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Interaction of Anthracyclines with Covalently Closed Circular DNA

SEYMOUR MONG,[1] VIRGIL H. DUVERNAY,*[1] JAMES E. STRONG* AND STANLEY T. CROOKE*,†

*Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030, and †Bristol Laboratories, Syracuse, New York 13201

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SUMMARY

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The effects of four anthracyclines, adriamycin, carminomycin, aclacinomycin, and marcellomycin, on covalently closed circular PM-2 DNA were studied. At a concentration of 5–10 μM each anthracycline induced conformational changes in PM-2 DNA demonstrated by production of diffuse staining patterns on agarose gels. No significant PM-2 DNA degradation was observed using alkaline sucrose gradient centrifugation, agarose gel electrophoresis analysis nor by a PM-2 DNA fluorescence assay using alkaline denaturation. At higher concentrations (200 μM) and in the presence of reducing agents and high temperature, the anthracyclines were able to degrade PM-2 DNA. Bleomycin A_2 was used to test whether the conformational changes induced by anthracyclines were due to random breakage, and Bleomycin A_2 was shown to degrade the anthracycline bound PM-2 DNA confirming that the anthracyclines did not degrade PM-2 DNA. Further, these results suggest that anthracyclines bind to PM-2 DNA, and induce significant conformational changes that do not affect Bleomycin A_2-induced DNA degradation.

INTRODUCTION

The interaction of anthracyclines with DNA has been the subject of many studies employing a variety of methodologies including equilibrium dialysis (1, 2), spectrophotometric methods (1, 3, 4) and fluorescence techniques (5–9). Binding of anthracyclines to DNA has been reported to produce alterations in the secondary structure of DNA (4, 6, 10, 11). As a result, it is generally accepted that a major portion of the anthracyclines' interaction with DNA involves intercalation between two adjacent base pairs of the native DNA duplex (1).

Recently adriamycin (ADM)[2] was reported to degrade covalently closed circular (CCC) PM-2 DNA in the presence of $NaBH_4$ (12), and it has been suggested that DNA degradation may be responsible for the cytotoxicity of these agents. This was based upon a fluorescence assay system in which CCC PM-2 DNA was exposed to anthracyclines, then heat and alkali denaturation. Since single-stranded DNA is unable to bind ethidium bromide (EB), only intact CCC PM-2 DNA could bind EB under those conditions and a decrease of EB-induced fluorescence indicated degradation of CCC PM-2 DNA. However, since the concentration of ADM employed was relatively high, the fluorescence decrease could also have resulted from inhibition of EB binding by anthracyclines or alkali-labile damage induced by the anthracyclines and/or the heating and cooling (13).

To further investigate the interaction of anthracyclines with DNA, we have studied the interactions of PM-2 DNA with four different anthracyclines, ADM, carminomycin (CAM), marcellomycin (MAR), and aclacinomycin (ACM) using the following techniques: the fluorescence assay after alkaline denaturation, nondenaturing agarose gel electrophoretic separation of PM-2 DNA isomers and alkaline sucrose density gradient centrifugation analysis. The studies to be reported here show that at low concentrations (5–50 μM), none of these anthracycline antibiotics break PM-2 DNA without the addition of $NaBH_4$. However, conformational alterations in PM-2 DNA can be detected. Under the conditions previously reported (12), i.e., 200 μM ADM, 33 mM $NaBH_4$, plus heat denaturation, anthracyclines induced DNA degradation.

MATERIALS AND METHODS

Materials. Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide) was purchased from Sigma Chemical Company (St. Louis, Mo.). Agarose-ME

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[2] The abbreviations used are: CCC PM-2 DNA, covalently closed circular PM-2 DNA; ADM, adriamycin; CAM, carminomycin; ACM, aclacinomycin; MAR, marcellomycin; $BLMA_2$, bleomycin A_2; EB, ethidium bromide; 2-ME, 2 mercaptoethanol.

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was purchased from Miles Laboratories (Elkhart, Ind.). Bleomycin A_2 ($BLMA_2$), ADM, CAM, MAR, and ACM were obtained from Bristol Laboratories (Syracuse, N. Y.).

PM-2 DNA isolation. CCC PM-2 DNA was isolated as previously described (14, 15). It was purified by centrifugation on cesium chloride and ethidium bromide gradients, then dialyzed extensively against 0.15 M NaCl prior to use. Each batch of PM-2 DNA was analyzed for integrity and purity by agarose gel electrophoresis. Only PM-2 DNA samples that contain greater than 80% CCC circular DNA were employed in analyses.

Ethidium bromide fluorescence assay after alkaline denaturation. The reaction of various drugs with PM-2 DNA was performed, except as otherwise indicated, by adding drug to 25 μg of PM-2 DNA in 0.5 ml of 50 mM borate buffer plus 0.015 M NaCl at pH 9.5. After incubation with the anthracyclines, the PM-2 DNA in the reaction mixture was denatured by mixing 0.1 ml of the reaction mixture with 0.9 ml of the denaturation buffer (40 mM Na_3PO_4, 100 mM NaCl, 10 mM EDTA, pH 12.1, adjusted with 0.15 M NaOH). Following denaturation, 0.1 ml ethidium bromide solution (22 μg/ml in denaturation buffer) was added.

Fluorescence of the ethidium bromide–DNA mixture was determined using a spectrophotofluorometer (Aminco–Bowman) at 530 nm excitation and 590 nm emission. The change in concentration of CCC PM-2 DNA was determined by the percentage decrease in fluorescence, after subtraction of background fluorescence relative to control reactions not containing drugs. Control reactions were also performed with or without 2-mercaptoethanol (2-ME), and no decrease in fluorescence was detected due to addition of 2-ME alone. The specificity of this technique has been demonstrated by addition of up to 100-fold greater concentration of calf thymus DNA than the amount of PM-2 DNA used in this assay without an increase in ethidium bromide fluorescence.

Agarose gel electrophoresis. The products of drug–PM-2 DNA interactions were electrophoretically separated on 1% agarose gels under nondenaturing conditions. The $BLMA_2$ reaction was terminated by addition of 50 μl of reaction mixture to 10 μl of 50 mM EDTA. Bromophenol blue (0.1% w/v) in 75% glycerol (20 μl) was then added and 30 μl of the resultant mixture was electrophoresed on 1% agarose gels in a horizontal slab gel apparatus (Aquebogue Machine Shop, Aquebogue, N. Y.). Electrophoresis was performed in a buffer containing 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA, pH 7.8, for 12 hr at 5 V/cm at 22°. After electrophoresis, the gels were incubated in electrophoresis buffer containing 0.5 μg/ml ethidium bromide for staining. Under these conditions the order of anodal migration for the conformational forms of DNA was: CCC DNA (form I), double-stranded broken linear (form III), and single-stranded broken open circular DNA (form II) (16).

Alkaline sucrose density gradient centrifugation. The reaction mixture containing PM-2 DNA (25 μg) and drug was applied directly to a 5–20% alkaline sucrose gradient (0.3 M NaOH, 0.7 M NaCl and 1 mM EDTA) and centrifuged for 7.5 hr at 23,000 rpm using a SW27 rotor (Beckman Instruments, St. Louis, Mo.). The gradients were fractionated with an ISCO density gradient fractionator (Lincoln, Neb.), into 1.2-ml fractions and the absorbance profile at 254 nm was recorded.

Ethidium bromide fluorescence assay and heat denaturation procedure. Anthracyclines were incubated with CCC PM-2 DNA as previously described (12). Briefly, anthracyclines (200 μM) were incubated with CCC PM-2 DNA (60 μg) in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.2, with 33 mM $NaBH_4$ for varying periods of time (5–90 min). Aliquots (40 μl) were mixed with 960 μl of a 20 mM potassium phosphate buffer at pH 11.8 which contained 0.5 mM EDTA and 0.4 μg/ml ethidium bromide and the fluorescence was recorded (preheated fluorescence). Then the solution was heated to 96° for 2–3 min, then cooled rapidly by immersion in ice, and the fluorescence was recorded after the solution reached room temperature (postheating fluorescence).

RESULTS

The results shown in Fig. 1 confirmed the observations reported previously by Lown *et al.* (12), i.e., at high concentration (200 μM) of ADM, and in the presence of 33 mM $NaBH_4$, ADM produced a decrease of fluorescence indicative of PM-2 DNA degradation. However, at lower concentration and without $NaBH_4$, a less pronounced fluorescence decrease was observed. Figure 2 demonstrates that all four anthracyclines induced a concentration-dependent fluorescence reduction in the alkaline denaturation fluorescence assay system. This assay required 100 μM of four anthracyclines to produce a 20–25% decrease of fluorescence. Little qualitative or quantitative differences were detected among the anthracyclines studied. These results suggest that DNA breakage induced by low concentration of anthracyclines under these conditions was probably insignificant. Alkaline sucrose gradient sedimentation was used to investigate whether

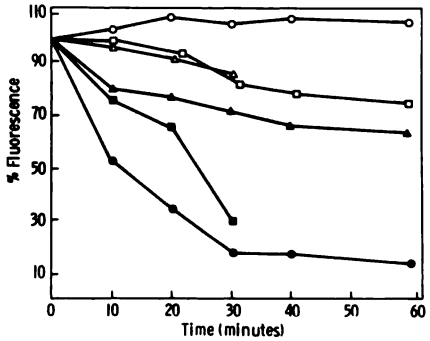

FIG. 1. *The effects of ADM on PM-2 DNA binding of ethidium bromide*

Reactions were performed in 50 mM phosphate buffer at pH 7.2. 1.2 A_{260} units/ml of CCC PM-2 DNA was incubated with 200 μM ADM and 33 mM $NaBH_4$ (○); 200 μM ADM (△); or 200 μM ADM and 20 mM 2-ME (□), for indicated periods of time. Reaction mixture, 40 μl, was mixed with 0.96 ml of phosphate buffer (20 mM, pH 11.8) that contained 0.5 μg/ml of EB and 0.4 mM EDTA. The fluorescence was recorded before (open symbols) and after (filled symbols) the heating and cooling cycle. The results presented are the means of the experiments performed with triplicate samples. The standard deviations were smaller than the symbols employed.

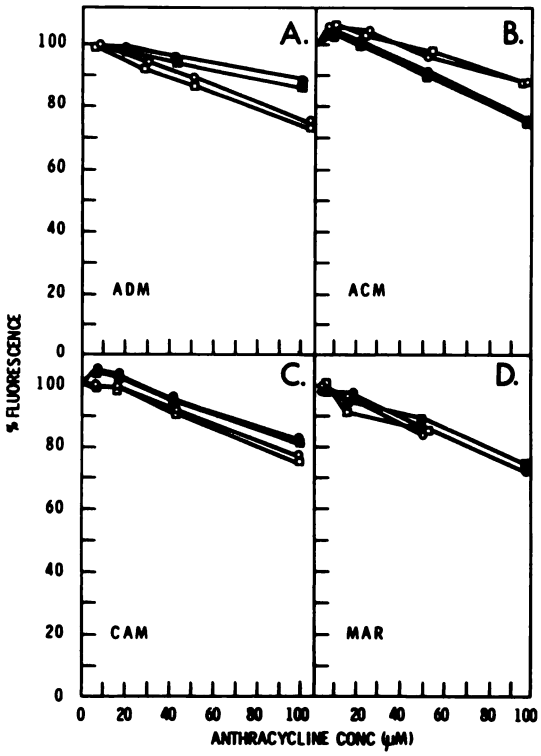

FIG. 2. *The effect of anthracyclines on PM-2 DNA in the fluorescence assay*

PM-2 DNA was incubated with each drug and 2-ME for 30 min (—○—) or 60 min (—□—), or without 2-ME for 30 min (—●—) or 60 min (—■—). (A) ADM, (B) ACM, (C) CAM, (D) MAR. The results presented are means of five experiments performed in triplicate. Standard deviations are smaller than the symbols employed.

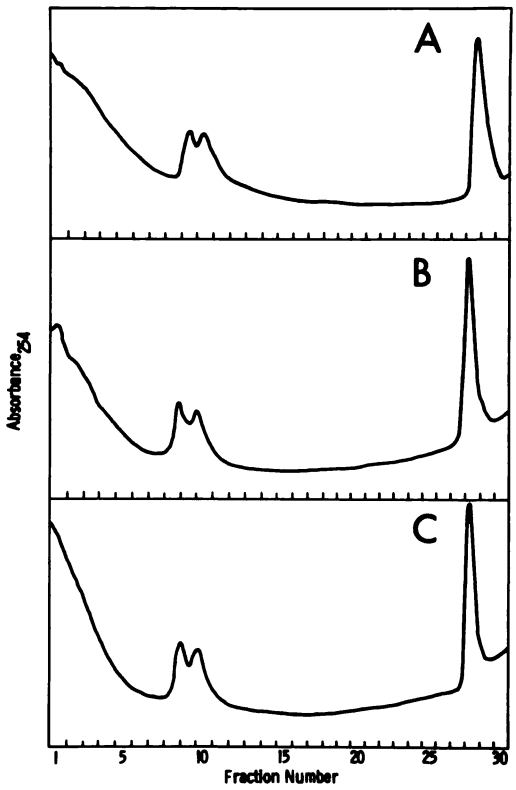

FIG. 3. *Alkaline sucrose density gradient centrifugation analysis of ADM-treated PM-2 DNA*

CCC PM-2 DNA, 50 μg, was incubated with (A) distilled water, (B) 25 μM ADM, and (C) 50 μM of ADM in the presence of 2-ME for 60 min and then centrifuged in 5–20% alkaline sucrose gradients. Sedimentation is from left (top) to right (bottom). The doublet peaks represent the single-stranded linear (24 S) and linear forms (27 S), and the rapidly migrating peak represents the intact CCC PM-2 DNA (76 S).

these effects were caused by DNA breakage. As shown in Fig. 3, the slow sedimenting doublet peaks in the gradient profile represent the complementary strands of denatured relaxed form DNA species, i.e., the single-stranded linear and the circular forms with sedimentation coefficients of 24 and 27 S, respectively (17). The more rapidly sedimenting peak represents the denatured CCC PM-2 DNA with a sedimentation coefficient of 76 S (17). Since there is little change in the relative amount of these different forms of PM-2 DNA after treatment with ADM, (as a representative example) these results suggest that at concentrations of 25 or 50 μM of anthracyclines, little evidence of induction of alkaline-labile sites or DNA breakage was detected in PM-2 DNA following treatment with four anthracyclines.

Figure 4 shows that under nondenaturing conditions the PM-2 DNA–anthracycline interaction was detected at relatively low concentrations (5–10 μM) as a diffuse staining pattern after agarose gel electrophoresis. The diffuse staining pattern is compatible with conformational alterations in the superhelical density of CCC PM-2 DNA induced by intercalative binding of a variety of agents (18, 19). The concentrations of the anthracyclines required to induce the diffuse staining pattern and to reduce the electrophoretic mobility of PM-2 DNA correlated with their binding affinities to DNA (20), i.e., MAR and ADM binding affinity coefficients were greater

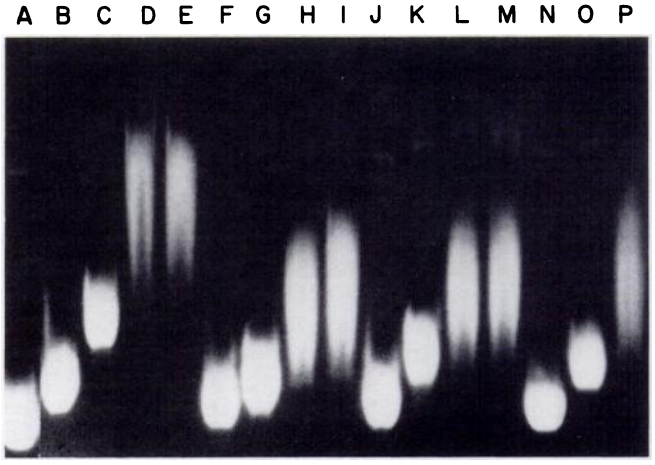

FIG. 4. *Effects of anthracyclines on agarose gel electrophoretic patterns of PM-2 DNA*

Reaction conditions, electrophoresis, and staining were performed as described in MATERIALS AND METHODS. No 2-ME was present. From left to right: (A) control PM-2 DNA; (B) 5 μM MAR; (C), 25 μM MAR; (D) 50 μM MAR; (E) 100 μM MAR; (F) 5 μM CAM; (G) 25 μM CAM; (H) 50 μM CAM; (I) 100 μM CAM; (J) 5 μM ACM; (K) 25 μM ACM; (L) 50 μM ACM; (M) 100 μM ACM; (N) 5 μM ADM; (O) 25 μM ADM; (P) 50 μM ADM.

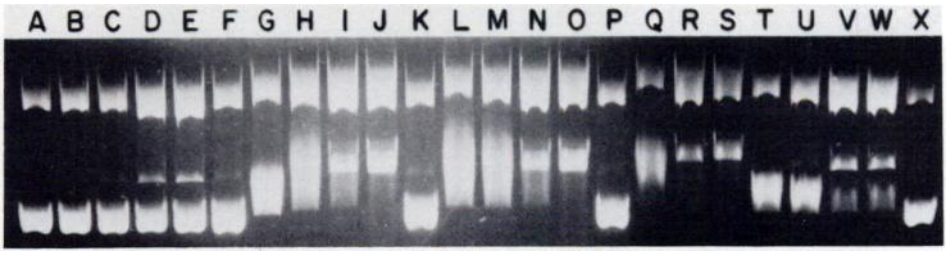

FIG. 5. *Effects of anthracycline pretreatment of PM-2 DNA on degradation induced by $BLMA_2$*

CCC PM-2 DNA was incubated with anthracyclines in the presence of 20 mM 2-ME with anthracyclines for 30 min (25 μm) as described in MATERIALS AND METHODS and then incubated with increasing concentrations of $BLMA_2$. The products of the reactions were separated by agarose gel electrophoresis as described in MATERIALS AND METHODS. (A) Control PM-2 DNA; (B) 5 nM $BLMA_2$; (C) 10 nM $BLMA_2$; (D) 25 nM $BLMA_2$; (E) 50 nM $BLMA_2$; (F) control PM-2 DNA; (G–J) PM-2 DNA treated with 25 μM CAM and post-treated with 0, 10, 50, or 100 nM $BLMA_2$. (K) control PM-2 DNA; (L–O) PM-2 DNA treated with 25 μM ADM and then post-treated with 0, 10, 50, or 100 nM $BLMA_2$. (P) control PM-2 DNA, (Q–S) PM-2 DNA treated with MAR and then post-treated with 10, 25, and 50 nM $BLMA_2$. (T–W) PM-2 DNA treated with 25 μM then post-treated with 0, 12, 25, or 50 nM $BLMA_2$. (X) control PM-2 DNA.

than those for ACM and CAM, and they induced a diffuse staining pattern at lower concentrations.

That the diffuse staining patterns observed were not due to random breaks was confirmed by the following technique. PM-2 DNA was treated with each of the anthracyclines (25 μM), then the PM-2 DNA–anthracycline complex was treated with increasing concentrations of $BLMA_2$, an agent that induces single- and double-stranded breaks. Figure 5 shows that agarose gel electrophoresis demonstrated that each of the anthracyclines induced a diffuse staining pattern at the concentration tested. Post-treatment of the anthracycline–PM-2 DNA complex with $BLMA_2$ resulted in the induction of the form II and form III species of PM-2 DNA reflective of typical $BLMA_2$-induced single-stranded and double-stranded breakage degradation of PM-2 DNA (14, 21). Thus, the diffuse staining patterns observed could not have resulted from random degradation.

The degradation products induced by bleomycin A_2 in DNA pretreated with anthracyclines appear slightly more diffuse than typical degradation products on agarose gels. This is probably due to the presence of some unbroken molecules that are partially relaxed due to the anthracyclines treatment.

DISCUSSION

The studies presented in this report demonstrate that the four anthracyclines investigated interact with CCC PM-2 DNA. Employing conditions equivalent to those previously reported (12), we were able to confirm that ADM degrades PM-2 DNA. However, we observed that at lower concentrations and under the conditions employed in the present study, the anthracyclines did not cause DNA degradation but induced superhelical conformational changes similar to those induced by intercalative-type binding (18, 19). All of the anthracyclines studied induced observable conformational changes at concentrations of 5–10 μM and no qualitative differences among the anthracyclines in the type of diffuse staining pattern were detected by agarose gel electrophoresis. The stronger binding anthracycline MAR, demonstrated by fluorescence quenching studies (22), seemed to produce a more pronounced effect than the other three drugs studied.

Since some mammalian DNA has been shown to be in the superhelical state (23), and the superhelical density changes were observed at concentrations and conditions more likely to occur in the cells than those required to demonstrate DNA degradation, the DNA superhelical conformational changes might be important as one of the possible mechanisms of the cytotoxic effects.

Although previous studies have shown that anthracyclines may be categorized into two classes on the basis of their selectivity for nucleolar RNA synthesis inhibition (18, 24), all anthracyclines studied behaved similarly in the experiments in this study. Thus the nucleolar RNA inhibitory selectivity of ACM and MAR cannot be explained on the basis of these results. More careful titrations of these and other anthracyclines are being performed currently in this laboratory using equilibrium gel electrophoresis to determine the minimum concentrations necessary to induce superhelical conformational changes in PM-2 DNA precisely. Definitive conclusions relative to comparison of the effects of the anthracyclines on supercoiled DNA must await these studies.

In conclusion, anthracyclines can interact with PM-2 DNA at concentrations of 5–10 μM and induce superhelical conformational changes without breakage of the DNA. At high concentration (200 μM) in the presence of reducing agents and after heating, anthracyclines can be shown to induce DNA degradation. Thus, the conformational alterations, presumably due to intercalative binding, occur at concentrations and under conditions making these effects more likely to be responsible for the cytotoxicity of anthracyclines than DNA breakage.

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REFERENCES

1. Zunino, F., R. Gambetta, A. DiMarco and A. Zaccara. Interaction of daunomycin and its derivatives with DNA. *Biochim. Biophys. Acta* **277**: 489–498 (1972).
2. Arlandini, E., A. Vigevandi and F. Arcamone. Interaction of new derivatives of daunorubicin and adriamycin with DNA. II. *Farmaco* **32**: 314–323 (1977).
3. Calendi, E., A. DiMarco, M. Reggiani, B. Scarpinato and L. Valentini. On

physico-chemical interactions between daunomycin and nucleic acid. *Biochim. Biophys. Acta* **103**: 25–49 (1965).
4. Gabbay, E. J., D. Crier, R. Finegele, R. Reineri, S. W. Pearee and W. P. Wilson. Interaction specificity of the anthracyclines with deoxyribonucleic acid. *Biochemistry* **15**: 2062–2069 (1976).
5. Tsou, K. C. and K. F. Yip. Effect of deoxyribonuclease on adriamycin-polynucleotide complexes. *Cancer Res.* **36**: 3367–3373 (1976).
6. Zunino, F., R. Gambetta, A. DiMarco, A. Veleich, A. Zaccara, F. Quadrifoglio and V. Crescenzi. The interaction of adriamycin and its β-anomer with DNA. *Biochim. Biophys. Acta* **476**: 38–46 (1977).
7. DiMarco, A., A. M. Cazazza, T. Dasida, A. Necco, G. Patesi, P. Rivolta, A. Weleich, A. Zaccara and F. Zunino. Changes of activity of daunorubicin, adriamycin and sterieoisomers following the introduction of removal of hydroxyl groups in the amino sugar moiety. *Chem.-Biol. Interact.* **19**: 291–302 (1977).
8. Plumridge, T. and J. Brown. Spectrophotrometric and fluorescence polarization studies of the binding of ethidium, daunomycin, and mepacrine to DNA and to poly (I·C). *Biochim. Biophys. Acta* **479**: 441–449 (1977).
9. Lerman, L. S. Structure considerations in the interaction of DNA and acridines. *J. Mol. Biol.* **3**: 18–30 (1961).
10. Waring, M. Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: Evidence for molecular models involving intercalation. *J. Mol. Biol.* **54**: 247–279 (1978).
11. Fecchinetti, T., A. Montovani, R. Cantoni and M. Salmona. Intercalation with DNA is a prerequisite for Daunomycin, Adriamycin and its congeners in inhibiting DNAse I. *Chem.-Biol. Interact.* **20**: 97–102 (1978).
12. Lown, J. W., S. Sim, K. C. Majumdar and R. C. Chang. Strand scission of DNA by bound adriamycin and daunorubicin in the presence of reducing agents. *Biochem. Biophys. Res. Commun.* **76**: 705–709 (1976).
13. Strong, J. E. and S. T. Crooke. Mechanism of action of tallysomycin, a third generation bleomycin. *Cancer Res.* **38**: 3322–3326 (1978).
14. Salditt, M., S. W. Braunstein, R. D. Comerini-Otero and R. M. Franklin. Structure and synthesis of lipid containing bacteriophage. X. Improved technique for production of bacteriophage PM-2. *Virology* **48**: 259–264 (1972).
15. Strong, J. E. and R. R. Hewitt. Investigation of human plasma DNase by DNA-polyacrylamide gel electrophoresis, in *Isozymes III* (C. Market, ed). Academic Press, New York, 413–485 (1975).
16. Johnson, P. H. and L. I. Grossman. Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-strand DNAs. *Biochemistry* **16**: 4217 (1977).
17. Thielmann, H. W. Detection of strand breaks in ϕx 174 RFI and PM-2 DNA reacted with ultimate and proximate carcinogens. *Z. Krebsforsch. Klin. Onkol.* **90**: 37–69 (1977).
18. Legerski, R. J., H. B. Gray and D. L. Robberson. A sensitive endonuclease probe for lesion in deoxyribonucleic acid helix structure produced by carcinogenic or mutagenic agents. *J. Biol. Chem.* **254**: 8740–8746 (1978).
19. Espejo, R. T. and J. Lebowitz. A simple method for the determination of superhelix density of closed circular DNAs and for observation of their superhelix density heterogeneity. *Anal. Biochem.* **72**: 95–103 (1976).
20. DuVernay, V. M., J. M. Essery, T. W. Doyle, W. T. Bradner and S. T. Crooke. The antitumor effects of anthracyclines: The importance of the carbomethoxy-group at position-10 of marcellomycin and rudolfomycin. *Mol. Pharmacol.* **15**: 341–356 (1979).
21. Lloyd, R. S., C. W. Haidle and R. R. Hewitt. Bleomycin-induced alkaline-lapile damage and direct strand breakage of PM-2 DNA. *Cancer Res.* **38**: 3191–3196 (1978).
22. DuVernay, V. D., J. Pachter and S. T. Crooke. Studies on the DNA binding of several anthracyclines. *Biochemistry* (in press).
23. Finch, J. T., L. C. Lutter, D. Rhodes, R. S. Brown, B. Rushton, M. Levitt and A. Klug. Structure of nucleosome core particles of chromatin. *Nature* **269**: 29–33 (1977).
24. Crooke, S. T., V. H. DuVernay, L. Galvan and A. W. Prestayko. Structure activity relationships of anthracyclines relative to effects on macromolecular synthesis. *Mol. Pharmacol.* **14**: 290–298 (1978).

Send reprint requests to: Seymour Mong, Department of Pharmacology, Baylor College of Medicine, Houston, Tex. 77030.