70% solubilization). Figure 3 illustrates a paper chromatogram of the same reaction mixtures used for the alkaline sucrose gradients shown in Fig. 1. The ^{32}P counts did not move from the origin of the chromatogram; the tritium counts (DNA labeled in

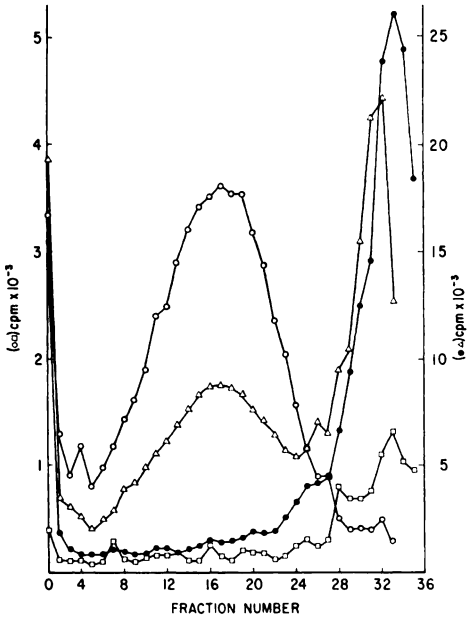

FIG. 1. *Effect of bleomycin on fragmentation and solubilization of DNA labeled with [³H]thymine and ³²P*

Alkaline sucrose gradients were used; sedimentation is from right to left. The reaction mixtures contained 106 μg/ml of [³H]thymine, ³²P-labeled DNA, 25 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin, ³H counts; □, 12 mg/ml of bleomycin, ³H counts; △, control, no bleomycin, ³²P counts; ●, 12 mg/ml of bleomycin, ³²P counts.

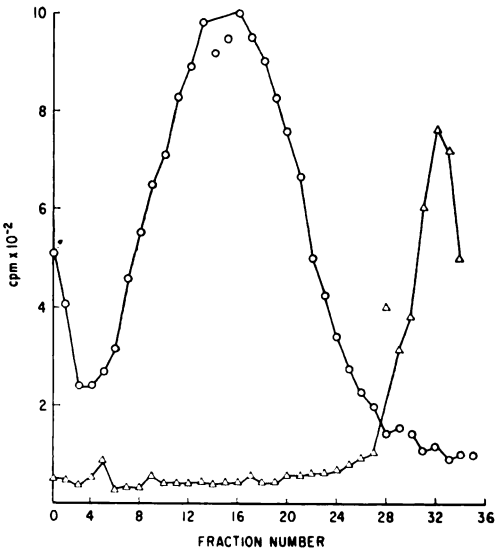

FIG. 2. *Effect of bleomycin on fragmentation and solubilization of DNA labeled in the 2-deoxyribose moiety*

Alkaline sucrose gradients were used; sedimentation is from right to left. The reaction mixtures contained 53 μg/ml of [*2-deoxyribose*-5-³H)thymidine-labeled DNA, 12 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin; △, 12 mg/ml of bleomycin.

the base moiety) did migrate, co-chromatographing with authentic thymine. In this solvent system free bases, mononucleosides, mononucleotides, deoxyribose, deoxyribose phosphate, and inorganic phosphate all migrated from the origin; no other DNA breakdown products migrated.

Figure 4 depicts a paper chromatogram obtained with DNA labeled in the deoxyribose moiety. That the DNA was labeled specifically in the deoxyribose portion of thymidine is shown by the migration of radioactivity from the enzyme hydrolysate to the position expected for thymidylic acid. Hydrolysis of DNA by DNase I and venom phosphodiesterase yields nucleotides. No radioactivity migrated from the acid-hydrolyzed DNA; therefore the label must have been incorporated solely as [5-³H]2-deoxyribose. After reaction with bleomycin this DNA did not exhibit a peak in the position expected for either nucleosides or nucleotides. The chromatogram represented by Fig. 5 shows that radioactivity released from bleomycin-treated DNA labeled with uracil co-chromatographed with authentic cytosine. The radioactivity of this DNA after acid hydrolysis also co-chromatographed with cytosine. *B. subtilis* converts uracil to cytosine before incorporation into DNA. Figure 6 shows the results of a chromatogram of bleomycin-treated DNA labeled with guanine. In this case some of the guanine was incorporated into adenine by *B. subtilis*, as shown by the peaks of the radioactivity released after acid hydrolysis. Again the radioactivity from the bleomycin-treated DNA co-chromatographed both with the acid-hydrolyzed sample and with authentic guanine and adenine. Similar results were observed for DNA labeled with adenine (Fig. 7). The bleomycin-treated DNA co-chromatographed with the acid-hydrolyzed sample and with authentic adenine. In Figs. 5–7 the

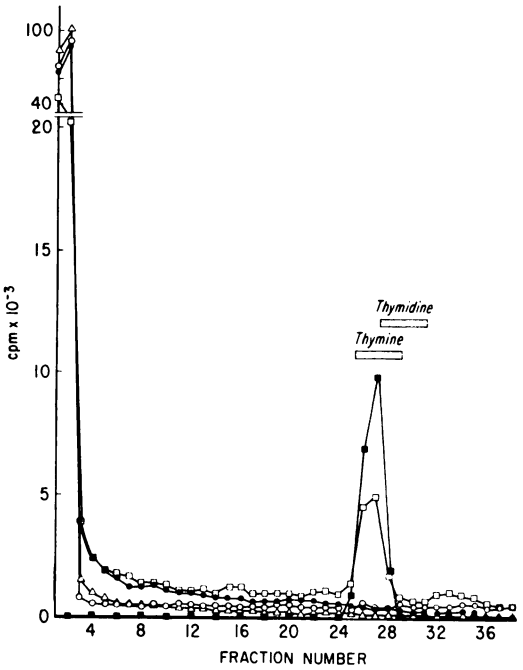

FIG. 3. *Paper chromatogram of [³H]thymine, ³²P-labeled DNA treated with bleomycin*

The reaction mixtures contained 106 μg/ml of ³H]thymine, ³²P-labeled DNA, 25 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin, ³H counts; □, 12 mg/ml of bleomycin, ³H counts; △, control DNA, no bleomycin, ³²P counts; ●, 12 mg/ml of bleomycin, ³²P counts; ■, acid-hydrolyzed, [³H]thymine-labeled DNA. One-tenth milliliter of each reaction mixture was spotted. The stippled bars indicate the positions of the authentic labeled compounds.

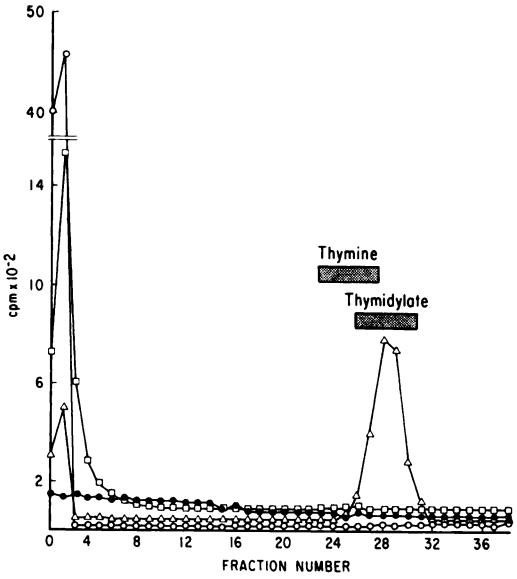

FIG. 4. *Paper chromatogram of [2-deoxyribose-5-³H]thymidine-labeled DNA treated with bleomycin*

The reaction mixtures contained 21 μg/ml of [2-deoxyribose-5-³H]thymidine-labeled DNA, 30 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin; □, 33 mg/ml of bleomycin; △, DNase I-venom phosphodiesterase-hydrolyzed DNA; ●, acid-hydrolyzed DNA. One-tenth milliliter of each reaction mixture was spotted. The stippled bars indicate the positions of the authentic labeled compounds.

nucleotides always migrated two or three fractions further than the bases. Similar chromatographic results were obtained using the solvent systems butanol–NH_4OH–H_2O (86:1:13) and ethyl acetate–2-propanol–H_2O (65:22.5:12.5).

Finally, when a reaction mixture containing 300 μg/ml of [³H]thymidine, 12 mg/ml of bleomycin, and 15 mM 2-mercaptoethanol was incubated, followed by paper chromatography, all the radioactivity co-chromatographed with authentic [³H]thymidine. No radioactivity co-chromatographed with authentic [³H]thymine. Similarly, when a reaction mixture containing 0.85 μg/ml of [³H]-thymidine 5′-triphosphate, 24 mg/ml of bleomycin, and 30 mM 2-mercaptoethanol was incubated, followed by paper chromatography, all the radioactivity again co-chromatographed with authentic [³H]thymidine 5′-triphosphate.

DISCUSSION

The fact that 80% of the tritium counts of Fig. 1 (base-labeled) became soluble in trichloracetic acid after reaction with bleomycin could mean that the bases had been removed from the DNA, and not necessarily that the backbone of the DNA had been broken sufficiently for the production of oligonucleotides small enough to be acid-soluble. In general, fragments containing fewer than 10 nucleotides are soluble in trichloracetic acid. However, since the ³²P counts also became solubilized to the extent of 25%, it is apparent not only that bases were removed but that the DNA backbone had been cleaved. That the ³²P counts were

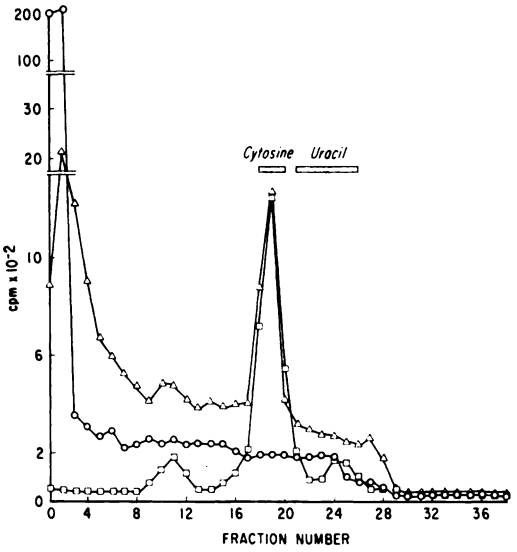

FIG. 5. *Paper chromatogram of [³H]cytosine-labeled DNA treated with bleomycin*

The reaction mixtures contained 103 μg/ml of [³H]cytosine-labeled DNA, 25 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin; △, 25 mg/ml of bleomycin; □, acid-hydrolyzed DNA. One-tenth milliliter of each reaction mixture was spotted. The stippled bars indicate the positions of the authentic labeled compounds.

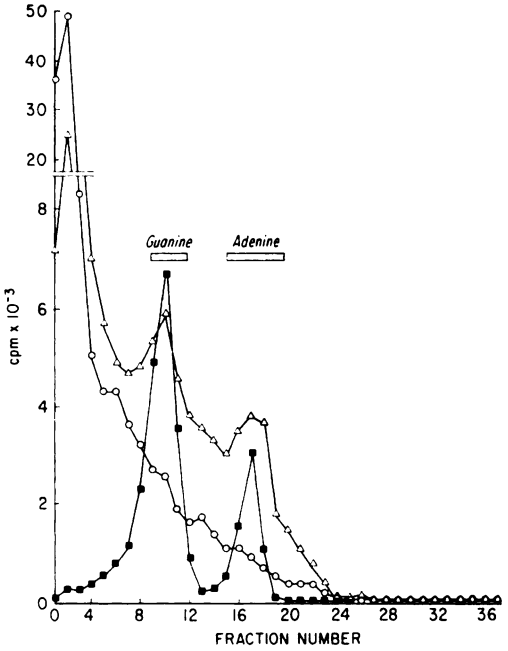

FIG. 6. *Paper chromatogram of [¹⁴C]guanine-adenine-labeled DNA treated with bleomycin*

The reaction mixtures contained 90 μg/ml of [¹⁴C]guanine-adenine-labeled DNA, 33 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin; △, 50 mg/ml of bleomycin; ■, acid-hydrolyzed DNA. One-tenth milliliter of each reaction mixture was spotted. The stippled bars indicate the positions of the authentic labeled compounds.

not solubilized to the same extent as the tritium counts can be explained by the fact that *B. subtilis* DNA preparations are contaminated with small amounts of phosphorus-containing teichoic acids, which are not solubilized by bleomycin (14, 15). The results of Fig. 3 also support the contention that the backbone of DNA was indeed broken, since 70% of this DNA, labeled exclusively in the deoxyribose portion of thymidine, became trichloracetic acid-soluble after reaction with bleomycin.

When DNA labeled with ^{32}P and [³H]thymine was allowed to react with high concentrations of bleomycin and subjected to paper chromatography (Fig. 3), only one peak, corresponding to [³H]thymine, was observed. Since none of the ^{32}P counts migrated from the origin, it is assumed that no free nucleotides, deoxyribose phosphate, or inorganic phosphate were released as a result of the action of the drug.

The results shown in Fig. 4 establish that when *B. subtilis* was grown in the presence of [*2-deoxyribose*-5-³H]thymidine the tracer was incorporated directly as the deoxyribose of thymidine and not interconverted, since enzyme hydrolysis yeielded only one peak in the position expected for thymidylic acid while no peaks were observed after acid hydrolysis. Similarly, no peaks were observed after reaction of this DNA with bleomycin. Thus no detectable amount of thymidine or deoxyribose was released. Similar experiments could not be performed easily for the other nucleosides, since these specifically labeled compounds were not available. However, we have no chromatographic evidence of any nucleoside being released after bleomycin action.

The results of paper chromatograms (Figs. 5–7), using DNA preparations labeled with cytosine, guanine, and adenine, are similar

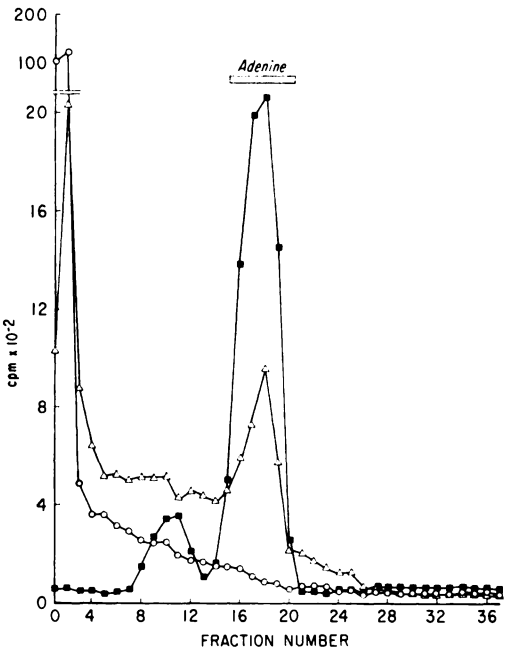

FIG. 7. *Paper chromatogram of [³H]adenine-labeled DNA treated with bleomycin*

The reaction mixtures contained 45 μg/ml of [³H]adenine-labeled DNA, 33 mM 2-mercaptoethanol, and bleomycin as follows: ○, control, no bleomycin; △, 50 mg/ml of bleomycin; ■, acid-hydrolyzed DNA. One-tenth milliliter of each reaction mixture was spotted. The stippled bar indicates the position of the authentic labeled compound.

to the results obtained using thymine-labeled DNA. The peaks observed after bleomycin treatment always co-chromatographed with the acid-hydrolyzed DNA as well as with the authentic bases. Thus there is strong evidence that all four bases of DNA were released after bleomycin treatment. Unfortunately the amounts of bases released were insufficient to perform spectral analyses. Although about 80% of the DNA became soluble in trichloracetic acid, only a small proportion of this amount (5–10%) was released as free base. Our results are consistent with the idea that bleomycin acts as an alkylating agent. It is likely that the drug acts primarily to remove bases from the DNA, with the subsequent spontaneous cleavage of the phosphodiester backbone. It is known that DNA which is depurinated by alkylating agents is labile and can spontaneously degrade under mild conditions (16). This cannot be definitely established for bleomycin-treated DNA at this time, because of the rapid reaction rate. The failure of bleomycin to release base from nucleotide and nucleoside is also consistent with the alkylating agent hypothesis (16). Whether or not bleomycin acts as an alkylating agent is still open to question, since alkylated bases have not been observed. However, the DNA, like alkylated DNA, would still be labile and subject to spontaneous degradation after removal of bases.

We are currently studying the nature of the trichloracetic acid-soluble material, other than free-base, as well as the small molecular weight, acid-insoluble material which remains after bleomycin action. This small molecular weight, acid-insoluble material seems to be resistant to further action of bleomycin.

The fact that high bleomycin concentrations are required to demonstrate the release of free bases is, at least partially, the result of the inability to detect small amounts of radioactivity. Considerable quenching of the radioactivity released occurred in this paper chromatographic system. In addition, DNA of higher specific activity should require less antibiotic to demonstrate the release of free bases. However, the use of high bleomycin concentrations seems justified, since this drug has been used in rather high concentrations in the treatment of human malignancies (17).

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REFERENCES

1. H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, *J. Antibiot.* (*Tokyo*) **19**, 200 (1966).
2. H. Umezawa, Y. Suhara, T. Takita and K. Maeda, *J. Antibiot.* (*Tokyo*) **19**, 210 (1966).
3. T. Takita, Y. Muraoka, K. Maeda and H. Umezawa, *J. Antibiot.* (*Tokyo*) **21**, 79 (1968).
4. T. Takita, K. Maeda, H. Umezawa, S. Omoto and S. Umezawa, *J. Antibiot.* (*Tokyo*) **22**, 237 (1969).
5. H. Umezawa, M. Ishizuka, K. Kimura, J.

Iwanaga and T. Takeuchi, *J. Antibiot.* (*Tokyo*) **21**, 592 (1968).
6. M. Takeuchi and T. Yamamoto, *J. Antibiot.* (*Tokyo*) **21**, 631 (1968).
7. T. Ichikawa, *Progr. Antimicrobial Anticancer Chemother.* **2**, 288 (1970).
8. S. Shastri, R. E. Slayton, J. Wolter, C. P. Perlia and S. G. Taylor, *Cancer* **28**, 1142 (1971).
9. C. W. Haidle, *Mol. Pharmacol.* **7**, 645 (1971).
10. H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka and H. Umezawa, *J. Antibiot.* (*Tokyo*) **22**, 446 (1969).
11. K. Nagai, H. Suzuki, N. Tanaka and H. Umezawa, *J. Antibiot.* (*Tokyo*) **22**, 569 (1969).
12. K. Nagai, H. Suzuki, N. Tanaka and H. Umezawa, *J. Antibiot.* (*Tokyo*) **22**, 624 (1969).
13. J. Eigner and P. Doty, *J. Mol. Biol.* **12**, 549 (1965).
14. F. E. Young, D. J. Tipper and J. L. Strominger, *J. Biol. Chem.* **239**, PC3600 (1964).
15. F. E. Young and A. P. Jackson, *Biochem. Biophys. Res. Commun.* **23**, 490 (1966).
16. W. C. Ross, "Biological Alkylating Agents," pp. 44, 83. Butterworths, London, 1962.
17. C. Taketa, Y. Shimosato, A. Nagano, K. Washizu, S. Matsuura, I. Ono and S. Ebihara, *Jap. J. Clin. Oncol.* **1**, 41 (1971).