Biochemical Pharmacology, Vol. 30, pp. 231–240.

0006-2952/81/0201-0231 $02.00/0

INTERACTIONS OF A NEW ANTITUMOR AGENT, 1,4-DIHYDROXY-5,8-BIS[[2-[(2-HYDROXYETHYL)AMINO]-ETHYL]AMINO]-9,10-ANTHRACENEDIONE, WITH NUCLEIC ACIDS

JAN KAPUSCINSKI,* ZBIGNIEW DARZYNKIEWICZ, FRANK TRAGANOS and MYRON R. MELAMED

Investigative Cytology Laboratory, Memorial Sloan–Kettering Cancer Center, New York, NY 10021, U.S.A.

(*Received* 19 *February* 1980; *accepted* 24 *July* 1980)

Abstract—1,4-Dihydroxyl-5,8-bis [[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione (DHAQ), a new antitumor drug (NSC 279836), suppressed cell progression at the G_2 phase *in vitro* at concentrations of 1–10 ng/ml. Cells blocked in G_2 contained increased amounts of RNA. The drug bound to both DNA and RNA in the nuclear chromatin with a preference for the nucleolus and to RNA in the cytoplasm of cells *in situ*. Spectroscopic evidence indicated that DHAQ interacts by intercalation with both natural and synthetic nucleic acids, although electrostatic interactions also play a part in the binding. All A and B structure nucleic acids intercalated DHAQ though there was no clear base specificity; interactions with polymers containing only A–T base pairs were somewhat weaker than with other polymers, and alternating polymers exhibited stronger affinity than did homopolymer pairs. Electrophoretic dye titration with SV 40 DNA provided additional proof that DHAQ binds to DNA by intercalation. The intrinsic association constant of the drug (K_i) was $1.8 \times 10^6\,M^{-1}$, its binding site size (n) was 5 nucleotides, and the unwinding angle (ϕ) was 26.5°. Because DHAQ precipitated single-stranded polymers, it is possible that the drug interacted electrostatically with the anionic exterior of single-stranded nucleic acids with subsequent stacking. The data indicated that the pharamacological effect of DHAQ may be related to drug intercalation into double-stranded nucleic acids which may impair DNA transcription and RNA processing.

1,4-Dihydroxy-5,8-bis [[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione (DHAQ, NSC 279836) is a polycyclic aromatic compound first synthesized by Zee-Cheng and Cheng [1]. DHAQ demonstrated antitumor activity in a variety of *in vivo* experimental mouse tumor systems [2, 3]. In addition, recent *in vitro* experiments have shown that DHAQ blocks progression of cells at the G_2 phase of the cycle, inhibits CHO cell clonogenicity, and induces profound changes in nuclear chromatin structure in the dose range of 1–10 ng/ml [4]. Depending upon the cell type, significant differences in sensitivity to the drug were observed; while Friend erythroleukemia (FL) cells were blocked in G_2 by 10 ng/ml DHAQ, normal human lymphocytes stimulated by mitogens were refractory to the drug at concentrations up to 5 μg/ml [4].

DHAQ, as well as other aminoanthraquinones, binds tightly to DNA and stabilizes its structure against thermal denaturation and inhibits thymidine and uridine incorporation. Based on these findings, an intercalation mode of binding has been proposed for the drug [1, 2].

In the present study, further attempts were made to investigate the mechanism of action of the drug. Preferential binding of DHAQ to cell constituents *in situ* was explored as a possible means of identifying intracellular targets of the drug. The interaction of DHAQ with natural nucleic acids and with synthetic polynucleotides was also investigated. The relationship between the intracellular site and mode of binding of DHAQ and the effects of the drug observed *in vitro* on whole cells is discussed.

*Address all correspondence to: Dr. Jan Kapuscinski, Memorial Sloan–Kettering Cancer Center, 1275 York Av., New York, N.Y. 10021, U.S.A.

MATERIALS AND METHODS

Materials. DHAQ was provided by Dr. David Abraham of the Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Stock solutions of the drug (0.2 mM) were freshly prepared by solubilizing DHAQ (1 mg/ml) in 0.1 M HCl diluted with buffer and adjusted to pH 7.0. Polyvinyl-sulfate (potassium salt) and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer were purchased from the Sigma Chemical Co. (St. Louis, MO). Ethidium bromide (EB) and acridine orange (chromatographically purfied, AO) were obtained from Polysciences, Inc. (Warrington, PA) and agarose from Sekem (Rockland, ME).

The synthetic polynucleotides poly(dA), poly(rA), poly(dT), poly(rU), poly(rC), and poly(rI) were obtained from Miles Laboratories Inc. (Elkhart, IN); poly(dC) and poly[d(G-C)]·poly[d-(G-C)] from the Grand Island Biological Co. (Grand Island, NY); and poly(rG), poly(dG), poly[d(A-T)]·poly[d(A-T)] and poly(dG)·poly(dC) from P-L

Biochemicals (Milwaukee, WI). Poly(dA)·poly(dT) was prepared by heating an equimolar mixture of poly(dA) and poly(dT) to 45° for 10 min, followed by slow cooling.

Calf thymus DNA (Type I) and wheat germ RNA (Type V) were purchased from the Sigma Chemical Co. Denatured DNA was prepared by heating native calf thymus DNA at 100° for 10 min in a solution of 0.1 N NaCl, 5 mM HEPES, and 1 mM EDTA–Na, pH 7.0, followed by rapid cooling to 0°. MS2 RNA and 23S RNA were obtained from Miles Laboratories. SV 40 DNA (Form I and Form II) was purchased from the Bethesda Research Laboratory, Inc. (Rockville, MD). Pancreatic DNase I and RNase A (RASE) were obtained from the Worthington Biochemical Corp. (Freehold, NJ). All other chemicals were high grade commercial products.

Cell system. Friend leukemia (FL) cells, strain 745, were obtained from the Medical Research Institute, Camden, NJ. The cells, grown in suspension as described previously [4], were routinely passaged twice weekly by addition of 1×10^6 cells in 1.0 to 9.0 ml of medium. The cells were split 1:3 with fresh, pre-warmed medium on 3 successive days prior to addition of drug to ensure asynchronous growth.

Exponentially growing CHO cells, described previously in detail [5], were used in experiments to study DHAQ binding. The cells growing on coverslips were fixed either by immersion of coverslips into 70% ethanol, or into a mixture of ethanol–acetone (1:1, v/v) at 4°. Following fixation, cells were slowly hydrated by sequential rinsing in alcohol solutions of a decreasing alcohol concentration and then treated with 1 mg/ml of DNase I or 10^3 units per ml of RNase A or with both enzymes together dissolved in Hanks' balanced salt solution (HBSS) for 30 min at 37°. Control slides were incubated in HBSS alone. After incubation, cells were treated with 100 μg/ml of DHAQ for 30 min at 24°, rinsed in HBSS, and analyzed by microscopy.

Flow cytometry: simultaneous assay of DNA and RNA. This staining reaction and its specificity have been described in previous publications [6, 7], Briefly, under these staining conditions, AO intercalates into double helical nucleic acids (predominantly DNA in this case) fluorescing green (530 nm) in blue light [8], whereas it 'stacks' in polymeric form on single-stranded nucleic acids (in this case RNA), with a shift in maximum emission to red (640 nm) [9].

FL cells in suspension, taken directly from culture, were made permeable by the addition of 0.2 ml of cell suspension to 0.4 ml of a solution containing 0.08 N HCl, 0.15 M NaCl and 0.1% Triton X-100. The cells were stained with acridine orange (AO) 30 sec later by adding 1.2 ml of a solution containing 0.2 M Na_2HPO_4–0.1 M citric acid buffer (pH 6.0), 1 mM EDTA–Na, 0.15 M NaCl and 6 μg/ml AO.

Exponentially growing cells were incubated in the absence or presence of 10 ng/ml DHAQ for 30 min, washed, and resuspended in fresh medium. After an additional 23.5 hr of culture, the relative DNA and RNA content of 5×10^3 cells was determined by flow cytometric analysis of AO stained cells (see below).

The green (DNA) and red (RNA) fluorescence and the green fluorescence pulsewidth of individual AO stained cells were obtained as described previously [10, 11] by use of an FC-200 flow cytometer (Ortho Diagnostic Instruments, Westwood, MA) interfaced to a Nova 1220 minicomputer (Data General Corp., Southboro, MA). The computer drawn displays were obtained from a Tektronix 4010-I graphics display terminal (Tektronix, Inc., Beaverton, OR).

Spectrophometry. Spectrophotometric measurements and thermal denaturation experiments were carried out using a 1 cm path-length cuvette in either a Zeiss PM6 spectrophotometer equipped with a thermoelectric control unit, or in a Perkin–Elmer 576–55 spectrophotometer. The measurements were performed in 25 mM HEPES, 0.1 M NaCl, and 1 mM EDTA-Na (pH 7.0) (HNE), unless stated otherwise.

Nucleic acid–DHAQ complexes were obtained by adding appropriate aliquots of the drug solutions into dissolved nucleic acids during extensive stirring. Since under certain conditions the nucleic acid–DHAQ complexes were insoluble, prior to spectrophotometric measurements, the samples were centrifuged at 2000 rpm for 10 min, and only samples having no visible precipitate were analyzed.

Spectrophotometric titrations were performed directly in the cuvette, by stepwise addition of the 10–50 μl aliquots of the DHAQ stock solution (0.2 mM), dissolved in 50 mM HEPES, 20 mM Na–acetate, 30 mM NaCl, and 1 mM EDTA (pH 7.0), into a 3 ml solution of calf thymus DNA (C_{DNA} = 50 μM) in the same buffer. After stirring, the absorption was measured at 682 nm ($t = 25°$) to calculate the concentration of free (C_F) and bound drug (C_B) assuming the molar absorptions to be $\varepsilon_F = 8.36 \times 10^3$ and $\varepsilon_B = 17.26 \times 10^3$ respectively. ε_B was estimated by several measurements of the DNA–drug complexes with a large excess of DNA (i.e. the ratio of dye to nucleotide, D/P, was less than 0.005) and extrapolating the results to D/P = 0. Following the titration experiments, solutions were tested for insoluble precipitate as described above.

The Scatchard plot of these data was compared with the theoretical plots computed according to the excluded site model and the McGhee–von Hippel treatment [12, 13].

$$\frac{r}{C_F} = K_i \frac{(1 - r \cdot n)^n}{1 - [(n-1)\, r]^{n-1}} \qquad (1)$$

where:

$r = \dfrac{C_B}{C_{DNA}}$ (moles of bound dye per mole nucleotide)

K_i = intrinsic association constant

n = binding site size (nucleotides)

The best fit theoretical isotherm (equation 1) and experimental points from the titration experiment were computed on a Nova 1220 computer.

Electrophoretic dye titration. Electrophoretic dye titration was carried out according to the method described by Espejo and Lebowitz [14] and DeLeys and Jackson [15]. The gels (1% agarose) in the same buffer as for the titration exeriments (17×0.5 cm gel tubes) contained DHAQ at various concentra-

tions (0–150 μg/ml). Aliquots of 20 ng of SV 40 (Form I and Form II) in 20 μl of buffer (HNE + 6% (w/v) sucrose] were applied; the tracing dye (bromophenol blue) was added only to control gels, without DHAQ. Electrophoresis was carried out at 60 V (4.2 mA/tube) at 25° for 4 hr, at which time the tracing dye migrated about 7.5 cm. The gels were then removed from the tubes, rinsed three times with 0.1 M NaCl (for 1 hr), and stained in 0.5 μg/ml EB solution. After subsequent rinsing with 0.1 M NaCl the bands were located by illuminating gels with a "Black Light" lamp (General Electric, F15T8-82B) and photographed with a Polaroid camera (Land Film, type 55). The critical free drug concentration (C_f) was estimated as the concentration at which circular closed DNA most nearly co-migrated with the nicked DNA. Known values of the intrinsic association constant (K_i) and the binding site size (n) allow one to determine the critical binding ratio (r') according to the McGee–von Hippel equation (Equation 1) using an iterative computer program. Since the superhelical density (σ_0) of SV 40 DNA is known and is defined by Equation 2 of Bauer and Vinograd [16],

$$\sigma_0 = \frac{-10\ r'}{180} \tag{2}$$

the unwinding angle (ϕ) for DHAQ can be estimated.

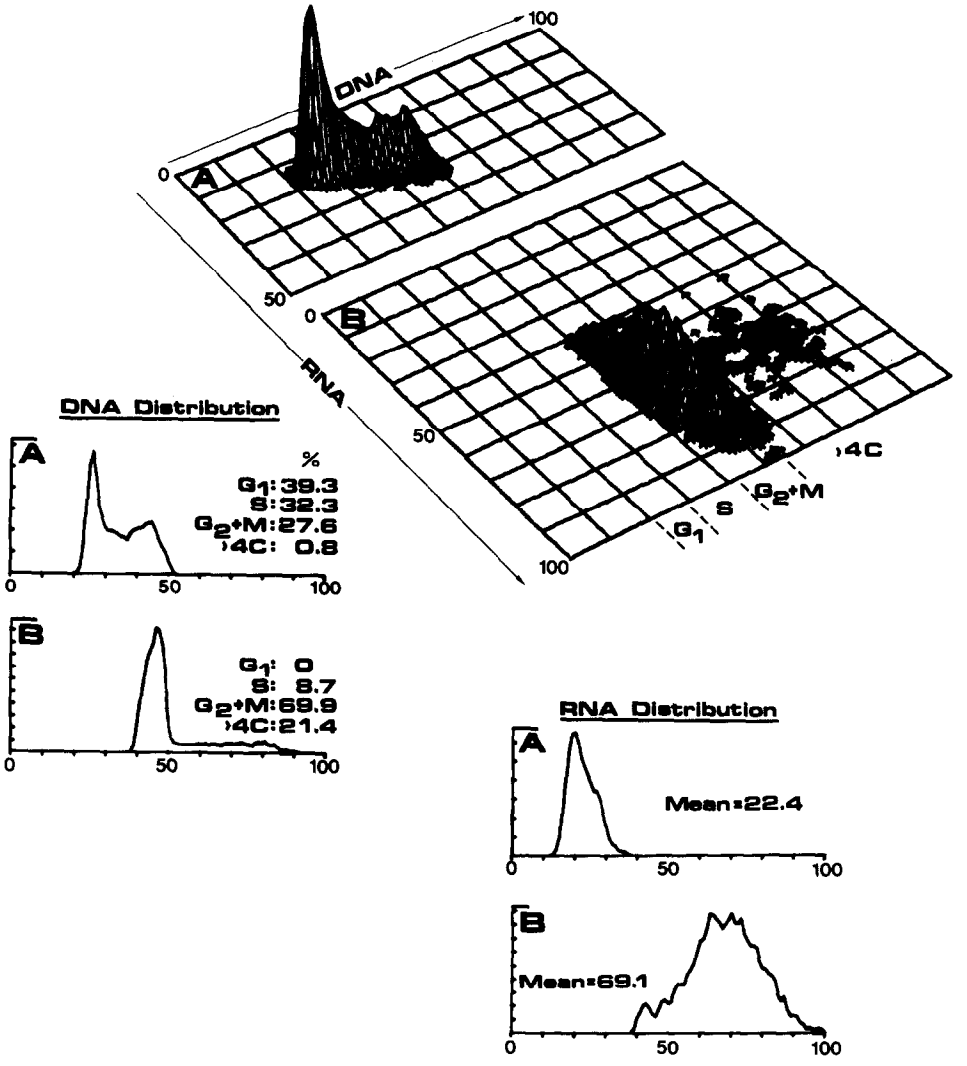

Fig. 1. DNA and RNA distribution of Friend leukemia cells treated with DHAQ. In each set of histograms, (A) represents control and (B) drug-treated cells. Top: Two-parameter frequency histograms representing relative values of DNA and RNA in populations of FL cells untreated (A) and treated (B) with 10 ng/ml DHAQ. The heights of the peaks and ridges represent the relative cell number with respective DNA and RNA values indicated along the ordinate and abscissa, in arbitrary units, respectively. Left: Single parameter (DNA) frequency distribution histograms of the same cells (A, untreated; B, DHAQ-treated), which permits the discrimination of cells in various cell cycle phases. The percentages were calculated using an interactive computer program. Right: RNA frequency distribution histograms of control (A) and DHAQ-treated (B) cells. The mean red fluorescence (RNA content) of the two populations is indicated.

RESULTS

Effect of DHAQ on FL Cells in vitro. Previous studies have shown that brief treatment of FL cells in culture with low concentrations of DHAQ has a dramatic effect on cell progression through the cycle [4]. Figure 1 illustrates the effects of cytostatic concentrations of DHAQ on FL cells. The DNA distribution of control cultures (Fig. 1A, DNA histogram) was typical of exponentially growing cells. Drug-treated cells, however, accumulated in G_2 phase (Fig. 1B, DNA histogram). In addition, DHAQ treatment resulted in a significant increase in cells with greater than a 4C content of DNA (Fig. 1B, top). While there was a significant increase in cell volume of DHAQ-treated cells, the RNA content of cells blocked by the drug more than doubled (Fig. 1, RNA histograms).

In situ *DHAQ interactions*. DHAQ solutions are strongly blue. Casual microscopic observation of living cells from cultures treated with high drug concentrations (5.0 μg/ml) revealed that cell nuclei, especially nucleoli, stain blue. A weak cytoplasm counterstaining was also apparent. This observation suggested that it is possible to investigate *in situ* the binding sites for the drug. Next, experiments were performed on permeablized, fixed cells that could be treated with enzymes prior to staining with DHAQ to analyze the chemical nature of those binding sites. Strong binding of DHAQ to nucleoli was observed (Fig. 2). Especially deeply stained were peripheral parts of nucleoli resulting in a characteristic ring-shaped staining pattern. Some nucleoli stained more homogenously. A fine, granular staining of cytoplasm, as well as a diffuse, very weak staining of extranucleolar nuclear chromatin, was also apparent.

RNase treatment alone was adequate to preclude drug binding to cytoplasm and to diminish, but not preclude, staining of nucleoli. DNase somewhat lowered the stainability of nucleoli and nuclear chromatin. Combined treatment of cells with both DNase and RNase almost totally abolished cell staining with DHAQ.

Characteristics of DHAQ. DHAQ has a molecular weight of 444.5 and consists of navy blue crystals soluble in acids and certain organic solvents. The presence of four amino groups is responsible for its basic character (Fig. 3). At pH 7.0 the drug is predominantly in the monocation form (Fig. 4). pK_I and pK_{II} are close to each other within the pK range of 6.5 to 8.0 (Fig. 4).

The absorption spectrum showed the presence of four distinct peaks ($\lambda_A = 242$, $\lambda_B = 276$, $\lambda_C = 610$, and $\lambda_D = 662$ nm). The amplitudes of the peaks varied depending on drug concentration and pH (Fig. 4) and, to a lesser extent, on ionic strength (not shown). The molar absorption of band D was particularly sensitive to drug concentration, suggesting association of drug molecules and drug–drug interactions at higher concentrations. Concentrations of DHAQ solutions were estimated spectrophotometrically at the isobestic point, i.e. 682 nm ($\varepsilon_i = 8.36 \times 10^3\ cm^{-1}M^{-1}$). At that wavelength drug solutions in the concentration range between 1×10^{-6} and 5×10^{-5} M (in 0.1 M NaCl, pH 7.0) obeyed the Beer–Lambert law.

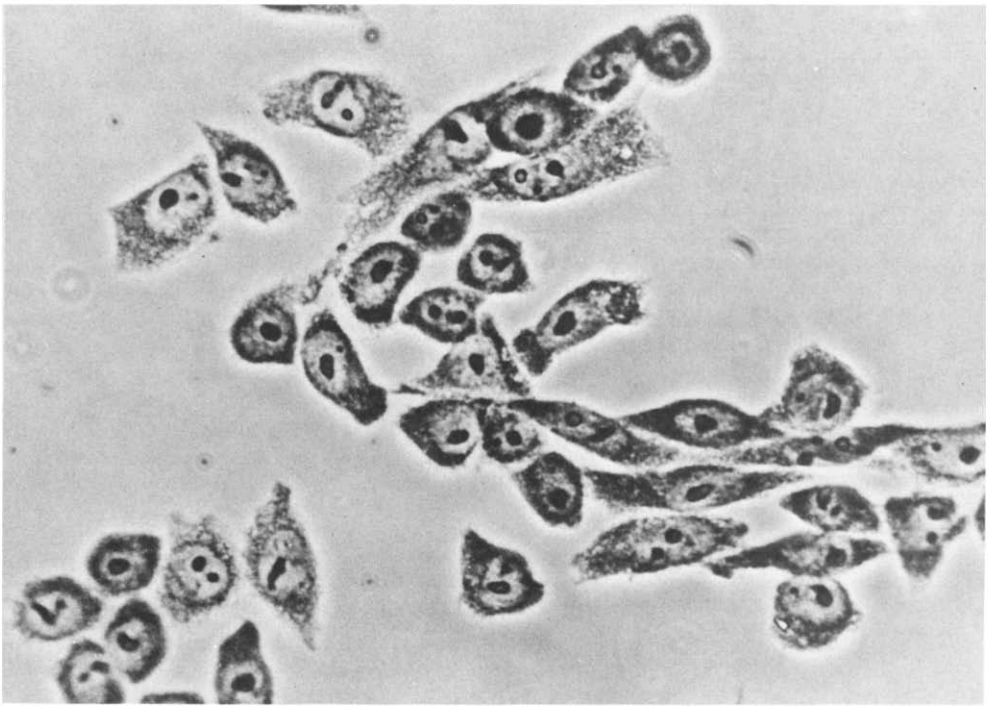

Fig. 2. Exponentially growing CHO cells stained with DHAQ. The staining procedure has been described in the text. Note the preferential drug localization in nucleoli (peripheral, ring-shaped staining of some nucleoli) and in the cytoplasm.

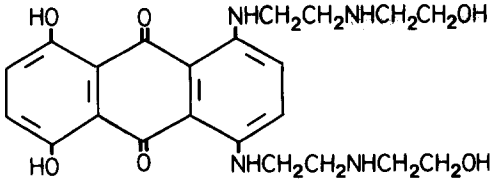

Fig. 3. Structure of 1,4-dihydroxy,5,8-bis [[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione (DHAQ).

Drug interactions with natural nucleic acids. The natural ribopolymers (tRNA, MS2 RNA, 23S RNA) precipitated upon interaction with DHAQ at low ionic strength. In solutions with NaCl at concentrations above 0.5 M, however, these natural ribopolymers were soluble in the presence of drug, and their absorption spectra exhibited a large red shift of the C and D peaks, as well as an increase of the relative absorption of the D band ($\varepsilon_D/\varepsilon_C$ ratio).

Natural, double-stranded DNA (calf thymus) reacted with DHAQ, resulting in complexes that were soluble in 0.1 M NaCl. The changes in the absorption spectra of the dye in these complexes were similar to, but more extensive than, those observed in RNA–DHAQ complexes (for example see Fig. 5A). Thus, there was an increase in the relative intensity of the absorption at the D band which was similar to that observed in free drug solutions during a decrease of DHAQ concentration (compare Fig. 4).

This increase in the $\varepsilon_D/\varepsilon_C$ ratio was responsible for the lack of a clear isobestic point in the spectra of solutions containing DNA–DHAQ complexes and free DHAQ, which impeded measurement of the

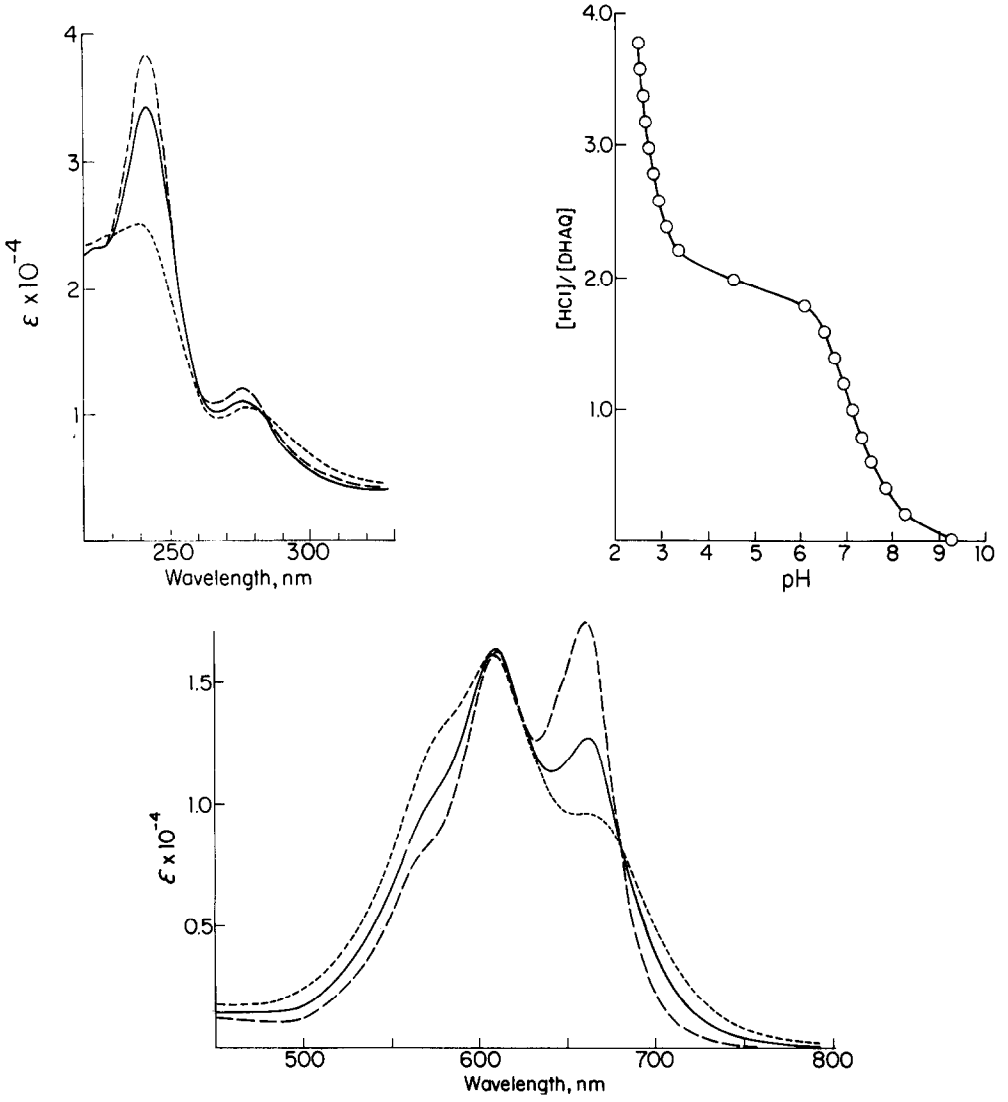

Fig. 4. Visible adsorption spectra of DHAQ in 0.1 M NaCl, 5 mM HEPES, pH 7.0. The spectra were obtained with DHAQ concentrations of 5×10^{-6} M (——), 5×10^{-5} M (——) in a 1.0 cm path-length cuvette, and 5×10^{-4} M (– – –) in a 0.1 cm path-length cuvette. Upper left: Ultraviolet absorption spectra of 5×10^{-5} M DHAQ in a 0.02 M acetate buffer, pH 5.0 (——), 5 mM HEPES buffer, pH 7.0 (——), and 0.02 M phosphate buffer, pH 10.0 (– – –). Upper right: pH-metric titration of DHAQ (22 mg in 10 ml of 50% ethanol with 2 N HCl).

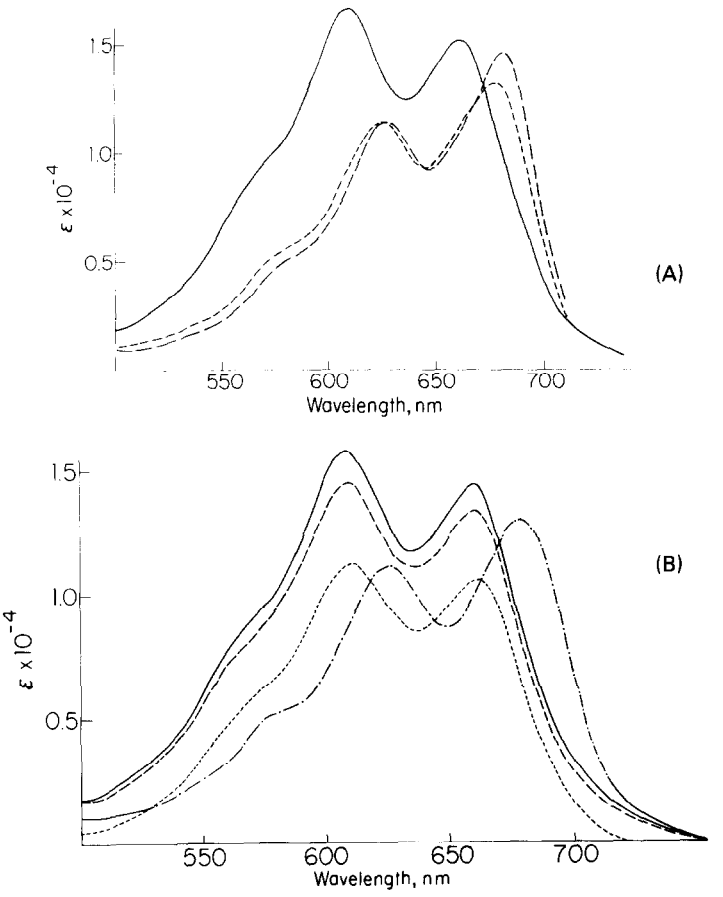

Fig. 5. Visible spectra of DHAQ (1×10^{-5} M) and its complex with nucleic acids (5×10^{-4} M) in 25 mM HEPES, pH 7.0. The NaCl concentration was 1.0 M in every case but poly(rI), where it was 0.1 M. Panel A: Spectra of DHAQ alone (——), and in complex with MS2 RNA (– – –) or calf thymus DNA (— —). Panel B: Spectra of DHAQ alone (——), and in complex with poly(rC) (— —), poly(rU) (– – –), or poly(rI) (— · —).

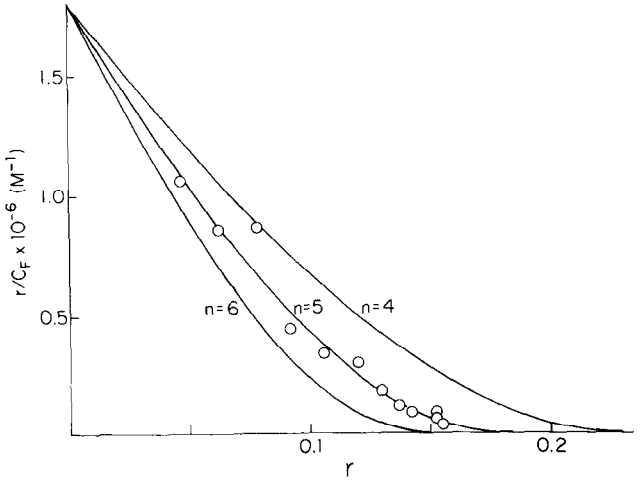

Fig. 6. Scatchard plot of the spectrophotometric titration ($\lambda = 682$ nm) of calf thymus DNA according to the McGhee–von Hippel treatment [13]. The open circles are experimental points obtained from the titration. Solid lines represent theoretical curves calculated from equation 1 (see Materials and Methods). The best fit was obtained for $n = 5$, and $K_i = 1.8 \times 10^6\ M^{-1}$, where n is the binding site size in number of nucleotides and K_i is the intrinsic association constant. The two other curves were drawn with the same K_i value but with $n = 4$ and $n = 6$.

association constant (K_i). However, during titration in a large excess of calf thymus DNA, the increase in absorption at 682 nm (isobestic point of the free drug) was found to be proportional to the concentration of the DNA–DHAQ complex. The molar extinction of the complex at that wavelength was approximately 2-fold higher than that of the free drug. Based on that observation, the intrinsic association constant ($K_i = 1.8 \times 10^6\ M^{-1}$) and the binding site size ($n = 5$ nucleotides) could be calculated (see Materials and Methods). A good correlation of the observed data with the theoretical curve was observed (Fig. 6), despite uncertainty in the titration due to the lack of a well-defined isobestic point.

The interaction of DHAQ with denatured calf thymus DNA was similar to that observed for natural ribopolymers (not shown). To determine the contribution of dye binding to single-stranded regions of denatured DNA and natural RNAs, the interaction of DHAQ with synthetic single-stranded polyanions was examined.

Interactions between DHAQ and single-stranded polyanions. All ribo- and deoxyribopolymers studied (as listed in Materials and Methods), with the sole exception of poly(rI) which will be discussed later, precipitated in the presence of DHAQ. A subtle blue precipitate was observed at D/P = 1 in 0.1 M NaCl at pH 7.0. The precipitation was diminished or precluded at higher ionic strength. At 1.0 M NaCl some of the polymer–DHAQ complexes were fully soluble and at that ionic strength it was possible to record their absorption spectra. All spectra exhibited strong hyperchromicity and a minor (1–3 nm) red shift of the C and D bands (for example, see Fig. 5B). However, as opposed to double-stranded natural DNAs, no evidence of a significant change in relative intensity of the absorption bands (D and C) was apparent. Spectral changes in the u.v. range were difficult to interpret due to the overlap of the absorption bands of nucleic acids and DHAQ.

Polyvinyl sulfate precipitated upon interaction with DHAQ, as did single-stranded polynucleotides. The solubility of the polyvinyl sulfate–DHAQ complex was very low (even at increased ionic strength), which precluded analysis of its absorption spectrum.

In contrast to other polynucleotides, poly(rI) reacted with DHAQ, resulting in complexes that were fully soluble in 0.1 M NaCl but insoluble in 1.0 M NaCl. Furthermore, these complexes exhibited a large red shift of the C and D bands (~20 nm) as well as a significant increase in relative intensity of the absorption of the D band (Fig. 5).

DHAQ binding to synthetic nucleic acid duplexes. Differential spectra of DHAQ complexes with synthetic polynucleotide duplexes were investigated. The most interesting part of the spectra was between 650 and 725 nm (Fig. 7) because this region reflects a red shift of the D band as observed in natural nucleic acids and poly(rI). This shift is one characteristic of drug intercalation. With the sole exception of poly(dA)·poly(dT), all other DNA synthetic polymers studied had maximum differential absorption within the wavelength range of 650–725 nm, in 0.1 M NaCl. Poly(dA)·poly(dT) also showed a maximum, although less prominent than for other duplexes, but only at high ionic strength (1.0 M NaCl, not shown). A pronounced maximum was also observed in the case of the synthetic RNA analogue, poly(rA)·poly(rU).

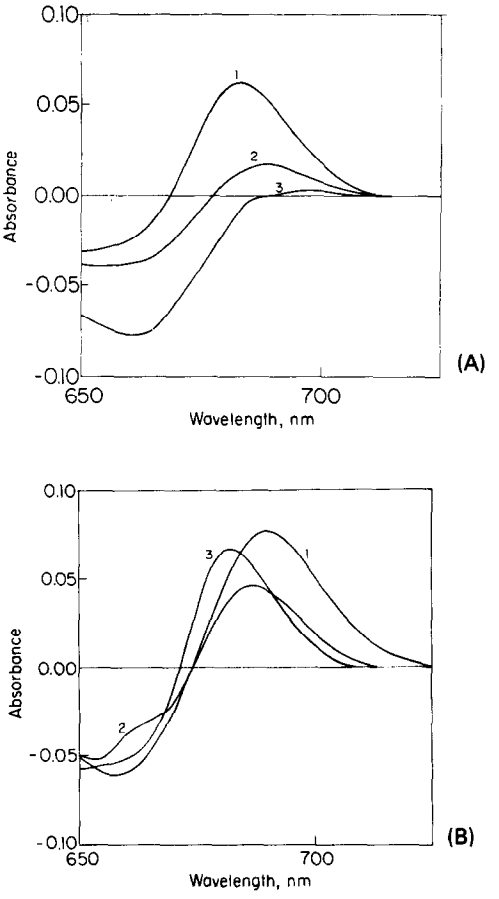

Fig. 7. Differential (visible) absorption spectra (versus free dye) of complexes of DHAQ (1×10^{-5} M) with synthetic double-stranded DNA and RNA (5×10^{-4} M) in 0.14 M NaCl, 25 mM HEPES, and 1 mM EDTA, pH 7.4. Panel A: Spectra 1–3 represent the difference spectra of DHAQ complexed with poly[d(A-T)]·poly[d(A-T)], poly(rA)·poly(rU), and poly(dA)·poly(dT), respecively, versus free dye. Panel B: Spectra 1–3 represent the difference spectra of DHAQ complexed with poly[d(G-C)]·poly[d(G-C)], poly(dG)·poly(dC) and poly[d(I-C)]·poly[d(I-C)], respectively, versus free dye.

Unwinding of closed, circular, double-stranded DNA by DHAQ. The increase in the relative absorption of the D band, the red shift of C and D band absorption, the high value of the association constant, and the stabilization of the duplex structure [2, 17] are all features characteristic of the intercalative mechanism of binding. The presence, however, of long alkyl groups in the 1 and 4 position of the aromatic rings of DHAQ could hinder intercalation. Additional evidence, therefore, has been sought to confirm the intercalating mechanism of the drug binding. Toward this end, studies on the ability of the drug to unwind closed, circular, double-stranded DNA were performed.

The electrophoretic titration method was chosen

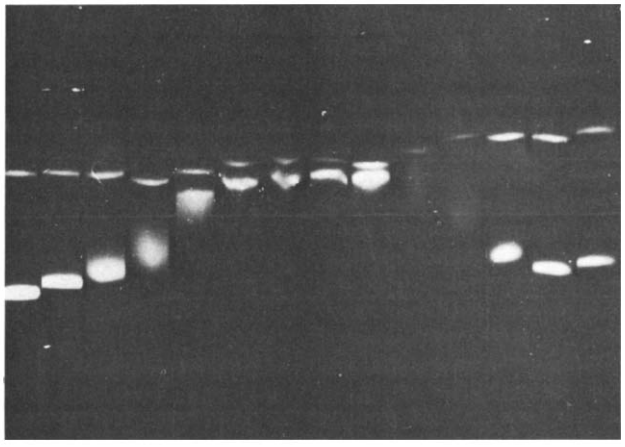

Fig. 8. Electrophoretic dye titration of SV 40 DNA. Electrophoresis was performed in gels containing 20 ng of SV 40 DNA (Form I and Form II) and increasing concentrations of DHAQ (from left to right 0, 2, 4, 6, 10, 12, 14, 16, 18, 20, 25, 50, 100 and 150 ng/ml). The upper, slower moving band represents nicked SV 40 DNA.

to assay the hydrodynamic properties of closed, circular, double-stranded SV 40 DNA upon reaction with DHAQ. As can be seen in Fig. 8, a clear pattern of intercalation was evident. To calculate the unwinding angle, the superhelical density value ($\sigma = -0.061$) of SV 40 was used. This value was obtained based on the experimental data of Espejo and Lebowitz [14] and applying the mathematical model of McGhee and von Hippel (equation 1), as well as using newer data related to the binding of ethidium bromide to nucleic acids, i.e. $\phi = 26°$ [18], $K_i = 2.6 \times 10^5$ M^{-1}, $n = 4$ [19]. The value of the unwinding angle for DHAQ was calculated to be 26.5°.

Thermal melting. Johnson *et al.* [2] presented evidence that DHAQ and several other aminoanthraquinones produced very large upward shifts in the thermal melting of calf thymus DNA [$\Delta T_m = 15.9°$, $C_{DNA} = 50\ \mu M$, D/P = 0.1, 10 mM phosphate buffer, pH 7.0, 5% dimethylsulfoxide (DMSO)]. Our findings confirm these results in the case of calf thymus DNA in low salt concentration and in the presence of DMSO, but not with poly[d(A-T)]·poly[d(A-T)] and poly(dA)·poly(dT) in higher salt (HNE buffer). In the latter cases, no significant changes in the T_m of nucleic acids were observed. Since aqueous solutions of the dye were thermally unstable, as evidenced by the fact that the absorption spectrum of the free drug changed irreversibly after heating at 80° for 10 min (not shown), no further attempts were made to study thermal denaturation of DHAQ–DNA complexes.

DISCUSSION

DHAQ is an extremely potent drug *in vitro*. Nanogram concentrations of the drug induced a well-defined block in the G_2 phase of the cycle (Fig. 1). Blocked cells were characterized by an increased RNA content. Submicrogram concentrations of DHAQ have been shown to be cytotoxic, reducing the clonogenecity of Chinese hamster ovary cells and resulting in the inability of suspension cultures (L1210 and FL cells) to exclude trypan blue [4]. Incorporation of [5-^{3}H]uridine by DHAQ-treated cells was found to be markedly suppressed [4]. The terminal point of drug action was determined to be in the G_2 phase, about 15 min prior to mitosis [4]. Of particular interest is the fact that normal human lymphocytes were considerably more resistant to the drug, surviving in cultures containing up to 10 μg/ml DHAQ [4].

The staining pattern of living and fixed cells with high concentrations of DHAQ, as presently seen, suggests that both DNA and RNA are cellular targets of the drug since cells almost completely fail to bind the drug if pretreated with both DNase and RNase. Of interest is the preferential nucleolar localization of DHAQ.

Examination of drug interaction with a variety of natural and synthetic polynucleotides in solution support the fact that nucleic acids are the intracellular sites of DHAQ action. It is clear from the present study, however, that DHAQ has two modes of binding to nucleic acids. Single-stranded homopolymers (as well as polyvinyl sulfate) precipitated upon interaction with DHAQ. This fact and the observation that binding of DHAQ, as well as of other aminoanthraquinone analogues, caused hypochromic changes in the drug spectrum upon interaction with single-stranded homopolymers indicates strong electrostatic interactions between the drugs and polymers, presumably with cooperative dye stacking [20].

In the present studies, various degrees of drug–polymer ionic interactions were seen, depending on the type of polymer. Electrostatic interactions were observable in the case of natural and synthetic DNA and RNA polymers, though there appears to be the same specificity with respect to DHAQ interaction with homopolymers. Thus, while the drug complexes with poly(rA) were totally insoluble, even in 2.0 M NaCl, the complexes with poly(rU) or poly(rC) were

soluble in 1.0 M, but not 0.1 M, NaCl. Furthermore, the spectral changes of the drug were much more pronounced in the case of poly(rU) (Fig. 5). Although the nature of the specificity is not clear, it is possible that interaction between bases and dye molecules may occur. This type of partial intercalation was recently demonstrated in the case of AO interaction with synthetic polynucleotides or 5′-AMP [21].

Johnson *et al.* [2] proposed DHAQ binding by intercalation to DNA. This hypothesis was based on the fact that binding of DHAQ resulted in the stabilization of the DNA double helix against thermal denaturation. They also noted inhibition of incorporation of labeled thymidine and uridine into TCA-insoluble material of L1210 cells. Our results support this hypothesis. Namely, changes in the drug spectrum (red shift), the high affinity constant of the drug, and, in particular, unwinding of SV 40 DNA all indicate that DHAQ binds by intercalation to double-stranded nucleic acids. Interactions of DHAQ with poly(rI) may also be related to the intercalative mechanism of drug binding since there is a tendency for poly(rI) to form a four-stranded helix in solutions of high ionic strength [22]. This phenomenon has also been observed for the dye-poly(rI) interaction of DAPI, 4′,6-diamidine-2-phenylindole, i.e. a molecule for which strong evidence exists for intercalation [23].

Not much specificity in DHAQ binding to nucleic acids by intercalation was evident. The drug intercalated into both A structure [natural RNA, poly(rA)·poly(rU)] as well as B structure [natural DNA, synthetic DNA duplexes, poly(rI)] nucleic acids. Likewise, no clear base specificity was observed as the drug interacted with all polymers containing A, C, G, I and T. However, DHAQ interactions with polymers containing only A–T base pairs were somewhat weaker than with other polyanions. In addition, alternating polymers exhibited stronger affinity than homopolymer pairs. Similar variations in affinity were observed in the case of DAPI [23] and EB [24] interactions with alternating polymers and homopolymers pairs. The weakest interactions were observed when DHAQ reacted with poly(dA)·poly(dT). Perhaps this phenomenon may be responsible for the odd value calculated for the binding site size ($n = 5$). Assuming that poly(dA)·poly(dT) sequences occur in native DNA and that DHAQ binding to these sequences was very weak, the calculated mean n value would be overestimated and in the absence of these sequences the actual binding site size may be represented by two base pairs ($n = 4$) as in the case of EB [19].

Although it is apparent that DHAQ binds by intercalation, the structure of the intercalative complex is not clear. The presence of alkyl residues in the 1 and 4 position (Fig. 3) makes it impossible to accommodate the drug molecule in such a way that the long axis of the aromatic rings will be parallel to the long axis of the base pairs. For a discussion of the influence of the aminoalkyl groups on drug activity, see Johnson *et al.* [2]. Taking into consideration the mode of binding of some intercalating antibiotics which structurally resemble DHAQ (i.e. daunomycin, adriamycin, see Refs. 1–3 and 25–29), one is led to conclude that the alkyl–amino residues of DHAQ may bind electrostatically to the outside of the double helix, while only a portion of the aromatic system of the molecule intercalates between base pairs.

The two modes of drug binding, i.e. intercalation and ionic interaction (probably with the phosphate backbone of nucleic acids), may occur simultaneously under certain conditions of D/P ratio and ionic strength. In addition, there may be competition as to the extent of binding by either of the mechanisms. Such competition has been observed for other intercalating probes [21, 30]. Intercalation of the drug is expected to be preferred at low D/P ratios. The fact that the drug was more effective at very low concentrations, and thus low D/P ratios, *in vitro*, supports the notion that the strong binding sites (i.e. intercalation) may be responsible for its pharmacological effect. The high association constant of DHAQ intercalating sites ($K_i = 1.8 \times 10^6$ M^{-1}) compared to those of other antitumor intercalating agents (e.g. ethidium bromide, $K_i = 0.26 \times 10^6$ M^{-1}; daunomycin, $K_i = 0.5 \times 10^6$ M^{-1}), is additional evidence to support the fact that the intercalation mode of binding predominates at pharmacological doses.

Preferential accumulation of DHAQ in nucleoli, binding of the dye to chromatin and to cytoplasmic RNA, its high affinity for DNA and RNA in solution, and its suppression of RNA synthesis coupled with increased RNA accumulation per cell [4], all point out that both, DNA transcription as well as RNA processing, are influenced by the drug. In fact, these effects would be anticipated if the drug intercalated both into DNA and double-stranded RNA. In the first case, partial intercalation of the molecule, a part of which may protrude into the groove (i.e. as in the case of daunomycin), is expected to impede transcription. Intercalation into a double-stranded region of RNA (i.e. HnRNA or rRNA), on the other hand, may affect it processing, or turnover rate, and thus be responsible for the cellular accumulation of RNA, even when the overall rate of its synthesis is diminished.

Acknowledgements—The authors wish to thank Mrs. Lisa Coico for her technical assistance and Miss Robin Nager for her assistance in the preparation of the manuscript. This work was supported by Grant CA23296-02 from the National Cancer Institute and, in part, by NCI Core Grant CA-08748.

REFERENCES

1. R. K-Y. Zee-Cheng and C. C. Cheng, *J. med. Chem.* **21**, 291 (1978).
2. R. K. Johnson, R. K-Y. Zee-Cheng, W. W. Lee, E. M. Acton, D. W. Henry and C. C. Cheng, *Cancer Treat. Rep.* **63**, 425 (1979).
3. R. E. Wallace, K. C. Murdock, R. B. Angier and F. E. Durr, *Cancer Res.* **39**, 1570 (1979).
4. F. Traganos, D. P. Evenson, L. Staiano-Coico, Z. Darzynkiewicz and M. R. Melamed, *Cancer Res.* **40**, 671 (1980).
5. Z. Darzynkiewicz, D. P. Evenson, L. Staiano-Coico, T. Sharpless and M. R. Melamed, *J. cell. Physiol.* **100**, 425 (1979).
6. Z. Darzynkiewicz, F. Traganos, T. Sharpless and M.

R. Melamed, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2881 (1976).
7. F. Traganos, Z. Darzynkiewicz, T. Sharpless and M. R. Melamed, *J. Histochem. Cytochem.* **25**, 46 (1977).
8. L. S. Lerman, *Proc. natn. Acad. Sci. U.S.A.* **49**, 94 (1963).
9. D. F. Bradley and M. K. Wolf, *Proc. natn. Acad. Sci. U.S.A.* **45**, 944 (1959).
10. T. K. Sharpless, in *Flow Cytometry and Sorting* (Eds. M. R. Melamed, P. F. Mullaney and M. L. Mendelsohn), p. 359. John Wiley, New York (1979).
11. T. Sharpless, F. Traganos, Z. Darzynkiewicz and M. R. Melamed, *Acta cytol.* **19**, 577 (1975).
12. H. M. Sobell, C. C. Tsai, S. C. Jain and S. G. Gilbert, *J. molec. Biol.* **114**, 333 (1977).
13. J. D. McGhee and P. H. von Hippel, *J. molec. Biol.* **86**, 469 (1974).
14. R. T. Espejo and J. Lebowitz, *Analyt. Biochem.* **72**, 95 (1976).
15. R. J. DeLeys and D. A. Jackson, *Biochem. biophys. Res. Commun.* **69**, 446 (1976).
16. W. Bauer and J. Vinograd, *J. molec. Biol.* **33**, 141 (1968).
17. J-B. LePecq, in *Methods of Biochemical Analysis,* Vol. 20 (Ed. D. Glick), p. 42. John Wiley, New York (1971).
18. J. C. Wang, *J. molec. Biol.* **89**, 783 (1974).
19. J-J. Lawrence and M. Daune, *Biochemistry* **15**, 3301 (1976).
20. J. C. Double and J. R. Brown, *J. Pharm. Pharmac.* **28**, 166 (1976).
21. V. von Tscharner and G. Schwarz *Biophys. Struct. Mechanism* **5**, 75 (1979).
22. C. H. Chou, G. J. Thomas, Jr., S. Arnott and P. J. Campbell Smith, *Nucl. Acids Res.* **4**, 2407 (1977).
23. J. Kapuscinski and W. Szer, *Nucl. Acids Res.* **6**, 3519 (1979).
24. B. C. Baguley and E-M. Falkenhaug, *Nucl. Acids Res.* **5**, 161 (1978).
25. J. C. Double and J. R. Brown, *J. Pharm. Pharmac.* **27**, 502 (1975).
26. H. Porumb, *Prog. Biophys. molec. Biol.* **34**, 175 (1978).
27. A. Di Marco, F. Arcamone and F. Zunino, in *Antibiotics,* Vol. III (Eds. J. W. Corcoran and F. E. Hahn), p. 101. Springer, New York (1975).
28. M. Waring, *J. molec. Biol.* **54**, 247 (1970).
29. H. Kersten and W. Kersten, *Inhibitors of Nucleic Acid Synthesis*, p. 67. Springer, New York (1974).
30. A. L. Stone and D. F. Bradley, *J. Am. chem. Soc.* **83**, 3627 (1961).