Jacob, G. S., Schaefer, J., & Wilson, G. E., Jr. (1985) *J. Biol. Chem. 260*, 2777–2781.
Kanamori, K., Legerton, T. L., Weiss, R. L., & Roberts, J. D. (1982) *J. Biol. Chem. 257*, 14168–14172.
Kandler, O. (1979) *Naturwissenschaften 66*, 95–105.
Kanodia, S., & Roberts. M. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5217–5221.
Kenealy, W. R., Thompson, T. E., Schubert, K. R., & Zeikus, J. G. (1982) *J. Bacteriol. 150*, 1357–1365.
König, H., & Kandler, O. (1979a) *Arch. Microbiol. 121*, 271–275.
König, H., & Kandler, O. (1979b) *Arch. Microbiol. 123*, 295–299.
Lapidot, A., & Irving, C. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1988–1992.
Lapidot, A., & Irving, C. S. (1979) *Biochemistry 18*, 1788–1796.
Legerton, T. L., Kanamori, K., Weiss, R. L., & Roberts, J. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1495–1498.
Meeks, J. C., Wolk, C. P., Lockau, W., Schilling, N., Shaffer, P. W., & Chien, W. S. (1978) *J. Bacteriol. 134*, 125–130.
Schaefer, J., Skokut, T. A., Stejskal, E. O., McKay, R. A., & Varner, J. E. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5978–5982.
Schaefer, J., Skokut, T. A., Stejskal, E. O., McKay, R. A., & Varner, J. E. (1981b) *J. Biol. Chem. 256*, 11574–11579.
Schubert, K. R., & Coker, G. T. III (1981) *Plant Physiol. 67*, 662–665.
Seeley, R. J., & Fahrney, D. E. (1983) *J. Biol. Chem. 258*, 10835–10838.
Stupperich, E., & Fuchs, G. (1981) *Arch. Microbiol. 130*, 294–300.
Stupperich, E., & Fuchs, G. (1984) *Arch. Microbiol. 139*, 8–20.
Taylor, C. T., Kelly, D. P., & Pirt, S. J. (1976) in *Proceedings of the Symposium on Microbial Production and Utilization of Gases (H_2, CH_4, CO)* (Schlegal, H. G., Gottschalk, G., & Pfenning, N., Eds.) pp 173–180, Akademie der Wissenschaften Zu Göttingen, E. Goltze, Göttingen.
Tyler, B. (1978) *Annu. Rev. Biochem. 47*, 1127–1162.
Vanderbilt, A. S., Gaby, N. S., & Rodwell, V. W. (1975) *J. Biol. Chem. 250*, 5322–5329.
Winters, J. C., & Kunin, R. (1949) *Ind. Eng. Chem. 41*, 460–463.
Zeikus, J. G. (1977) *Bacteriol. Rev. 41*, 514–541.
Zeikus, J. G., Fuchs, C. T., Kenealy, W. R., & Thauer, R. K. (1977) *J. Bacteriol. 132*, 604–613.

Proliferation Dependence of Topoisomerase II Mediated Drug Action†

Daniel M. Sullivan,‡ Bonnie S. Glisson,‡,§ Paula K. Hodges,§ Sherin Smallwood-Kentro,§ and Warren E. Ross*,‡,§
Departments of Medicine and Pharmacology, College of Medicine, University of Florida, Gainesville, Florida 32610
Received September 4, 1985

ABSTRACT: Topoisomerase II mediated DNA scission induced by both a nonintercalating agent [4′-demethylepipodophyllotoxin 4-(4,6-*O*-ethylidene-β-D-glucopyranoside) (VP-16)] and an intercalator [4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)] was studied as a function of proliferation in Chinese hamster ovary (CHO), HeLa, and mouse leukemia L1210 cell lines. Log-phase CHO cells exhibited dose-dependent drug-induced DNA breaks, while plateau cells were found to be resistant to the effects of VP-16 and *m*-AMSA. Neither decreased viability nor altered drug uptake accounted for the drug resistance of these confluent cells. In contrast to CHO cells, plateau-phase HeLa and L1210 cells remained sensitive to VP-16 and *m*-AMSA. Recovery of drug sensitivity by plateau-phase CHO cells was found to reach a maximum approximately 18 h after these cells regained exponential growth and was independent of DNA synthesis. DNA strand break frequency correlated with cytotoxicity in CHO cells; log cells demonstrated an inverse log linear relationship between drug dose (or DNA damage) and colony survival, whereas plateau-derived colony survival was virtually unaffected by increasing drug dose. Topoisomerase II activity, whether determined by decatenation of kinetoplast DNA, by cleavage of pBR322 DNA, or by precipitation of the DNA–topoisomerase II complex, was uniformly severalfold greater in log-phase CHO cells compared to plateau-phase cells.

DNA topoisomerase II [EC 5.99.1.3; for a review, see Gellert (1981), Liu (1983), and Wang (1985)] is present in both eukaryotes and prokaryotes and exists as a dimer with a subunit molecular mass of 131–180 kilodaltons (kDa),[1] requires magnesium cations and ATP for activity, shows DNA-stimulated ATP hydrolysis, requires the presence of a sulfhydryl reagent for full activity, and changes linking numbers of DNA in steps of two. This enzyme catalyzes DNA topoform interconversions by introducing a transient enzyme-bridged, double-stand break in one of the two crossing

† This work was supported in part by National Institutes of Health Grant CA-24586.
* Address correspondence to this author at the Department of Pharmacology, University of Florida.
‡ Department of Medicine.
§ Department of Pharmacology.

[1] Abbreviations: VP-16, 4′-demethylepipodophyllotoxin 4-(4,6-*O*-ethylidene-β-D-glucopyranoside); *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; CHO, Chinese hamster ovary; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)-aminomethane; kDa, kilodalton(s).

0006-2960/86/0425-2248$01.50/0

DNA segments. Liu et al. (1983) and Rowe et al. (1984) have shown that topoisomerase II cuts DNA with a four-base stagger and becomes covalently linked to the 5′ end of each broken strand via a phosphotyrosyl bond. This intermediate DNA–enzyme complex is isolable upon treatment with a protein denaturant and, presumably, represents the reaction intermediate of the breaking–rejoining mechanism. Several investigators have suggested that topoisomerase II is intimately involved in a variety of intracellular genetic processes that involve DNA topological isomerization. A novel enzyme role, and the one explored in this paper, involves the drug-induced DNA damage mediated by topoisomerase II.

Numerous intercalating and nonintercalating antitumor drugs have been found to induce DNA single- and double-strand breaks. Loike and Horwitz (1976a,b) first demonstrated DNA single-strand breaks induced by VP-16 in HeLa cells. Ross et al. (1978, 1979) and Ross and Bradley (1981) were the first to suggest that a topoisomerase binds covalently to DNA and potentially mediates the effects of adriamycin, VP-16, and ellipticine. Recent investigations (Tewey et al. 1984a,b; Nelson, E. M., et al., 1984; Chen et al., 1984) indicate that these drugs stabilize the formation of the DNA–topoisomerase II complex discussed above, thus resulting in the increased DNA scission seen with protein denaturants and the concomitant inhibition of catalytic activity (no rejoining). A correlation between DNA strand break frequency and cytotoxicity has been observed for VP-16-treated log-phase mouse leukemia L1210 cells (Wozniak & Ross, 1983) and lung adenocarcinoma cells treated with congeners of podophyllotoxin (Long et al., 1984).

No investigations have explored the drug–enzyme interaction with respect to the stage of cell proliferation, and only recently have studies examined topoisomerase II activity as a function of phase of growth. Enzyme activity in each of these studies was assayed in crude cellular extracts. Duguet et al. (1983) observed considerable increases in topoisomerase II activity in both regenerating rat liver following partial hepatectomy and concanavalin A stimulated guinea pig lymphocytes (Taudou et al., 1984). In both cases, enzyme activity increased dramatically when resting cells resumed exponential growth. In contrast, Tricoli et al. (1985) found no difference in topoisomerase II levels between log and confluent mouse embryo fibroblasts, and no change in enzyme activity was observed as a function of the phase of the cell cycle (G_1, S, M) in these same cells.

An initial report from this laboratory (Sullivan et al., 1985) showed marked differences in VP-16-induced DNA damage in log vs. confluent CHO cells. We have now extended these investigations to examine the drug–enzyme interaction and its cellular consequences as a function of proliferation in various cell models. By studying drug-induced DNA cleavage by topoisomerase II in whole cells, we have been able to examine the activity of the enzyme for the first time in its native environment.

Materials and Methods

Materials

Wild-type CHO cells and HeLA cells were grown in monolayer in α-MEM (Gibco Laboratories) with 5% and 10% fetal calf serum, respectively. RPMI 1630 medium (Gibco) with 20% fetal calf serum was used to grow mouse leukemia L1210 cells in suspension. Cells were grown at 37 °C in the presence of 5% CO_2 with penicillin (100 IU/mL) and streptomycin (100 μg/mL) added to all culture media. These three cell lines were cultured from initial seeding through plateau growth without replenishment of the media (starved). A culture of *Crithidia fasciculata* was kindly provided by Dr. Paul England (Johns Hopkins). These cells were grown in suspension at 27 °C in Difco brain heart infusion medium supplemented with hemin (20 μg/mL). Monolayer cells were harvested from culture flasks by incubation with 0.05% trypsin (2–3 mL, Gibco) for 30–60 s at 37 °C or by scraping with a rubber policeman in the presence of Hank's buffered salt solution (Gibco) with 0.02% EDTA. Cell numbers were determined by a Coulter counter and viability by trypan dye exclusion.

[^{14}C]Thymidine (57 mCi/mmol) and [α-^{32}P]dATP (3200 Ci/mmol) were obtained from ICN, Irvine, CA. [^{3}H]Thymidine (20 Ci/mmol) and Aquassure were from New England Nuclear. Safety-Solve was obtained from Research Products International Corp. Moravek Biochemicals, Inc., Brea, CA, supplied [^{3}H]VP-16 (200 mCi/mmol). VP-16 was obtained from Bristol-Myers Co., Syracuse, NY, and *m*-AMSA from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Two-micrometer poly (vinyl chloride) filters are a product of Millipore. *Hin*dIII restriction endonuclease and Klenow fragment of DNA polymerase I were obtained from Bethesda Research Laboratories. Tetrapropylammonium hydroxide is a product of RSA Corp., Ardsdale, NY. Dimethyl sulfoxide, deoxynucleotide triphosphates, ATP, proteinase K (type XI), Pronase, PMSF, aphidicolin, Ficoll, Nonidet P-40, DNase I, and hemin were all supplied by Sigma Chemical Co. All other reagents were of analytical grade.

Methods

Drug Treatment. Starved experimental CHO or HeLa cells were radiolabeled during log-phase growth with [^{14}C]thymidine. At different stages of cell growth (determined by counting parallel flasks), the growth medium was replaced with fresh medium, cells were incubated at 37 °C for 1 h, and duplicate experimental cell flasks were treated with various concentrations of drugs for 30–60 min at 37 °C. VP-16 and *m*-AMSA were dissolved in dimethyl sulfoxide, and control cells were treated with dimethyl sulfoxide alone. After incubation with the drug, the cells were washed 2–3 times with cold PBS, removed from the flasks, resuspended in cold media, and kept on ice to preclude repair of DNA strand breaks. Drug-induced DNA single-strand break frequency was then determined by alkaline elution.

Alkaline Elution. The alkaline elution technique for assaying DNA single-strand breaks has been described in detail (Kohn et al., 1976, 1981; Kohn, 1979). Briefly, 2.5×10^5 to 1×10^6 ^{14}C-labeled experimental cells were combined with 5×10^5 ^{3}H-labeled internal standard cells (mouse leukemia L1210 cells) that had received either 150 R (low strand break frequency) or 1500 R (high strand break frequency) from a ^{137}Cs source. The cell combination was layered onto a poly(vinyl chloride) filter and lysed with a solution of 2% SDS, 20 mM Na_2EDTA, and proteinase K (0.5 mg/mL). The DNA was then eluted from the filter with tetrapropylammonium hydroxide at pH 12.1. To measure low strand break frequency (0–1000 R equiv), the elution flow rate was 0.04 mL/min with a fraction interval of 3 h and a total elution time of 15 h. For high single-strand break frequency assays, the elution rate was 0.16 mL/min with a fraction interval of 5 min and a total elution time of 30 min.

Recovery of Drug Sensitivity by Plateau Cells. Recovery of drug sensitivity was followed by trypsinizing starved wild-type CHO plateau-phase cells (2×10^7/25 cm^2 flask) and seeding 1×10^6 total cells into 25 cm^2 flasks containing fresh media. VP-16-induced DNA strand breaks were quantified

by alkaline elution at various times posttrypsinization. DNA synthesis was concurrently measured by trichloroacetic acid (5%) precipitation of DNA from cells pulse labeled with [^{3}H]thymidine.

Uptake of [^{3}H]VP-16. To study epipodophyllotoxin uptake by CHO cells at various stages of growth, starved cells ($\geq 10^7$ cells total) were incubated with 17 μM [^{3}H]VP-16 for 1 h at 37 °C. The cells were then washed with cold PBS, removed from the flask by scraping with a rubber policeman in the presence of Hank's buffered salt solution with 0.02% EDTA, pelleted by centrifugation at 500*g* for 2–3 min (Dynac II centrifuge), and finally washed with cold PBS and recentrifuged. The pelleted cells were transferred to drying paper and dried overnight in an oven at 65 °C. After the pellet was weighed, it was dissolved in 250 μL of 1 N NaOH at 65 °C for 2 h in a capped vial, allowed to sit at room temperature for 20–30 min, and neutralized with 250 μL of 1 N HCl, and ^{3}H was determined by scintillation counting under 4.5 mL of Safety-Solve. Drug uptake for log- and plateau-phase cells was determined as cpm per milligram of cells.

Generation of G_1- and G_2/M-Enriched CHO Populations. Exponentially growing wild-type CHO cells were trypsinized from monolayer culture, seeded in spinner flasks, and after 24-h growth at 37 °C pelleted by centrifugation at 500*g* for 5 min and resuspended in 1% Ficoll (total number of cells 8 $\times 10^7$). An aliquot of cells was removed from this suspension (asynchronous population) and resuspended in 3% Ficoll. The remaining cells (7.5×10^7) were separated by velocity sedimentation on a Ficoll gradient of 2–4% in a Celsep apparatus (Du Pont Co.). After sedimentation for 2 h at room temperature, 25 25-mL fractions were collected and cells pelleted by centrifugation (as were asynchronous cells), washed with PBS, and recentrifuged. DNA histograms were generated from an aliquot of each fraction on an Epics "C" flow cytometer (Coulter Electronics) using propidium iodide staining and analyzed by using the Para I cell cycle analysis program. In this manner, G_1- and G_2/M-enriched populations (relative to asynchronous cells) were identified. The enriched populations were reseeded in monolayer and treated with VP-16, and DNA damage was assessed by alkaline elution.

Cytotoxicity Assays. Drug-induced cytotoxicity was assessed for both log- and plateau-phase CHO cells by colony-forming assays. Appropriate cells were treated with 2–50 μM VP-16 in dimethyl sulfoxide (control cells were treated with dimethyl sulfoxide alone) for 1–4 h, washed 2–3 times with PBS to remove the drug, and trypsinized, and 500–1000 viable cells were seeded into triplicate 100-mm plastic petri dishes with 10 mL of fresh media. Colonies were allowed to grow for 7–8 days, stained with 2% crystal violet in 25% ethanol, and counted, and the percent survival was determined by comparison with untreated controls.

Measurement of Topoisomerase II Catalytic Activity. (*A*) *Extraction of Topoisomerase II from the Nucleus.* All procedures were at 0 °C; fresh PMSF in 2-propanol was added to all buffers at a final concentration of 1 mM, and all centrifugations were in a Sorvall SS34 rotor unless otherwise noted. Starved CHO cells were grown in suspension cultures in spinner flasks at 37 °C. Resistance to VP-16-induced DNA damage in plateau cells was confirmed by alkaline elution, and 1×10^8 log- or plateau-phase viable cells were centrifuged at 5000 rpm for 10 min (Sorvall GSA rotor). The pellet was resuspended in 30 mL of PBS and sedimented at 4000 rpm for 10 min. The pellet was washed twice more with PBS and centrifuged as described previously. After the final wash, the cells were resuspended in 10 mL of 10 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, and 10 mM NaCl and allowed to sit at 0 °C for 10 min. A nonionic detergent (1 mL of 10% Nonidet P-40) was added and the mixture gently triturated and finally left at 0 °C for 15 min. The cells were then Dounce homogenized and centrifuged at 2500 rpm for 10 min, and the pellet was resuspended in 2 mL of buffer A [50 mM Tris (pH 7.5), 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, and 0.25 M sucrose]. The nuclei thus obtained were layered over 0.6 mL of buffer B (buffer A with 0.6 M sucrose) and sedimented at 7000 rpm for 10 min. The pellet was resuspended in 2 mL of buffer C (buffer A without $CaCl_2$ and with 5 mM $MgCl_2$), centrifuged at 7000 rpm for 10 min, and finally resuspended in 0.3 mL of buffer D (same as buffer C without sucrose). To this solution were added 30 μL of 0.2 M EDTA (pH 8.0) and 0.66 mL of buffer E [80 mM Tris (pH 7.5), 2 mM EDTA, 1 mM DTT, 0.53 M NaCl, and 20% glycerol (v/v)]. This mixture was gently triturated, left at 0 °C for 30 min, and centrifuged at 19000 rpm for 20 min. The supernatant from the last centrifugation contains topoisomerase II activity. The protein concentration of the nuclear extract was determined by the method of Bradofrd (1976). The enzyme solution was diluted with an equal volume of glycerol, and BSA and PMSF were added to final concentrations of 1 mg/mL and 0.5 mM, respectively. The enzyme is stable at −20 °C for 4–5 days.

(*B*) *Decatenation of Kinetoplast DNA.* Form I kinetoplast DNA (kDNA) was isolated from the mitochondria of *Crithidia fasciculata* according to established procedures (Simpson & Simpson, 1974; Englund, 1978). The decatenation assay was a modification of earlier reports (Marini et al., 1980; Miller et al., 1981; Duguet et al., 1983). A total assay volume of 20 μL contained 50 mM Tris (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA, 30 μg/mL BSA, 1 mM ATP, 1 μg kDNA, and various amounts of nuclear topoisomerase II extract from above. After incubation at 30 °C for 30 min in a water bath, 5 μL of 0.05% bromophenol blue and 2% SDS were added; the samples were electrophoresed on 1% agarose gels for 18 h at 20 V and 10 mA in 89 mM Tris–borate buffer (pH 8.0) with 2 mM EDTA. The gel was finally stained with ethidium bromide and photographed under UV illumination. Form I kDNA networks remain at the origin, but the monomer circles generated by topoisomerase II migrate into the gel.

(*C*) *Cleavage of Plasmid pBR322 DNA.* Supercoiled pBR322 DNA was isolated according to the procedure of Clewell (1972). Assays for topoisomerase II mediated DNA cleavage followed other methods (Miller et al., 1981; Rowe et al., 1984; Nelson, E. M., et al., 1984) with slight modification. A 50-μL assay contained the same buffer as for decatenation, 1 μg of pBR322 DNA, and various amounts of topoisomerase II and VP-16. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 5 μL of 10% SDS and 1 mg/mL proteinase K. The mixture was allowed to sit at 37 °C for 30 min, at which time 10 μL of 2% SDS and 0.05% bromophenol blue were added and the DNA was electrophoresed as described above. Topoisomerase II, in the presence of VP-16, causes double-strand breaks resulting in linear DNA (form III).

(*D*) *Quantitative Precipitation of the Covalent Topoisomerase II–DNA Complex.* Labeling of the 3′ end of pBR322 DNA with [α-^{32}P]dATP was done as described by Liu et al. (1983) and Nelson, E. M., et al. (1984) as was the quantitative precipitation of DNA–enzyme complexes. Briefly, 50-μL reaction mixtures contained various amounts of nuclear extract containing topoisomerase II, 3′ end-labeled pBR322 DNA, and VP-16 in the same buffer as the decatenation

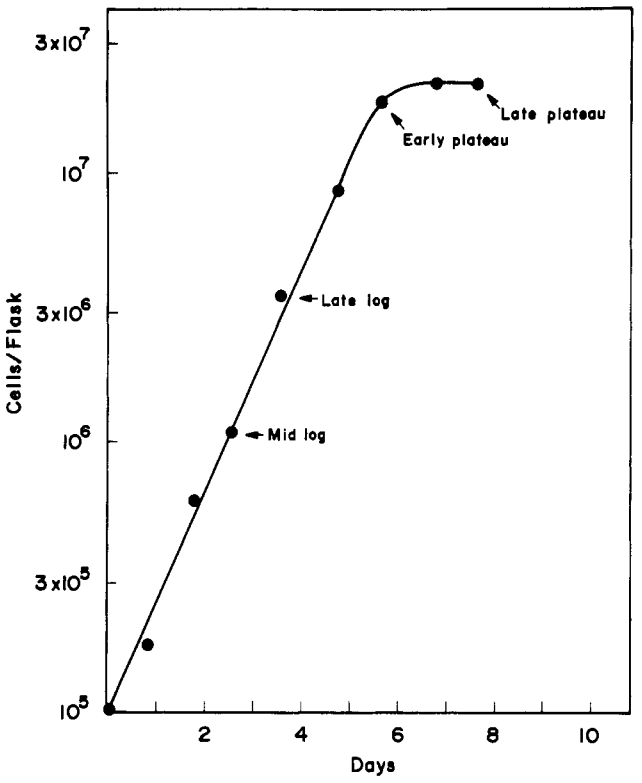


FIGURE 1: Growth curve of starved wild-type CHO cells. Cells were seeded at 1 × 10^5 total cells on day 0, and duplicate flasks were counted on subsequent days. Arrows indicate times at which parallel cells, which had been labeled with [^{14}C]thymidine for 48 h, were sampled for sensitivity to VP-16 and *m*-AMSA.

assays. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 100 μL of prewarmed 20% SDS, 0.02 M EDTA, 5 N NaOH, and 1 mg/mL herring sperm DNA and allowed to sit at 37 °C for 10 min. The enzyme–DNA complexes were then precipitated by addition of 50 μL of 2 M KCl, 1 N HCl, and 1 M Tris (pH 8.0). Precipitation was allowed to proceed for 10 min at 0 °C. The precipitate was collected in microcentrifuge tubes by centrifugation at 13000*g* for 15 min at 0 °C (Fisher Micro-Centrifuge Model 235A). The supernatant was aspirated and the pellet washed with 200 μL of 1 M Tris (pH 8.0), 2 M KCl, 0.2 M EDTA, and 4 mg/mL herring sperm DNA by heating at 65 °C for 10 min. The precipitate was collected by centrifugation as above, the supernatant aspirated, and the pellet suspended in 200 μL of H_2O and dissolved by heating to 65 °C. A 190-μL aliquot was diluted in 2 mL of H_2O and counted under 2 mL of Aquassure.

Results

Drug-Induced DNA Damage in Log vs. Plateau Growth. Starved wild-type CHO cells grown in monolayer were chosen as a model in which to study the effects of both an epipodophyllotoxin (VP-16) and an intercalating agent (*m*-AMSA) on topoisomerase II mediated DNA scission at various stages of cell proliferation. Figure 1 shows a typical growth curve for these cells. They were found to have a doubling time of 18 h, reach confluency in approximately 6 days (2 × 10^6 cells/mL), and maintain a viability of >85%. At different stages of growth (mid-log to late plateau; see Figure 1), cells were exposed to VP-16, and the DNA single-strand break frequency was quantified by alkaline elution (Figure 2A). These experiments demonstrated that sensitivity to VP-16-induced DNA cleavage is a function of proliferation. Log-phase cells exhibited the greatest DNA break frequency, while plateau-phase cells were resistant to the effects of VP-16. Similar results were obtained with the intercalating agent *m*-AMSA (Figure 2B).

To determine if the disparity in VP-16-induced DNA scission in log vs. confluent cells was a function of drug uptake, appropriate cells were exposed to [^{3}H]VP-16 as described under Methods. No difference in uptake of tritiated drug was found between log- and plateau-phase cells (data not shown).

DNA histograms, generated by flow cytometry, demonstrated that the majority of plateau-phase CHO cells have a

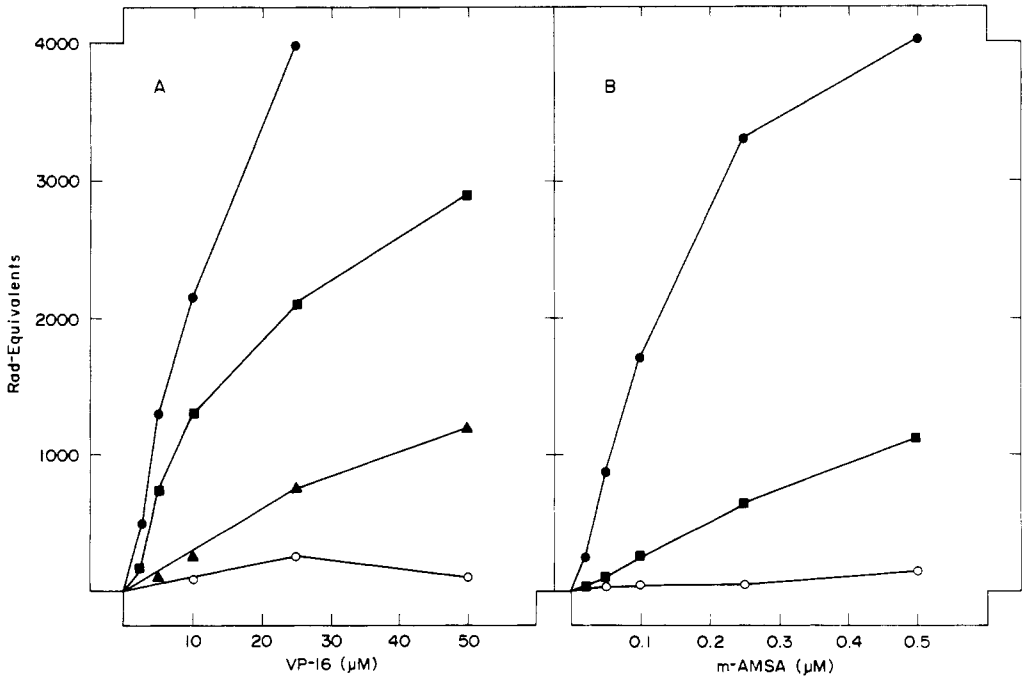


FIGURE 2: DNA single-strand break frequency in CHO cells induced by VP-16 and *m*-AMSA as a function of proliferation. Starved cells, at various stages of growth, were exposed to VP-16 (panel A) or *m*-AMSA (panel B) for 60 and 30 min, respectively. DNA damage was quantified by alkaline elution and is expressed as the equivalent radiation dose which would result in that degree of strand scission. (●) Mid log; (■) late log; (▲) early plateau; (○) late plateau.

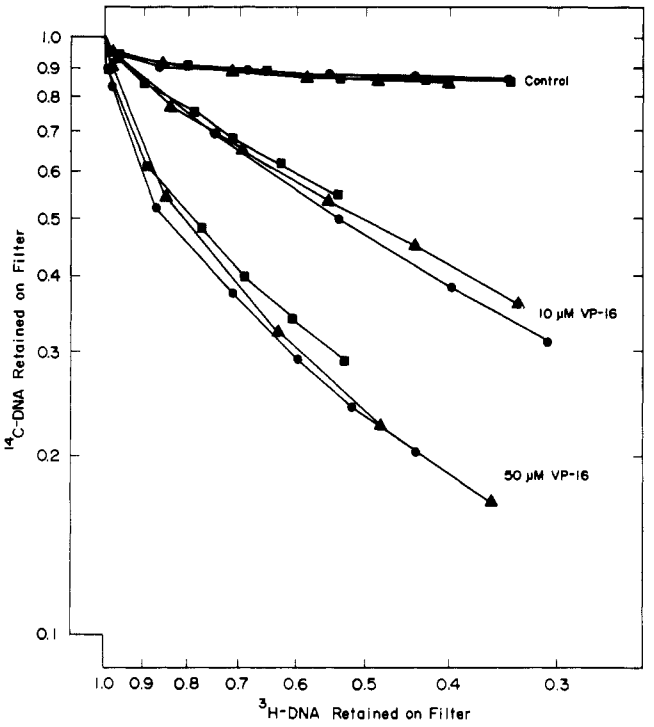


FIGURE 3: VP-16-induced DNA damage in asynchronous and enriched populations of CHO cells. G_1- and G_2/M-enriched populations were obtained by velocity sedimentation on a Ficoll gradient. DNA damage induced by 10 and 50 μM VP-16 was assessed by alkaline elution and is expressed as the fraction of unknown [^{14}C]DNA retained on the filter relative to the fraction of internal standard (^{3}H) retained. (●) Asynchronous cells; (■) G_1-enriched cells; (▲) G_2/M-enriched cells.

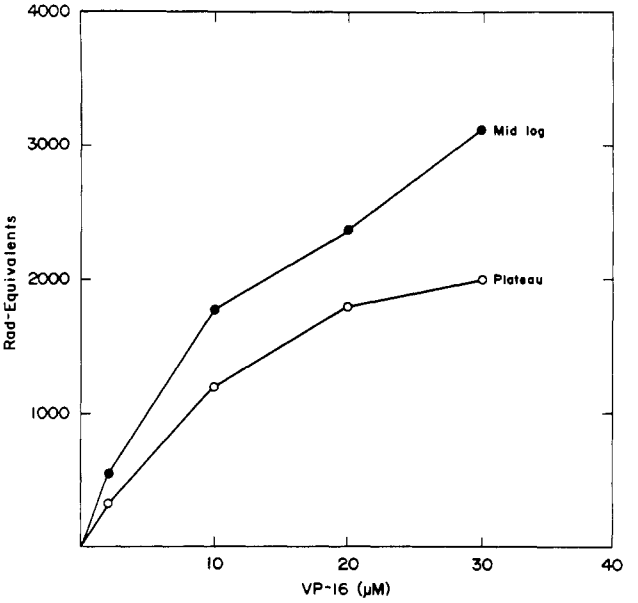


FIGURE 4: DNA single-strand break frequency induced by VP-16 in HeLa cells. Log and plateau HeLa cells were treated with VP-16 (2–30 μM) for 1 h, and DNA damage was assessed by alkaline elution. The marked reduction in VP-16 sensitivity seen in plateau-phase CHO cells (see Figure 2) was not observed in this model of malignant growth.

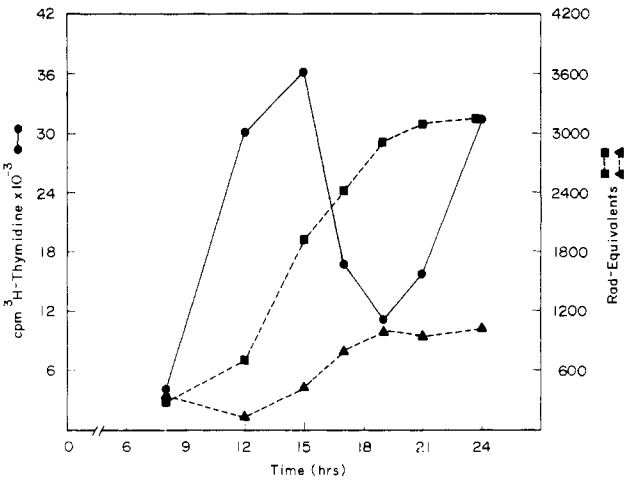


FIGURE 5: Recovery of VP-16 sensitivity and DNA synthesis by plateau-phase CHO cells. Confluent cells were trypsinized and seeded into several flasks, and [^{3}H]thymidine incorporation (●) as well as sensitivity to 10 (▲) and 50 μM (■) VP-16 was subsequently determined as described under Methods. Up to 8-h posttrypsinization, there was no appreciable DNA synthesis or drug-induced DNA damage.

G_1-like DNA content. To determine if a specific DNA content correlates with drug susceptibility, G_1- and G_2/M-enriched populations of CHO cells were isolated from asynchronous log-phase cells by velocity sedimentation on a Ficoll gradient. The enriched populations were 73% G_1 and 76% G_2/M, compared to the asynchronous cells which were 26% G_1, 32% G_2M, and 41% S. The three cell populations were then exposed to 10 and 50 μM VP-16, and DNA damage was assessed by alkaline elution (Figure 3). No difference in single-strand break frequency was observed among the three cell groups, suggesting that reduced DNA damage in plateau cells resulted from being in a true G_0-like state rather than from being trapped in a prolonged G_1 phase.

To study drug-induced DNA damage in a model more representative of malignant growth, log- and plateau-phase HeLa cells were treated with either VP-16 or *m*-AMSA, and DNA damage was assayed as before. A typical experiment, in which cells were treated with VP-16, is shown in Figure 4. Only small differences in DNA break frequency were observed between plateau- and log-phase cells; confluent cells remained sensitive to VP-16. Similar results were also observed for HeLa cells treated with *m*-AMSA and mouse leukemia L1210 cells treated with VP-16 (results not shown).

Recovery of Drug Sensitivity by Confluent Cells. Having established that plateau-phase CHO cells are markedly resistant to VP-16- and *m*-AMSA-induced DNA damage and that this phenomenon is independent of drug uptake and DNA content, experiments were next designed to investigate recovery of drug sensitivity by plateau cells. Plateau-phase cells were trypsinized and seeded into several flasks with fresh media, and the recovery of sensitivity to VP-16-induced DNA single-strand breaks as well as DNA synthesis was followed posttrypsinization (Figure 5). Up to 8-h posttrypsinization, there was no appreciable DNA synthesis or drug-induced DNA damage. Thymidine incorporation (DNA synthesis) was found to begin approximately 9-h posttrypsinization and peak at 15 h. Recovery of drug sensitvity was found to lag behind DNA synthesis and plateau at 18–24 h.

To determine if the return of DNA synthesis was a prerequisite for recovery of drug sensitivity, plateau-phase cells were trypsinized, seeded in new media, and assayed as in the previous experiment with the exception that a DNA polymerase inhibitor (aphidicolin) was added to the cells at 6-h posttrypsinization (Figure 6). In the absence of aphidicolin, thymidine incorporation was the same as in the previous experiment, whereas in the presence of 3 μg/mL aphidicolin, DNA synthesis was abolished. Recovery of VP-16 sensitivity, as measured by alkaline elution of damaged DNA, was unaffected by the presence or absence of aphidicolin. These results suggest that recovery of drug sensitivity by previously

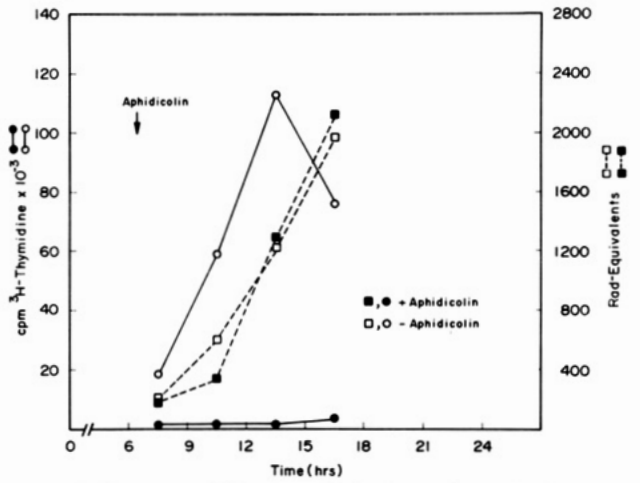


FIGURE 6: Recovery of VP-16 sensitivity by confluent CHO cells in the presence of a DNA polymerase inhibitor. Plateau cells were trypsinized and seeded into several flasks, and aphidicolin (3 μg/mL media) was added to half of the flasks 30 min before DNA synthesis and drug assays began. DNA synthesis (●, ○) was determined by trichloroacetic acid precipitation of DNA which had been exposed to [^{3}H]thymidine for 30 min. Recovery of sensitivity to 50 μM VP-16 (■, □) is expressed as rad equivalents. Time is hours posttrypsinization. This experiment demonstrated that inhibition of DNA synthesis did not affect recovery of VP-16 sensitivity.

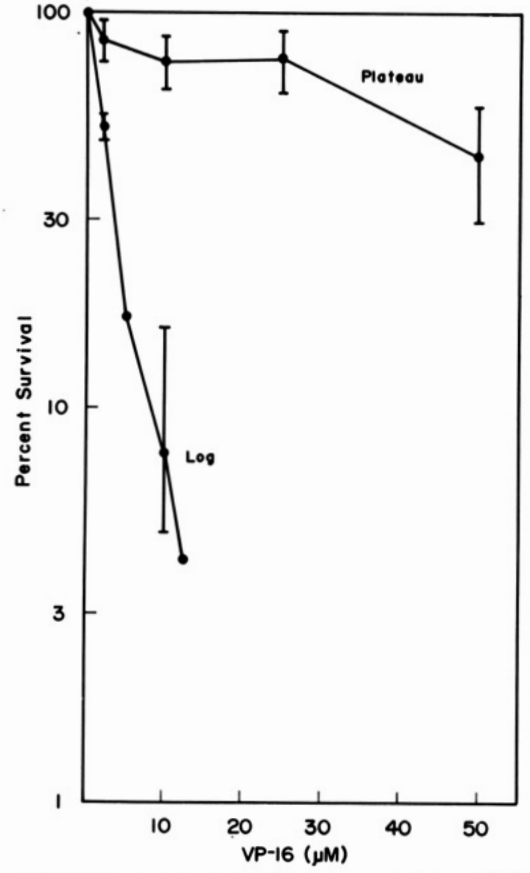


FIGURE 7: VP-16-induced cytotoxicity in log and plateau CHO cells. Log and confluent cells were incubated with VP-16 (2–50 μM) for 4 h at 37 °C, washed, and trypsinized, and colony-forming assays were done as described under Methods. Percent colony survival, relative to untreated log and plateau cell controls, was determined after 7 days growth in a CO_2 incubator.

resistant plateau-phase cells is independent of DNA synthesis.

Correlation of DNA Damage and Cytotoxicity. To examine the possible relationship between VP-16-induced DNA damage, stage of cell growth, and cell cytotoxicity, log- and plateau-phase cultures of CHO cells were treated with 2–50 μM VP-16 and colony-forming assays performed as described under Methods. This cytotoxicity assay revealed a marked difference between log and plateau cells (Figure 7). Treatment with 10 μM VP-16 for 4 h resulted in 8% and 85% survival for log- and plateau-derived colonies, respectively. A 25 μM drug treatment did not change plateau colony survival, whereas no surviving colonies from log-phase cells were found at this drug concentration. These experiments demonstrate that a relationship exists between drug-induced DNA single-strand break frequency (highest in log-phase cells) and cell cytotoxicity; drug concentration is directly related to single-strand break frequency and inversely related to percent survival.

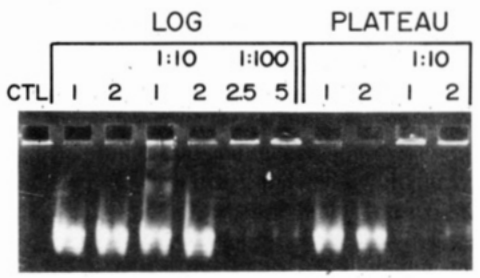


FIGURE 8: Decatenation of *Crithidia fasciculata* form I kDNA by topoisomerase II extracts from log and plateau CHO cells. Wild-type CHO cells were grown in spinner cultures and nuclear extracts of topoisomerase II obtained as described under Methods. Decatenation of kDNA networks by the enzyme was followed by agarose gel electrophoresis. Decatenated minicircles enter the gel, whereas form I substrate remains at the origin. Protein concentrations were made equivalent for log and confluent cell extracts. The second row of numbers refers to microliters of extract (where 1 μL undiluted = 0.78 μg of protein), and the top row refers to the dilution factor. CTL is control in the absence of extract.

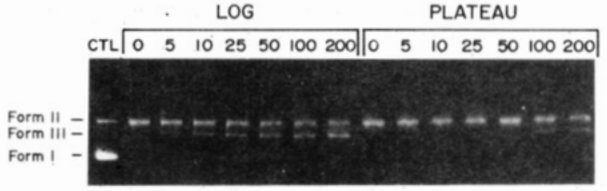


FIGURE 9: Cleavage of pBR322 DNA by topoisomerase II extracts from log and plateau CHO cells in the presence of VP-16. Topoisomerase II extracts from log and plateau cells were incubated with form I pBR322 DNA in the presence of 5–200 μM VP-16, and the formation of nicked circular (form II) and linear (form III) DNA was monitored by agarose electrophoresis. Numbers refer to micromolar VP-16. CTL is control assay in the absence of enzyme. Equivalent amounts of protein from log and plateau extracts were used; in this experiment, 7 μg of protein/enzyme assay.

Topoisomerase II Activity in Log vs. Plateau Cells. The putative mediator of VP-16- and *m*-AMSA-induced DNA damage is topoisomerase II. Having shown a significant difference between log and confluent cell growth with respect to drug-induced DNA damage and cell cytotoxicity, experiments were then carried out to examine the possible relationships of the above to topoisomerase activity.

(*A*) *Decatenation of kDNA.* Decatenation of form I kDNA to form minicircles is a reaction catalyzed specifically by topoisomerase II. Equivalent amounts of protein from crude nuclear extracts of log and plateau CHO cells were incubated with kDNA, and topoisomerase II activity was determined (Figure 8). From this experiment, it was observed that log-phase cells have approximately 4-fold greater decatenation activity when compared to plateau cells.

(*B*) *Cleavage of pBR322 DNA in the Presence of VP-16.* As in the decatenation assay, nuclear extracts from log- and

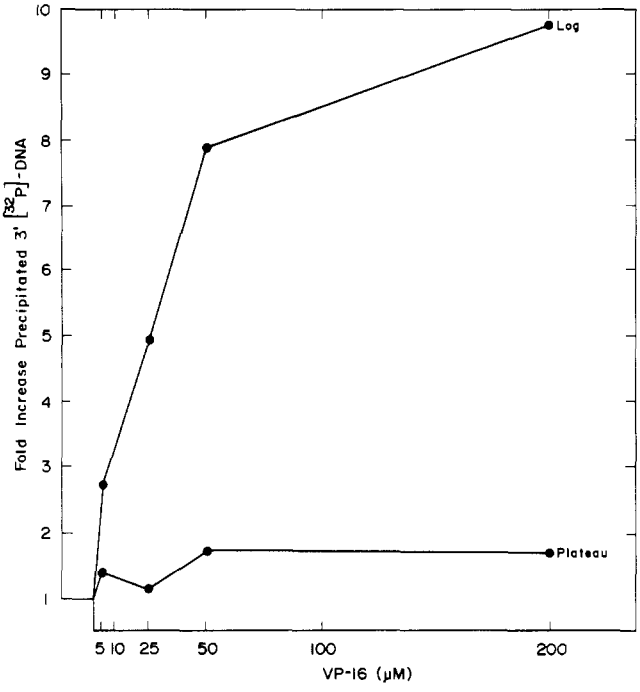


FIGURE 10: Quantitation of DNA–topoisomerase II complex induced by VP-16 in log vs. plateau CHO cells. Topoisomerase II nuclear extracts from log and plateau cells were incubated with pBR322 DNA (labeled with ^{32}P at the 3′ end) in the presence of 5–200 μM VP-16. Results are expressed as the fold increase in precipitable DNA–topoisomerase II complex over control (no VP-16). Equal amounts of protein from log and confluent nuclear extracts were used; in this experiment, 7 μg/enzyme assay.

plateau-phase cells were incubated with DNA substrate and the reaction products determined by agarose gel electrophoresis. In the present assay, however, increasing doses of VP-16 (5–200 μM) were included in the assay mixture (Figure 9). In the absence of VP-16, form I pBR322 DNA was converted totally to form II by both log and plateau nuclear extracts. When drug was included in the reaction mixture, the appearance of form III DNA became evident. The amount of linear DNA increased with increasing concentrations of VP-16 and appeared at much lower concentrations when log-phase nuclear extract was used. That is, in log cells form III is first seen at 5 μM VP-16 and in plateau cells at approximately 100 μM drug. These experiments showed that log-phase CHO cells were approximately 20-fold more sensitive to the topoisomerase-mediated effects of VP-16.

(*C*) *Precipitation of Topoisomerase II–DNA Complexes.* Liu et al. (1983) have shown that topoisomerase II binds covalently via a phosphotyrosyl bond to the 5′ end of DNA. This complex can be isolated by denaturation with SDS. In the following experiments, pBR322 DNA was labeled at the 3′ end with ^{32}P and incubated with topoisomerase extracts from log- and plateau-phase cells in the presence of VP-16, and the DNA–enzyme complex was "frozen" with SDS and finally precipitated with KCl. In the absence of VP-16, little cleavage was observed, and there was no quantitative difference between log and plateau cells (Figure 10). The amount of DNA–enzyme complex was stimulated by addition of VP-16 to the reaction mixture; complex formation in log-phase CHO cells was stimulated 3–7-fold over plateau stimulation. These results demonstrate that significantly more DNA–topoisomerase II complex is generated from log-phase cells treated with VP-16 than from confluent cells similarly treated. These experiments also show that the cleavage of pBR322 DNA observed in the previous experiments was topoisomerase II mediated because it is both protein linked and polar with respect to DNA.

Discussion

The results of these investigations indicate that log- and plateau-phase CHO cells differ markedly in their susceptibility to drug-induced DNA damage and cytotoxicity; the basis for this is most likely an alteration in topoisomerase II activity. DNA strand breaks, induced by both an intercalator (*m*-AMSA) and a nonintercalator (VP-16), decrease in frequency as cells progress to quiescence. Cells in plateau phase are essentially resistant to VP-16 and *m*-AMSA. This difference in drug sensitivity between rapidly dividing and quiescent cells was not found to result from changes in viability (plateau cells remained >85% viable) nor was it a function of differences in drug uptake (tritiated VP-16 was taken up equally by log and plateau cells). Experiments with G_1- and G_2/M-enriched populations of CHO cells demonstrated similar drug sensitivity as asynchronous cells, suggesting that quiescent cells are drug resistant because of changes in topoisomerase activity associated with entering a true G_0 state. Quiescence, as defined in our study, is probably due to serum starvation and not confluency for two reasons. First, decreased topoisomerase II activity was also seen in suspension cultures at plateau phase, and second, feeding cells allows for further cell division (Nelson, J. M., et al., 1984).

Studies with HeLa and mouse leukemia L1210 cell lines did not demonstrate the dramatic proliferation dependence in drug sensitivity seen with CHO cells. These cell lines retained VP-16 and *m*-AMSA sensitivity in the plateau phase. This difference in drug sensitivity between CHO and HeLa/L1210 cell lines probably resides in failure of the latter to enter G_0 and may provide part of the basis for therapeutic selectivity since many normal tissues exist in a differentiated G_0-like state. Furthermore, the probability that topoisomerase activity may be regulated differently along a spectrum of neoplastic growth suggests the possibility of selectively manipulating the enzyme in such a way as to improve the therapeutic index.

To determine when plateau-phase CHO cells regained drug sensitivity and to see if this was coordinately regulated with DNA synthesis, confluent cells were released into fresh media, and DNA synthesis and VP-16-induced DNA damage were followed for 24 h. DNA synthesis peaked at approximately 15 h and was followed by return of drug sensitivity, which reached a maximum approximately 20 h after cells were reseeded. An analogous situation has been reported by Duguet et al. (1983), who followed topoisomerase I and topoisomerase II activity in rat liver nuclei during the process of regeneration following partial hepatectomy. They found that when quiescent cells (prehepatectomy liver) were allowed to resume loglike growth (regenerating liver), there were marked increases in topoisomerase II activity with peak activity occurring 40-h posthepatectomy. This same lab (Taudou et al., 1984) has also shown considerable increases in topoisomerase II activity in nuclei from concanavalin A stimulated lymphocytes. In both cases, the increased activity was paralleled by increased activity of DNA polymerase α. Miskimins et al. (1983) stimulated quiescent fibroblasts with epidermal growth factor and found that topoisomerase II activity increased first in the cytoplasm and later in the nucleus. The above observations are in agreement with our data that topoisomerase II activity is diminished in quiescent cells. At odds with these results are the experiments of Tricoli et al. (1985) which failed to demonstrate any significant differences in topoisomerase II activity between log/plateau and between G_1/S/M stages of mouse embryo fibroblasts. The basis for these discrepant results is unclear, but it is of note that Tricoli's study employed whole

cell lysates rather than distinguishing between nuclear and cytoplasmic activity. Indeed, a particular strength of our alkaline elution experiments is that they assay topoisomerase II activity in situ rather than in an extract containing other proteins which might affect enzyme activity in vitro.

In summary, our studies and those of others suggest that following stimulation of quiescent cells there is an increase in nuclear topoisomerase II activity which is not evident until the conclusion of the initial G_1 phase. Once this has occurred, however, enzyme activity remains generally elevated during subsequent cell cycle phases.

Noguchi et al. (1983) have suggested that the multienzyme "replitase" complex in mammalian cells, of which topoisomerase II is a component (Nagata et al., 1983; Jazwinski & Edelman, 1984), is coordinately regulated in such a fashion that inhibition of one enzyme may affect the activity of others in the complex. In our experiments, inhibition of DNA polymerase α with aphidicolin did not affect topoisomerase II activity as might be predicted by a coordinately regulated model. It is important to note, however, that enzyme activity in these experiments is defined by drug-induced DNA cleavage. The possibility that this does not reflect every aspect of topoisomerase II function cannot be excluded.

We have measured topoisomerase II activity in log and plateau CHO cells by three different methods in the absence and presence of VP-16. Decatenation of kDNA in the absence of antitumor drug revealed approximately a 4-fold difference between log and plateau cells. Cleavage of pBR322 DNA and precipitation of the topoisomerase II–DNA complex, both in the presence of VP-16, demonstrated a 20-fold and 6-fold difference, respectively. Log-phase cells always demonstrated more enzyme activity, and this activity was stimulated by VP-16 severalfold more than confluent cells. Neither our data nor those of others indicate whether alterations in enzyme activity associated with proliferation are the result of qualitative or quantitative changes in topoisomerase II. It is known that this enzyme can serve as a substrate for tyrosine kinases in vitro (Tse-Dinh et al., 1984; Ackerman et al., 1985), but whether this occurs in vivo and what consequences would result from such posttranslational modifications are unknown. Alternatively, intracellular topoisomerase II content may decrease during quiescence, resulting in the need for new enzyme synthesis prior to DNA synthesis. A final possibility is that another intracellular factor which varies with proliferative status may alter the presumed interaction between drug and topoisomerase. Further studies are required to resolve these issues.

A positive correlation between strand break frequency and cytotoxicity has been observed for log-phase L1210 cells treated with VP-16 (Wozniak & Ross, 1983) and lung adenocarcinoma cells treated with VP-16 and VM-26 (Long et al., 1984). Furthermore, inhibition of topoisomerase-mediated DNA cleavage appears to block cytotoxicity (Wozniak et al., 1984; Rowe et al., 1985). In this paper, we demonstrate for the first time that changes in topoisomerase II activity which accompany perturbations of cell growth may also affect drug sensitivity. These data reiterate the unique role that topoisomerase II occupies as a cancer chemotherapy target. It is perhaps the only such target known whose activity varies directly with drug sensitivity, i.e., the more enzyme that is available, the greater the drug effect. As a consequence, efforts to understand the regulation of enzyme activity are of critical importance in fully exploiting the enzyme therapeutically.

ACKNOWLEDGMENTS

We appreciate the preparation of the typescript by Angelique Fair.

Registry No. *m*-AMSA, 51264-14-3; VP-16, 4375-07-9; topoisomerase, 80449-01-0.

REFERENCES

Ackerman, P., Glover, C. V., & Osheroff, N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3164–3168.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 13560–13566.

Clewell, D. B. (1972) *J. Bacteriol. 110*, 667–676.

Duguet, M., Lavenot, C., Harper, F., Mirambeau, G., & DeRecondo, A.-M. (1983) *Nucleic Acids Res. 11*, 1059–1075.

Englund, P. T. (1978) *Cell (Cambridge, Mass.) 14*, 157–168.

Gellert, M. (1981) *Annu. Rev. Biochem. 50*, 879–910.

Jazwinski, S. M., & Edelman, G. M. (1984) *J. Biol. Chem. 259*, 6852–6857.

Kohn, K. W. (1979) *Methods Cancer Res. 16*, 291–345.

Kohn, K. W., Erickson, L. C., Ewig, R. A. G., & Friedman, C. A. (1976) *Biochemistry 15*, 4629–4637.

Kohn, K. W., Ewig, R. A. G., Erickson, L. C., & Zwelling, L. A. (1981) in *DNA Repair: A Laboratory Manual of Research Techniques* (Friedberg, E. C., & Hanawalt, P. C., Eds.) pp 379–401, Marcel Dekker, New York.

Liu, L. F. (1983) *CRC Crit. Rev. Biochem. 15*, 1–24.

Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., & Chen, G. L. (1983) *J. Biol. Chem. 258*, 15365–15370.

Loike, J. D., & Horwitz, S. B. (1976a) *Biochemistry 15*, 5435–5443.

Loike, J. D., & Horwitz, S. B. (1976b) *Biochemistry 15*, 5443–5448.

Long, B. H., Musial, S. T., & Brattain, M. G. (1984) *Biochemistry 23*, 1183–1188.

Marini, J. C., Miller, K. G., & Englund, P. T. (1980) *J. Biol. Chem. 255*, 4976–4979.

Miller, K. G., Liu, L. F., & Englund, P. T. (1981) *J. Biol. Chem. 256*, 9334–9339.

Miskimins, R., Miskimins, W. K., Bernstein, H., & Shimizu, N. (1983) *Exp. Cell Res. 146*, 53–62.

Nagata, K., Guggenheimer, R. A., & Hurwitz, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4266–4270.

Nelson, E. M., Tewey, K. M., & Liu, L. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1361–1365.

Nelson, J. M., Todd, P., & Metting, N. F. (1984) *Cell Tissue Kinet. 17*, 411–425.

Noguchi, H., Reddy, G. P. V., & Pardee, A. B. (1983) *Cell (Cambridge, Mass.) 32*, 443–451.

Ross, W. E., & Bradley, M. O. (1981) *Biochim. Biophys. Acta 654*, 129–134.

Ross, W. E., Glaubiger, D. L., & Kohn, K. W. (1978) *Biochim. Biophys. Acta 519*, 23–30.

Ross, W. E., Glaubiger, D. L., & Kohn, K. W. (1979) *Biochim. Biophys. Acta 562*, 41–50.

Rowe, T. C., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 9177–9181.

Rowe, T. C., Kupfer, G., & Ross, W. (1985) *Biochem. Pharmacol. 34*, 2483–2487.

Simpson, A. M., & Simpson, L. (1974) *J. Protozool. 21*, 774–781.

Sullivan, D. M., Smallwood, S., Hodges, P., & Ross, W. E. (1985) *Am. Fed. Clin. Res. 33*, 459A.

Taudou, G., Mirambeau, G., Lavenot, C., Garabedian, A., Vermeersch, J., & Duguet, M. (1984) *FEBS Lett. 176*, 431–435.

Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984a) *J. Biol. Chem. 259*, 9182–9187.
Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984b) *Science (Washington, D.C.) 226*, 466–468.
Tricoli, J. V., Sahai, B. M., McCormick, P. J., Jarlinski, S. J., Bertram, J. S., & Kowalski, D. (1985) *Exp. Cell Res. 158*, 1–14.
Tse-Dinh, Y.-C., Wong, T. W., & Goldberg, A. R. (1984) *Nature (London) 312*, 785–786.
Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665–697.
Wozniak, A. J., & Ross, W. E. (1983) *Cancer Res. 43*, 120–124.
Wozniak, A. J., Glisson, B. S., Hande, K. R., & Ross, W. E. (1984) *Cancer Res. 44*, 626–632.

Substrate Specificity of Formylglycinamidine Synthetase†

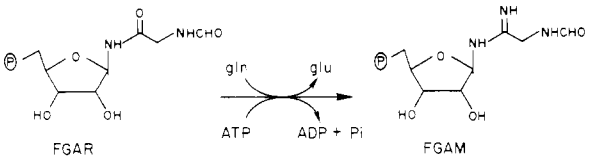
F. J. Schendel and J. Stubbe*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received September 25, 1985; Revised Manuscript Received December 2, 1985

ABSTRACT: Formylglycinamidine ribonucleotide (FGAM) synthetase, which catalyzes the conversion of formylglycinamide ribonucleotide (FGAR), glutamine, and ATP to FGAM, ADP, glutamate, and P_i, has been purified to homogeneity (sp act. 0.20 μmol min^{-1} mg^{-1}) from chicken liver by an alternative procedure to that of Buchanan et al. [Buchanan, J. M., Ohnoki, S., & Hong, B. S. (1978) *Methods Enzymol. 51*, 193–201] (sp act. 0.12 μmol min^{-1} mg^{-1}). A variety of new analogues of formylglycinamide ribonucleotide have been prepared in which the formylglycinamide arm (R = CH_2NHCHO) has been replaced by R = CH_3, CH_2OH, CH_2Cl, CH_2NH_3, $CH_2NHCOCH_3$, $CH_2NHCOCH_2Cl$, $CH_2NHCO_2CH_2Ph$, and L-$CHCH_3NHCHO$. These compounds have been characterized by ^1H and ^{13}C NMR spectroscopy. With compounds R = CH_3, CH_2OH, and $CH_2NHCOCH_3$ and ATP, in the presence or absence of glutamine, FGAM synthetase catalyzes the production of P_i at 4.5, 48, and 20%, respectively, the rate of production of P_i from formylglycinamide ribonucleotide. Only R = $CH_2NHCOCH_3$ causes glutaminase activity as well as ATPase activity and has been shown to be converted to the amidine analogue. Both FGAR (R = CH_2NHCHO) and the FGAR analogue (R = $CH_2NHCHOCH_3$) in the presence of ATP and FGAM synthetase and in the absence of glutamine form a complex isolable by Sephadex G-50 chromatography. FGAM synthetase is thus highly specific for its formylglycine side chain. [^{18}O]-β-FGAR was prepared biosynthetically, and FGAM synthetase was shown by ^{31}P NMR spectroscopy to catalyze the transfer of amide ^{18}O to inorganic phosphate.

Formylglycinamidine ribonucleotide (FGAM)[1] synthetase catalyzes the fourth step in the purine biosynthetic pathway conversion of formylglycinamide ribonucleotide (FGAR), glutamine, and ATP to FGAM, P_i, and ADP (eq 1). This



(1)

enzyme has been previously purified 300-fold to homogeneity from chicken liver and found to consist of a single polypeptide chain of M_r 133 000 (Mizobuchi & Buchanan, 1968a; Buchanan et al., 1978). Many detailed mechanistic studies have been undertaken by Buchanan and co-workers and have been recently reviewed (Buchanan, 1982). FGAM synthetase has also been purified 56-fold from Erlich ascites tumor cells by Chu and Henderson (1972). Mechanistic studies on this partially purified protein indicate considerable differences between it and the liver enzyme.

Elegant studies by Buchanan and co-workers have elucidated the role of glutamine in this enzyme-catalyzed amidine production. Their studies with [^{14}C]glutamine indicated that in the absence of other substrates the enzyme forms a 1:1 complex with glutamine ($t_{1/2}$ = 125 min). Futher studies indicate that a covalent thio ester of glutamine is formed enzymatically with liberation of "NH_3". This "NH_3" is the putative nucleophile in the FGAR → FGAM conversion and remains associated with a specific locus in the active site. Hydrolysis of the enzyme glutamyl moiety in the absence of other substrates is 0.5% the rate of turnover for the overall reaction (Mizobuchi & Buchanan, 1968b).

Intriguingly, a stable complex is also formed between ATP, FGAR, and enzyme with a stoichiometry of 0.7 mol of substrate/mol of enzyme in the absence of glutamine ($t_{1/2}$ = 62 min at 0 °C). Furthermore, there is evidence that the terminal phosphoanhydride bond of ATP is cleaved to ADP and a phosphate moiety (perhaps E∼PO_3) during this complex formation (Mizobuchi et al., 1968).

† This research was supported by Grant 32191 from the U.S. Public Health Service. J.S. is the recipient of an R. H. Romnes award from the University of Wisconsin and an NIH Research Career development award (AM 01222).

* Author to whom correspondence should be addressed.

[1] Abbreviations: FGAM, formylglycinamidine ribonucleotide; FGAR, formylglycinamide ribonucleotide; GAR, glycinamide ribonucleotide; AIR, aminoimidazole ribonucleotide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PRPP, 5-phosphoribosyl pyrophosphate; PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FID, free induction decay; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/86/0425-2256$01.50/0