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CELLULAR TARGETS OF ADRIAMYCIN-INDUCED DAMAGE IN *ESCHERICHIA COLI*

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Abstract—The cellular targets of adriamycin (ADR) activity were studied in *Escherichia coli* by following colony forming ability and various cellular functions. The parameter exhibiting the best correlation with mortality was inhibition of RNA synthesis. Total DNA synthesis was inhibited to a lesser extent, but may reflect a concurrent inhibition of replication and stimulation of DNA repair activity. Protein synthesis, membrane function and rate of oxygen consumption were affected later. No extensive DNA fragmentation was observed. The inhibition of RNA synthesis was independent of the stringent response and of inhibition of DNA synthesis induced by nalidixic acid. ADR activated the SOS repair system, and the lesions induced by the drug could be repaired by *recA* dependent functions. These results indicate that the primary activity of ADR was directed against the DNA and interfered with the DNA template function.

The antitumour drug, adriamycin (ADR),* has been extensively studied since its discovery was reported in 1969 [1]. ADR is in wide use in cancer chemotherapy but severe limitations on its use are imposed by the cardiotoxicity produced by the drug. On the cellular and molecular levels, the activities of ADR are extremely versatile, spanning a number of subcellular systems and several modes of interaction.

ADR has a high affinity for DNA [2–5] and the DNA–ADR interactions have been reported to result in DNA strand breaks [2, 3, 6–8], protein–DNA cross links [2, 3, 8], increased sister chromatid exchange [9], chromosome abberations [10], inhibition of DNA replication, transcription [2, 3, 5, 11–16] and repair [14, 17], possibly by interference with the template function of DNA [3, 5, 11–13]. Protein synthesis has been reported to be inhibited as well [13, 18].

The drug associates strongly with membranes [2, 3, 11, 19, 20] and causes a variety of disturbances of membrane structure and function, including lipid peroxidation [2, 3, 21–23], altered permeability to cations [2, 3, 12, 24, 25] and increased membrane fluidity [26]. Furthermore, ADR may interfere with mitochondrial electron transport [23, 27, 28].

Involvement of free radicals has been suggested in the mechanism of action of ADR. The drug may be reduced enzymatically in the mitochondria [27, 29, 30], in the endoplasmic reticulum [2, 6, 12, 21, 30, 31] or in the nuclei [6, 32], and subsequently oxidized, leading to the formation of superoxide radicals, hydrogen peroxide [2, 11, 12, 29] and hydroxyl radicals, particularly when iron is present [11, 33, 34]. It has been suggested that oxygen derived free radicals participate in the mechanisms of several of the above mentioned cellular and biochemical effects, contributing to both cardiotoxicity and antitumoral activity [2, 11, 21, 22, 35].

While the wide variety of ADR-induced effects on the cellular components and functions has suggested numerous possible sites for the cytotoxic action of the drug, the actual cellular target(s), at which the interaction with ADR results in the antitumoral or cardiotoxic effects, has not yet been identified. Moreover, it is not known which type of interaction between ADR and the primary cellular target causes the lethal effect and which interactions are of secondary importance.

With a view to clarifying the mechanism of action of ADR, a comparative investigation of the cellular targets of ADR activity was undertaken. In the present study, *E. coli* bacterial cells were used as a model system to discriminate between the various subcellular targets of ADR, and to determine which effects resulted in cytotoxicity and which were of secondary importance in this context.

MATERIALS AND METHODS

Chemicals and solutions. Adriamycin, supplied as a 1:5 (w/w) mixture with lactose, was produced by Farmitalia Carlo Erba, Italy. Stock solutions of 1×10^{-3}–4×10^{-3} M were prepared in triple distilled water and kept in the dark at 4°. ^{14}C-uridine, ^{14}C-thymidine (both approx. 50 Ci/mole) and ^{14}C-leucine (approx. 350 Ci/mole) were obtained from Amersham, U.K. Firefly lantern extract (FLE-50), leucine, HEPES, proteinase K (EC 3.4.21.14), pancreatic RNase (ribonucleate 3′-pyrimidinooligonucleotidohydrolase, EC 3.1.27.5), lysozyme (mucopeptide *N*-acetylmuramoyl hydrolase, EC 3.2.1.17), agarose, SDS, ATP, CM and nalidixic acid were from Sigma. Lambda DNA Hind III digest was

* Abbreviations used: ADR, adriamycin; CM, chloramphenicol; SSB, DNA single strand breaks; DSB, DNA double strand breaks; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; AP, apurinic and apyrimidinic (sites); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid.

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from Biolabs, U.S.A., uridine and thymidine were from Calbiochem, TCA was from Merck, Tris was from Serva and sodium arsenate was from Mallinckrodt. All chemicals used were analytical grade. Triple distilled water was used throughout. TE buffer, pH 7.6, contained 10 mM Tris–HCl and 1 mM EDTA. TEN buffer, pH 7.6, was a TE buffer supplemented with 0.1 M NaCl. TAE buffer, pH 8.0, contained 0.04 M Tris, 0.02 M acetic acid and 2 mM EDTA. Dilution buffer was composed of 1 mM phosphate buffer, pH 7.4, 1 mM $MgSO_4$ and 0.05% (w/v) gelatin. Scintillation cocktail contained 3 g/l PPO and 0.1 g/l dimethyl-POPOP, dissolved in toluene.

Bacterial strains and culture conditions. *E. coli* B SR 9 was used for most experiments. *E. coli* K 12 mutant strains used were Hfr KL 16, 9101 (*xthA*, derived from KL 16), KL 16 *recA*, a *relA phe* strain and its parent *phe* strain. All strains were grown in Davis mineral medium. *E. coli* K 12 strains were supplemented with thiamine (10 mg/l), and the media for the *phe* strains also contained phenylalanine (20 mg/l). Growth media contained 1% glycerol or 0.5% glucose as the carbon/energy source while all reaction media contained 0.5% glucose. Cultures were grown for at least two generations, to approx. 10^9 cells/ml, which was determined to be late logarithmic phase. Cells were suspended in the reaction media at 1–2 × 10^8 cells/ml (unless otherwise stated) and preincubated for 20–30 min before addition of the drug.

Survival. At various time intervals after drug addition, samples were taken and diluted serially in gelatin-containing dilution buffer (1 mM phosphate, pH 7.4, 1 mM $MgSO_4$, 0.05% w/v gelatin). The first dilution was 1:100, but even a 1:10 dilution was found to stop any further ADR-induced mortality (defined as loss of colony forming ability). In contrast, a 10–100-fold dilution in gelatin-free buffer slowed, but did not stop, the killing process. Viable cell number (mean of quadruplicate seedings) was determined by a standard clonogenic assay on TB (tryptone broth) or LB (L broth, Difco) agar plates, incubated overnight at 37°.

Cellular ATP content. Samples (approx. 2 × 10^7 cells) were added to preheated test tubes containing water, and kept in a boiling water bath for 5 min. The test tubes were allowed to cool to room temperature and 1 ml of the sample was added to a clear glass scintillation vial, containing 1 ml filtered sodium arsenate buffer, 0.1 M, pH 7.4 with 40 mM $MgSO_4$, and 1 ml phosphate buffer, 10 mM, pH 7.4 with 4 mM $MgSO_4$. Fifty microlitres of firefly lantern extract reagent was added and bioluminescence was determined immediately in the ^3H channel of a Packard Tri-carb liquid scintillation spectrometer, with the coincidence circuit disconnected. The results used were the average of the first two successive 6 sec determinations. Standard ATP curves were constructed and used to convert values of bioluminescence to ATP concentration (adapted from [36]).

Active transport of leucine. The initial rate of transmembrane transport was determined by incubating cells (1–2 × 10^8 cells/ml) with ^{14}C-leucine in the presence of CM (0.1 mg/ml). One millilitre samples were transferred to test tubes containing 0.1 mg CM and 4 × 10^{-6} M (final concentration) ^{14}C-leucine (5.7 Ci/mole), and incubated for 20 sec at 37°. The cells were collected on Schleicher and Schull membrane filters, and washed twice with 2 ml HEPES buffer, (5 mM, pH 7.4) preheated to 37° [37].

Steady state intracellular leucine pool. The steady state intracellular leucine pool was determined as described for leucine transport, except that cell samples were labelled for 4 min. At this time cell leucine content was found to have reached a steady state, and did not increase with prolonged incubation.

Oxygen consumption. O_2 consumption studies were performed essentially as described by Gianni *et al.* [38], using a Clark-type electrode on a Yellow Springs oxygraph, equipped with a water jacketed cell for temperature control. The solutions were equilibrated with air at 37° and assumed to contain the same concentration of dissolved O_2 as water (207 μM at 37°). Between experiments the oxygraph cell was washed with HCl (1 M), followed by at least three water rinses. Approx. 2 × 10^8 cells/ml were exposed to ADR and samples were diluted fivefold in dilution buffer, directly in the measuring cell. The oxygen concentrations measured were recorded and the rates of O_2 consumption were calculated.

DNA breakage. DNA single and double strand breaks were detected by agarose gel electrophoresis, slightly modified from [39]. One millilitre cells (4 × 10^8 cells/ml from a logarithmic growing culture) were diluted 10-fold in dilution buffer, sedimented and suspended in 1 ml TE buffer, pH 7.6. For single strand DNA extraction 0.5 ml of a solution containing 2% phenol, 70% ethanol, 20 mM sodium acetate, pH 5.3 and 2 mM EDTA was added to the cells. The suspension was frozen in liquid nitrogen (5 min) and sedimented in a table top type centrifuge (10 min, 15,000 rpm). The pellet was dried, dissolved in 50 μl 0.2 M NaOH, with 10 mM EDTA and incubated for 1 hr at 37°. The solution was spun down (as above) to remove the debris, and the supernatant fluid containing the DNA was loaded onto 0.6% agarose gels. Two size markers were included: (i) chromosomal DNA from *E. coli* treated with nalidixic acid (50 μg/ml, 25 min) and extracted as above, with the addition of 0.75% SDS, yielding DNA fragments of approx. 100 kilobases (45–50 DSB per chromosome) [40]; (ii) commercial Lambda-DNA Hind III digest (largest fragment 23 kilobases). For double strand DNA extraction, spheroplasts were formed by addition of lysozyme (200 μg/ml) and kept on ice for 30 min. Cells were lysed by incubation with 0.1% SDS and 50 μg/ml proteinase K (30 min, 37°). The lysate was extracted three times with $CHCl_3$: isoamylalcohol (24:1), adjusted to 0.3 M NaCl and the nucleic acids were precipitated by the addition of 2 vol. of ethanol. After overnight holding at −20° the precipitate containing the purified nucleic acids was collected by centrifugation and dissolved in 0.2 ml TEN buffer (pH 7.6). Ten micrograms pancreatic RNase was added and the mixture was incubated for 1 hr at 37°. The DNA was applied to 0.8% neutral agarose gels containing ethidium bromide (0.5 μg/ml). Electrophoresis was carried out at room temperature in NaOH (30 mM)–EDTA (2 mM) or

TAE buffer (pH 8.0) for single and double strand DNA respectively. Alkaline gels were run at 2 V/cm for 16 hr and neutral gels were run at 2 V/cm for 5 hr. Alkaline gels were neutralized and subsequently stained with ethidium bromide (1 μg/ml). Both alkaline and neutral gels were washed in water for several hours to remove excess ethidium bromide, and photographed under ultraviolet illumination, using polaroid type 667 film. DNA-breaks were quantitated as described by Kohen *et al.* [39].

Biosynthesis of macromolecules. To detect changes in the rate of cellular macromolecule synthesis, cells ($1–2 \times 10^8$/ml) were pulse labelled with radioactive precursors, after various time intervals of exposure to ADR. DNA, RNA and protein synthesis were monitored by separately measuring the incorporation of ^{14}C-thymidine, ^{14}C-uridine and ^{14}C-leucine, respectively. One-millilitre samples of the culture were transferred to each of three test tubes containing either 1×10^{-8} mole ^{14}C-thymidine (5 Ci/mole), 1×10^{-8} mole ^{14}C-uridine (5 Ci/mole) or 4×10^{-9} mole ^{14}C-leucine (5.7 Ci/mole) at 37° and incubated for 2 min. Ten millilitre of cold 5% TCA were added and the samples were kept on ice for at least 10 min before collecting the cold TCA precipitable material on membrane filters. The filters were washed twice with 10 ml cold 5% TCA.

Assay of radioactivity. The filters, on which radioactive material had been collected, were placed in scintillation vials, oven dried, covered with scintillation cocktail and counted in a Kontron Betamatic liquid scintillation counter.

Filamentation. Samples of ADR-treated cells (10^8 cells/ml) were diluted 1:10 in cold dilution buffer, sedimented and suspended in ADR free growth medium. The cells were incubated 2 hr at 37°, sampled on microscope slides, dried, heat fixed and stained with fuchsin. The slides were examined in a light microscope at ×1000 magnification, and filamentation was determined qualitatively by comparison to untreated samples.

Data presentation. Throughout this work, typical results are presented; similar results were obtained in 3–6 repeats of each experiment.

RESULTS

Experiments were performed with *E. coli* cells either in Davis growth medium or in buffer, both with added glucose (as described). Because of the possibility that iron ions may participate in ADR activity, no iron was added and reagents used were essentially iron free, but chelex-cleaning of the solutions was not performed. Cells were exposed to ADR, and cell survival, membrane damage, DNA breaks, biosynthetic activity and O_2 consumption were measured. The results were evaluated for a possible causative relationship between the damage to any of the observed systems and cell death.

E. coli B cells were killed by ADR at concentrations as low as 10^{-6} M–10^{-5} M, whereas mortality of most *E. coli* K 12 strains occurred only when 10^{-4} M–10^{-3} M ADR was provided (data not shown). Growing cells (in Davis minimal medium) were 5–6 times as sensitive to ADR as resting cells (in glucose supplemented HEPES buffer or in nitrogen deficient Davis minimal medium, data not shown). Exposure to ADR resulted in 30–50% lower mortality rates in the absence of an energy source than when an energy source was provided in the same medium (data not shown).

Membrane damage

Damage to the cell membrane was measured in terms of intracellular ATP content, active leucine transport (uptake) and steady state intracellular leucine concentration, each of which would reflect certain changes in membrane function or integrity.

ATP balance. Membrane damage, with resulting increased permeability to protons and metal cations, would change the cross membrane distribution of these ions. Consequently, ATP would be consumed by membranal pumps, such as proton pumps and the Ca^{2+}/K^+ ATPase. *E. coli* cells were exposed to ADR and endogenous ATP was measured by monitoring bioluminescence of the luciferin–luciferase reaction. Results were calculated as percent of the respective initial values, measured before addition of ADR. The initial ATP content in the cells used was 4.5×10^{-6} mole/g dry weight, which was comparable to levels reported by Cole *et al.* [36]. Cells were preincubated 20–30 min in the reaction media, before the experiments commenced, in order to restore normal ATP levels affected by the washing at 4° [36].

Cells were treated with various ADR concentrations and in different reaction media. Changes in survival and endogenous ATP levels as a function

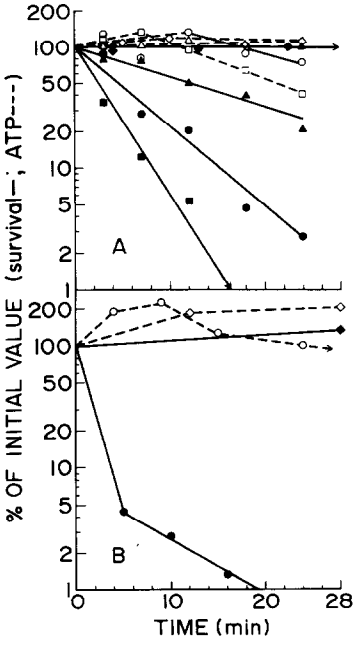

Fig. 1. Effect of ADR on bacterial survival and ATP content. *E. coli* cells were exposed to various ADR concentrations: 10^{-4} M ADR (squares); 2×10^{-5} M ADR (circles); 1×10^{-5} M ADR (triangles) and no ADR (rhombes), in 5 mM HEPES buffer (A) and Davis growth medium (B). The residual ATP content (open symbols) and surviving cell fraction (solid symbols) are presented as a function of exposure time to ADR.

of incubation time with the different ADR concentrations in HEPES buffer and in Davis growth medium are presented in Fig. 1A and 1B, respectively. When cells were exposed to 1×10^{-4} M or 2×10^{-5} M ADR, ATP levels decreased as compared to control values. This decrease was slight and much delayed compared to mortality. When 1×10^{-5} M ADR was given, no decrease in endogenous ATP levels was recorded, although mortality was still evident.

Active transport of leucine. Active transport across the cellular membrane is known to be highly sensitive to membrane damage [41]. Thus uptake of leucine was used as another marker for damage to membrane function. Cells were incubated with ADR and samples were taken at various time intervals. The samples were pulse labelled with ^{14}C-leucine in the presence of CM (0.1 mg/ml), and leucine uptake was determined as described in Materials and Methods. In the presence of CM, residual protein synthesis accounted for less than 10% of the leucine uptake measured. Cellular leucine accumulation presented saturation kinetics, reaching a plateau after 120–180 sec. The steady state intracellular concentrations were 40–200 times the extracellular concentration in the range 2×10^{5} M–4×10^{-6} M (data not shown).

Figure 2 presents the change in viability and in ^{14}C-leucine uptake in growing cells, as a function of incubation time with 1×10^{-5} M ADR. It is clearly seen that cell death precedes membrane damage as evidenced by ^{14}C-leucine uptake. Neither the initial rate of leucine uptake nor the ability to maintain a concentration gradient between the intracellular compartment and the medium was impaired by 1×10^{-5} M ADR until the majority of the cells had lost their viability. A moderate effect on leucine uptake was noted with higher concentrations of ADR, but at all ADR concentrations tested membrane damage clearly lagged behind mortality, both with growing and with resting cells (data not shown).

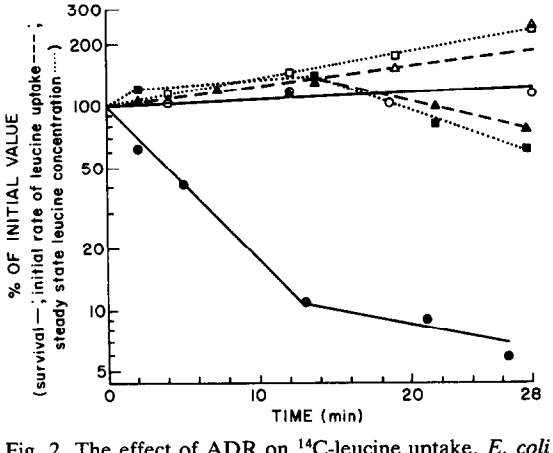

Fig. 2. The effect of ADR on ^{14}C-leucine uptake. *E. coli* cells were exposed to 1×10^{-5} M ADR in Davis growth medium and samples were pulse labelled with ^{14}C-leucine in the presence of CM (0.1 mg/ml). The surviving fraction (circles, solid lines), the initial rate of ^{14}C-leucine uptake (triangles, dashed lines) and the steady state intracellular leucine concentrations (squares, dotted lines) are presented as a function of exposure time to ADR. Solid symbols—1×10^{-5} M ADR; open symbols—no ADR.

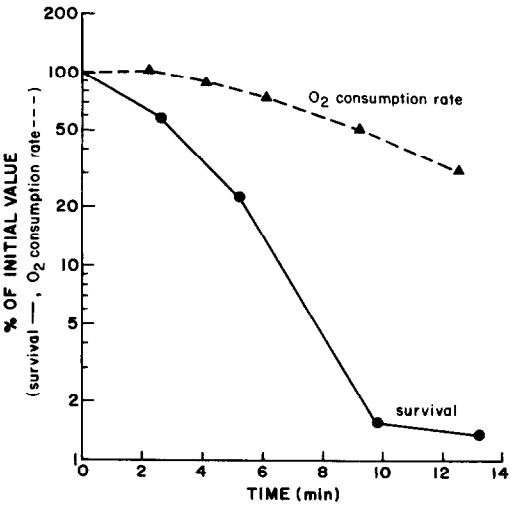

Fig. 3. The effect of ADR on *E. coli* oxygen consumption. *E. coli* cells were exposed to 4×10^{-5} M ADR in Davis growth medium. Samples were diluted directly into an oxygen measuring cell containing 4 volumes of dilution buffer. The oxygen concentration was recorded for 15–25 min using a Clark electrode, and rates of oxygen consumption were calculated, as percent of the respiratory rate of untreated cells. Oxygen consumption rates in untreated cells were 2.1 nmole $\times$ min^{-1} $\times$ 10^{8} cells^{-1}. Symbols: circles, solid line–survival; triangles, dashed line—rate of O_2 consumption.

Oxygen consumption

The possibility that ADR might interfere with the respiratory process, was investigated by monitoring the effect of ADR on bacterial oxygen consumption. 2×10^{-5} M ADR produces a moderate decrease in cellular oxygen consumption (Fig. 3), that is delayed and relatively small, compared to mortality.

DNA breakage

The ability of ADR to induce SSB and DSB in bacterial DNA *in vivo* was investigated as described by Kohen *et al.* [39]. DNA was extracted from cell samples, and both single and double strand DNA were electrophoresed on agarose gel, as described in Materials and Methods.

As previously reported [2, 42], ADR engaged in a photochemical reaction which could cause extensive DNA breaks during the DNA extraction procedure. In order to avoid the photochemical reaction, the samples were washed once, suspended in ADR free buffer and the DNA extraction performed in relative darkness. When these precautions were taken, agarose gel electrophoresis revealed no DNA damage: DNA from both untreated and ADR-treated (4×10^{-5} M) cells comigrated with the DNA size marker from nalidixic acid-treated cells, whereas the Lambda-DNA Hind III size markers were well separated from the chromosomal DNA (data not shown).

Biosynthesis of macromolecules

The effect of ADR on bacterial biosynthesis of macromolecules was investigated by pulse labelling

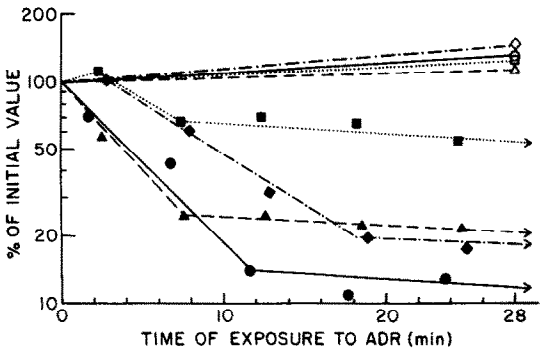

Fig. 4. The effect of ADR on biosynthesis of bacterial DNA, RNA and proteins. *E. coli* cells in Davis growth medium were exposed to 1×10^{-5} M ADR. After various time intervals, samples were pulse labelled separately with ^{14}C-uridine, ^{14}C-thymidine or ^{14}C-leucine. Cold TCA precipitable radioactivity was counted. The surviving fraction (circles, solid lines), the residual biosynthesis of RNA (triangles, dashed lines), DNA (squares, dotted lines) and protein (rhombes, dashed and dotted lines) are presented as a function of exposure time to ADR. Solid symbols—1×10^{-5} M ADR; open symbols—no ADR.

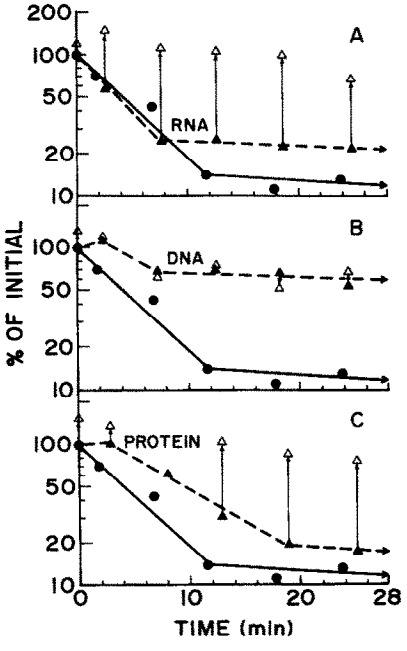

Fig. 5. The restoration of biosynthetic activity in ADR treated *E. coli* following removal of ADR. *E. coli* cells were exposed to 1×10^{-5} M ADR in Davis growth medium, washed once and suspended in fresh ADR free medium. The cells were then incubated for 30 min at 37°. Biosynthetic activity was determined as described in the legend to Fig. 4. The surviving fraction (circles, solid lines) and residual biosynthetic activity (triangles, dashed lines) as measured before (closed symbols) and after (open symbols) the recovery period are presented as a function of exposure time to ADR. (A) RNA synthesis; (B) DNA synthesis; (C) Protein synthesis. Vertical arrows denote the restoration of biosynthetic activity in the individual samples.

cells separately with ^{14}C-thymidine, ^{14}C-uridine or ^{14}C-leucine, and measuring acid precipitable radioactivity. The results were calculated as percent of the rate of synthesis before addition of ADR, and are presented in Fig. 4. RNA synthesis decreased at a rate closely similar to the decrease in survival. The inhibition of both protein and DNA synthesis appeared later. The total decrease in the rate of DNA synthesis was smaller than that in either RNA or protein synthesis. When ADR-treated cells were washed, suspended in Davis growth medium with glucose, and incubated for 30 min at 37°, both RNA and protein synthesis were restored, but DNA synthesis was not (Fig. 5 a–c). No increase in the surviving fraction was noted during this interval.

The possibility that the inhibition of RNA synthesis was of a secondary nature, due to the stringent response, was investigated by measuring the effect of ADR on RNA synthesis in an *E. coli* K 12 *relA* mutant (as opposed to the wild-type, the *relA* mutant does not stop RNA synthesis in response to amino acid starvation—the stringent response). In the absence of phenylalanine, the rate of leucine incorporation into acid insoluble material was 10% of the rate obtained in the presence of phenylalanine, in both the *relA* and the normal strains. As shown in Fig. 6, RNA synthesis was inhibited equally by ADR in the *relA* mutant and in its normal counterpart.

Similarly, it was investigated whether inhibition of RNA synthesis was a result of an inhibition of

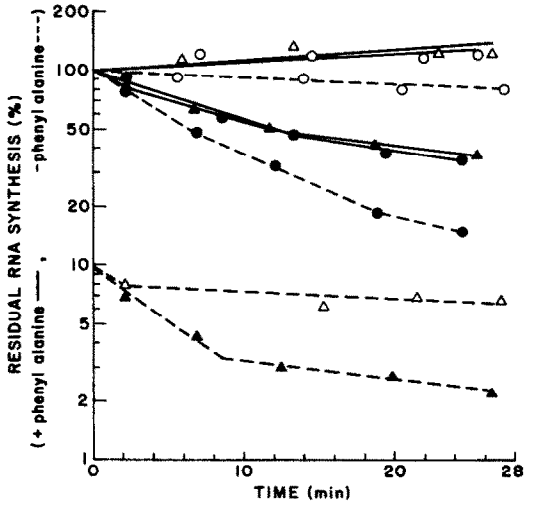

Fig. 6. The effect of ADR on RNA synthesis in a *phe* and a *relA phe* strain. Bacterial cells were washed and suspended in Davis growth medium, with or without phenylalanine. In the absence of phenylalanine, the rate of leucine incorporation into acid insoluble material was 10% of the rate obtained in the presence of phenylalanine in both strains. The cells were exposed to 2×10^{-4} M ADR and samples were pulse labelled with ^{14}C-uridine as described in Methods. The results are expressed as percent of the rate of RNA synthesis in phenylalanine supplemented cells prior to ADR addition. Symbols: triangles—*phe* strain; circles—*relA phe* strain. Filled symbols—2×10^{-4} M ADR; open symbols—no ADR. Solid lines—with phenylalanine; dashed lines—no phenylalanine.

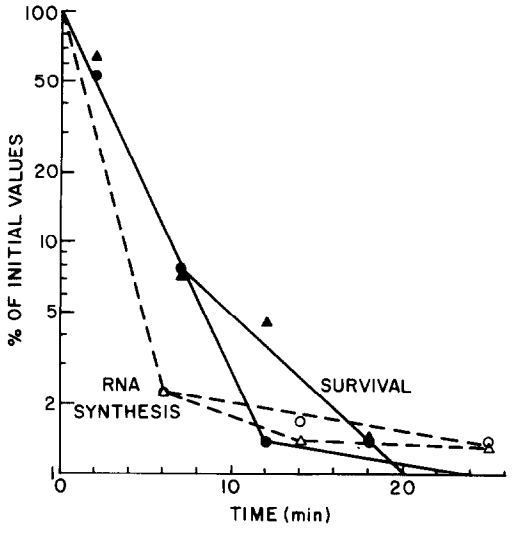

Fig. 7. The effect of ADR on cells treated with nalidixic acid. Cells were treated with nalidixic acid (10 μg/ml) and after 10 min 1.5×10^{-5} M ADR was added. Survival and RNA and DNA synthesis were monitored as described in Methods. Nalidixic acid alone reduced DNA synthesis by 90% and did not affect RNA synthesis. Survival (solid symbols, solid lines) and the rate of RNA synthesis (open symbols, dashed lines) were calculated as percent of the initial values. Circles—ADR only; triangles—nalidixic acid and ADR.

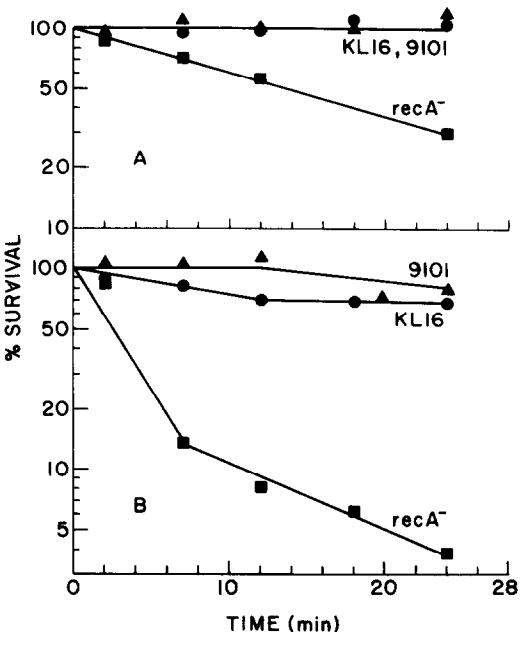

Fig. 8. The effect of ADR on DNA-repair deficient mutants of *E. coli*. Strains KL 16, KL 16 *recA* and 9101 (*xthA*) were exposed to 2×10^{-5} M ADR (A) or 2×10^{-4} M ADR (B), and their survival was determined. Circles—KL16; triangles—9101; squares—KL 16 *recA*.

replication. Nalidixic acid (10 μg/ml) was added to the cells 10 min before exposure to ADR. This concentration of nalidixic acid inhibited DNA synthesis by approx. 90% while it did not affect survival and RNA synthesis (data not shown). Untreated cells and cells treated with nalidixic acid responded equally to ADR (Fig. 7).

DNA-repair deficient mutants

The possibility that the effect of ADR on transcription and replication involved a direct interaction with the DNA template was investigated by use of DNA-repair deficient mutants. The *E. coli* K 12 strains KL 16 *recA* (deficient in recombination and in SOS repair), 9101 (*xthA*, deficient in repair of AP sites) and the parent strain, KL 16, were exposed to ADR and their survival was compared. Higher ADR concentrations were used with *E. coli* K 12, since it is less sensitive to ADR than *E. coli* B. The parent strain and the *xthA* mutant were unaffected by 2×10^{-5} M ADR while the *recA* strain exhibited a 70% decrease in survival after 24 min exposure (see Fig. 8A). When exposed to 2×10^{-4} M ADR the mortality rate of KL 16 *recA* was 9–10 times that of the parent strain, while the sensitivity of the *xthA* mutant was no different from that of KL 16 (Fig. 8B).

Filamentation

Following induction of SOS repair, filamentous growth has been reported in *E. coli* B but not in *E. coli* K 12 (except *lon* strains, which are defective in resumption of cell division after induction of SOS repair) [43]. When samples of *E. coli* B were exposed to 2×10^{-5} M ADR for 7 or 24 min and subsequently allowed 2 hr growth, nearly all cells were 3–4 times the normal cell length. No filamentation was observed in *E. coli* K 12 cells, even when treated with 2×10^{-4} M ADR.

DISCUSSION

The numerous activities of ADR and their wide variety, have provided many possible explanations for its mechanisms of action, but no conclusion as to which type of action causes the actual antitumoral and cardiotoxic effects. Our investigation was designed to evaluate the relative importance of the major cellular effects of ADR, with the aim of disclosing which was the lethal action of ADR on the cell. In most previous work on ADR the drug dose response of the factor under investigation has been determined. In the present study we chose to follow the time dependence of the damages induced by ADR rather than the dose dependence. A comparison of the kinetics of the various effects induced by ADR with cell mortality may reveal the time sequence for induction of the different types of cellular or functional damage and loss of reproductive ability. The choice of bacterial cells as a model system for this investigation seemed advantageous because of their relative simplicity as compared to Eukaryotic cells, while they still contained all major subcellular systems and functions reported to be affected by ADR. Additional advantages were the availability of a wide variety of well defined mutant strains, as well as the short generation time compared to Eukariotic cell cultures. ADR was active against

both growing cells and metabolically resting cells. Growing cells were 5–6 times more sensitive to ADR than resting cells, suggesting that functions active during cell growth are involved in ADR toxicity. These functions may include DNA replication, RNA synthesis and other metabolic or biosynthetic pathways active in growing cells. The nature of this difference between growing and resting cells is now under investigation.

The major types of ADR-induced cell damage reported for a wide variety of test systems, were shown to be inducible in the *E. coli* B model system as well. Thus membrane damage, DNA breaks, interference with respiration and inhibition of macromolecule biosynthesis were observed when *E. coli* cells were exposed to ADR. The various types of damage would differ in the ADR concentration required for their induction and in the kinetics of their development. Comparing the kinetics of damage induction with mortality at different ADR concentrations, proved to be a useful tool for evaluating the relative importance of each type of damage, and identifying the primary cellular target of ADR.

Membrane damage

The possibility that ADR may cause membrane damage or impairment of its functional integrity was investigated by monitoring parameters that depend on an intact membrane structure and its normal functioning. Thus, nonspecific structural damage to cell membranes, like that caused by free radicals in the paraquat/Cu^{2+} system [44], may result in leakage of protons or metal cations. The altered cross membrane electrochemical gradient would result in rapid utilization of ATP by proton or other pumps, such as the Ca^{2+}/K^{+} ATPase. More severe perforation of the membrane would cause leakage of larger molecules, such as amino acids. This situation would be reflected by a decreased ability to maintain a cross membrane leucine gradient. The active transport of leucine into the cell is highly sensitive to changes in membrane potential, pH and protonmotive force [41], and any change in these parameters would immediately be reflected by the leucine uptake rate. As reported above, neither cellular ATP levels (in agreement with the limited effect of ADR on ATP levels in rat liver cells and mitochondria [27]) nor the leucine uptake rate or its steady state level were affected by ADR before mortality had reached 90% or more. This strongly suggests that the membrane is not of primary importance as a target in ADR induced mortality in *E. coli*.

While this may seem to contradict reports on the activity of immobilized extracellular ADR [45–47], our results do not preclude a destructive effect of ADR on cell membranes, but simply indicate that this effect occurs too late to be of importance to cell death when ADR is free to enter the cells, as in our bacterial system. When ADR is immobilized, the otherwise secondary effect on cell membranes is expressed, due to limitations imposed by the experimental design.

Oxygen consumption

The possible interference of ADR with the respiratory chain may be reflected in O_2 consumption and cellular ATP levels. Electron abduction from the respiratory chain or inhibition of electron transport might cause a decrease in both cellular ATP levels and O_2 consumption. On the other hand, stimulated superoxide radical formation would result in an increased O_2 consumption. ATP levels were not affected by ADR until mortality was above 90%, and O_2 consumption was only moderately decreased by ADR. A concurrent decrease in cellular respiration and increased utilization of oxygen for superoxide radical generation may be obscured when net oxygen consumption is measured. In the absence of direct evidence for or against O_2^- formation, the unchanged intracellular ATP levels are taken to indicate that cellular respiration is not seriously impaired by ADR in the early stages of exposure. Thus, our findings suggest that the respiratory chain is not a primary target in ADR activity.

DNA breakage

A major cellular target reported for ADR activity is the DNA. Numerous authors have reported ADR induced SSB and DSB in DNA, both *in vivo* and *in vitro* [2, 3, 6–8]. In the present investigation no DNA strand breaks were observed, when precautions were taken to avoid photochemical effects. The method employed would not separate DNA fragments of 100 kilobases (45–50 breaks per chromosome) and above, as evidenced by the comigration of DNA from untreated cells and from cells treated with nalidixic acid, at drug concentrations known to cause up to 45–50 breaks per chromosome [40]. This resolution limit may cause a small amount of DNA breaks to go undetected. Nevertheless, extensive DNA fragmentation, as observed in cells treated with ascorbate/Cu^{2+} or 1–10 orthophenantroline/Cu^{2+} [48, 49] was not detected in ADR-treated cells. The possible ADR mediated induction of a small number of DNA breaks per chromosome is presently under investigation.

Macromolecule biosynthesis

An additional explanation for the cytotoxicity of ADR would be the drug interference with normal biosynthesis of macromolecules as has previously been found [2, 3, 5, 11–16]. The present results show that the kinetics of inhibition of RNA synthesis is closely and consistently similar to the kinetics of mortality. In contrast, the inhibition of DNA and protein synthesis lagged behing mortality (and inhibition of RNA synthesis). Furthermore, inhibition of protein synthesis by CM had little effect on ADR-induced mortality (data not shown). We may thus conclude that the effect of ADR on protein synthesis is of a secondary nature, as has been previously suggested [13, 50].

The close correlation between mortality and the inhibition of RNA synthesis suggests a direct role for this effect in ADR cytotoxicity. This conclusion is supported by the finding that the effect of ADR on RNA synthesis is not mediated by the stringent response, nor is it affected by inhibition (by nalidixic acid) of replication. It has previously been suggested that the drug mainly affects the initiation step of RNA synthesis [16, 51]. Rates of RNA and protein

synthesis were restored after removal of ADR and subsequent incubation for 30 min in drug free medium. This may reflect a general recovery from the ADR induced inhibition in all cells, indicating that inhibition by ADR is freely reversible, but it may also derive from accelerated synthesis in the surviving cells only. The lack of an increase in the rate of DNA synthesis and the absence of recovery of "dead" cells support the latter notion, i.e. since inhibition of RNA synthesis is a direct result of ADR activity, resumption of RNA synthesis would most probably depend on ADR removal or repair of the ADR induced lesions, which would also facilitate increased DNA synthesis, but this was not observed. It thus seems more probable that the recovery of RNA synthesis reflects accelerated activity in surviving cells only, the rate of RNA synthesis being much more flexible than that of DNA synthesis.

The role of the inhibition of DNA synthesis is not yet clear, because the onset of inhibition is delayed, and its extent is limited compared to mortality, while this inhibition is persistent in recovering cells in which RNA synthesis has been restored. The rate of DNA synthesis measured in this study comprised both replication and repair. In untreated cells, repair activity is negligible, but in ADR-treated cells inhibition of replication may be obscured by the onset of repair activity. Indications exist that this is indeed the case and replication is inhibited to a larger extent than suggested by the present results. This possibility is presently under investigation.

DNA repair

The use of two DNA repair deficient mutants of KL 16, *recA* and *xthA*, revealed that the *recA* controlled functions (i.e. recombination and/or SOS repair), but not the *xthA* gene product (exonuclease III–endonuclease II), are involved in the cellular defence against ADR induced damage. This suggests that ADR causes a potentially lethal DNA lesion or distortion, of a nature that activates, and can be relieved by, SOS or recombinational repair. On the other hand these lesions do not involve introduction of AP sites, since no dependence on endonuclease II function was noted. Additional enzymes, not investigated in this study, may be involved in the defence against ADR induced damage but the involvement of the *recA*-dependent functions suffices to indicate the DNA as the primary target for ADR.

The observation that *E. coli* B cells exhibited extensive filamentation following exposure to ADR, while *E. coli* K 12 did not, is consistent with the *lon* genotype of *E. coli* B, and with reports on filament formation following activation of the SOS repair pathway [43]. This supports our conclusion that induction of SOS repair is an early event triggered by exposure to ADR. The difference reported between *E. coli* B and K 12 in sensitivity to daunomycin [52, 53], may reflect the filamentous growth [43] occurring in *E. coli* B, but not in K 12 strains.

Our finding that RNA synthesis is inhibited earlier and to a larger extent than DNA synthesis is in conflict with results reported by authors who found that inhibition of DNA was larger than that of RNA synthesis [13, 50] or both were inhibited equally [2, 13] but in agreement with others [15, 54]. The apparent inconsistency in the activity of ADR may reflect differences between cellular characteristics and experimental conditions in the systems employed in different investigations.

The above conclusions do not preclude a direct action of ADR on other cell constituents and processes. Late occurring membrane damage and interference with respiration may be a result of a chain of events starting with inhibition of RNA synthesis, followed by decreased protein synthesis, with subsequent failure of replacement of vital protein components in the cell. On the other hand, it may be an independent action of ADR on the membrane and the respiratory chain. In any case, these effects are seemingly without influence on mortality in our bacterial model system and probably in other cell cultures as well. Their significance would become apparent under conditions where DNA is unavailable for ADR, as in red blood cells [19, 55] or when cells are exposed to immobilized ADR [45–47].

In summary, the present results confirmed the ability of ADR to induce the major types of cellular damage reported, i.e. membrane damage, DNA breaks, interference with respiration and inhibition of biosynthetic activity in the bacterial cell. By manipulating the reaction conditions and ADR concentrations, and monitoring the kinetics of damage development as compared to the kinetics of mortality, we have been able to show that membrane damage, DNA breakage, interference with energy production and respiration, and inhibition of protein synthesis are not primary events in ADR cytotoxic activity in *E. coli*. On the other hand, the close relationship between mortality and inhibition of transcription (and possibly also of replication) suggests that ADR exerts its cytotoxic activity against *E. coli* by interfering with normal DNA template function. Filament formation in *E. coli* B and the observation that *recA* mutants are much more sensitive to ADR than are wild-type cells, support the conclusion that, at least in the case of *E. coli* cells, the primary activity of ADR is directed against the DNA, and is of a nature that can be relieved by SOS or recombinational repair, but does not involve AP sites or extensive DNA fragmentation.

While the details of the mechanism of ADR cytotoxicity remain to be elucidated, we have defined the DNA as the primary site of action for ADR in *E. coli*. This may be useful in pinpointing the cellular targets for the antitumoral and/or the cardiotoxic activities of ADR in mammalian cells.

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REFERENCES

1. F. Arcamone, G. Cassinelli, G. Fantini, A. Grein, P. Orezzi, C. Pol and C. Spalla, *Biotechnol. Bioeng.* **11**, 1101 (1969).
2. L. Gianni, B. J. Corden and C. E. Myers, in *Reviews in Biochemical Toxicology* 5 (Eds. E. Hodgson, J. R.

Bend and R. M. Philpot), p. 1. Elsevier/North Holland, New York, (1983).
3. G. Aubel-Sadron and D. Londos-Gagliardi, *Biochimie* **66**, 333 (1984).
4. H. Porumb, *Prog. Biophys. molec. Biol.* **34**, 175 (1978).
5. M. F. Goodman, G. M. Lee and N. R. Bachur, *J. biol. Chem.* **252**, 2670 (1977).
6. V. Berlin and W. A. Haseltine, *J. biol. Chem.* **256**, 4747 (1981).
7. Y. C. Lee and J. E. Byfield, *J. natn. Cancer Inst.* **57**, 221 (1976).
8. W. E. Ross, D. L. Glaubiger and K. W. Kohn, *Biochim. biophys. Acta* **519**, 23 (1978).
9. B. Lambert, M. Sten, S. Söderhäll, U. Ringborg and R. Lewensohn, *Mutat. Res.* **111**, 171 (1983).
10. B. K. Vig, *Mutat. Res.* **39**, 189 (1977).
11. J. W. Lown, *Mol. Cell. Biochem.* **55**, 17 (1983).
12. R. C. Young, R. F. Ozols and C. E. Myers, *N. Engl. J. Med.* **305**, 139 (1981).
13. R. L. Momparler, M. Karon, S. E. Siegel and F. Avila, *Cancer Res.* **36**, 2891 (1976).
14. H. Fialkoff, M. F. Goodman and M. W. Seraydarian, *Cancer Res.* **39**, 1321 (1979).
15. F. Zunino, R. Gambetta, A. Di Marco, *Biochem. Pharmac.* **24**, 309 (1975).
16. R. Y. Chuang and L. F. Chuang, *Biochemistry* **18**, 2069 (1979).
17. W. E. Ross and M. C. Smith, *Biochem. Pharmac.* **31**, 1931 (1982).
18. E. Arena, F. Biondo, N. D'Alessandro, L. Dusonchet, N. Gebbia, F. Gerbasi, L. Rausa and R. Sanguedolce, *IRCS Libr. Compend* **2**, 1053 (1974).
19. R. B. Mikkelsen, P. S. Lin and D. F. H. Wallach, *J. mol. Med.* **2**, 33 (1977).
20. E. Goormaghtigh, R. Brasseur and J. M. Ruysschaert, *Biochem. biophys. Res. Commun.* **104**, 314 (1982).
21. J. Goodman and P. Hochstein, *Biochem. biophys. Res. Commun.* **77**, 797 (1977).
22. C. E. Myers, W. P. McGuire, R. H. Liss, I. Ifrim, K. Grotzinger and R. C. Young, *Science* **197**, 165 (1977).
23. E. J. F. Demant and P. K. Jensen, *Eur. J. Biochem.* **132**, 551 (1983).
24. T. Dasdia, A. Di Marco, M. Goffredi, A. Minghetti and A. Necco, *Pharmac. Res. Commun.* **11**, 19 (1979).
25. A. B. Combs, D. Acosta and K. Ramos, *Biochem. Pharmac.* **34**, 1115 (1985).
26. S. A. Murphree, T. R. Tritton, P. L. Smith and A. C. Sartorelli, *Biochim. biophys. Acta* **649**, 317 (1981).
27. K. J. A. Davies, J. H. Doroshow, T. M. Chan and P. Hochstein, in *Oxy Radicals and their Scavenger Systems*, Vol. II: *Cellular and Medical Aspects* (Eds. R. A. Greenwald and G. Cohen), p. 313 Elsevier/North Holland, New York (1983).
28. Y. Iwamoto, I. L. Hansen, T. H. Porter and K. Folkers, *Biochem. biophys. Res. Commun.* **58**, 633 (1974).
29. K. J. A. Davies, J. H. Doroshow and P. Hochstein, *FEBS Lett.* **153**, 227 (1983).
30. J. H. Doroshow, *Cancer Res.* **43**, 460 (1983).
31. N. R. Bachur, S. L. Gordon and M. V. Gee, *Cancer Res.* **38**, 1745 (1978).
32. N. R. Bachur, M. V. Gee and R. D. Friedman, *Cancer Res.* **42**, 1078 (1982).
33. D. A. Rowley and B. Halliwell, *Biochim. biophys. Acta* **761**, 86 (1983).
34. J. H. Doroshow, *Cancer Res.* **43**, 4543 (1983).
35. N. R. Bachur, S. L. Gordon, M. V. Gee and H. Kon, *Proc. natn. Acad. Sci. U.S.A.* **76**, 954 (1979).
36. H. A Cole, J. W. T. Wimpenny and D. E. Hughes, *Biochim. biophys. Acta* **143**, 445 (1967).
37. Y. Anraku, *J. biol. Chem.* **243**, 3128 (1968).
38. L. Gianni, J. L. Zweier, A. Levy and C. E. Myers, *J. biol. Chem.* **260**, 6820 (1985).
39. R. Kohen, M. Szyf and M. Chevion, *Analyt. Biochem.*, in press.
40. M. Snyder and K. Drlica, *J. molec. Biol.* **131**, 287 (1979).
41. B. D. Davis, R. Dulbecco, H. N. Eisen and H. S. Ginsberg, *Microbiology*, 3rd Edn, pp. 93–99. Harper & Row, Philadelphia (1980).
42. H. Eliot, L. Gianni and C. Myers, *Biochemistry* **23**, 928 (1984).
43. E. M. Witkin, *Bacteriol. Rev.* **40**, 869 (1976).
44. R. Kohen, P. Korbashi and M. Chevion, in *Proceedings of the 4th SOD International Conference, Rome, 1985* (Eds. J. Rotilio and J. V. Bannister).
45. T. R. Tritton and G. Yee, *Science* **217**, 248 (1982).
46. L. B. Wingard, Jr., T. R. Tritton and K. A. Egler, *Cancer Res.* **45**, 3529 (1985).
47. Z. A. Tölkes, K. E. Rogers and A. Rembaum, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2026 (1982).
48. L. E. Marshall, D. R. Graham, K. A. Reich and D. S. Sigman, *Biochemistry* **20**, 244 (1981).
49. S-H. Chiou, *J. Biochem.* **96**, 1307 (1984).
50. S. H. Kim and J. H. Kim, *Cancer Res.* **32**, 323 (1972).
51. V. Barthelemy-Clavey, C. Molinier, G. Aubel-Sadron and R. Maral, *Eur. J. Biochem.* **69**, 23 (1976).
52. A. Di Marco, in *Antibiotics* I: *Mechanism of Action* (Eds. D. Gottleib and P. D. Shaw), p. 190. Springer, Berlin (1967).
53. T. Tønnesen and J. D. Friesen, *Mol. Gen. Genet.* **124**, 177 (1973).
54. J. J. Wang, D. S. Chervinsky and J. M. Rosen, *Cancer Res.* **32**, 511 (1972).
55. C. E. Myers, L. Gianni, C. B. Simone, R. Klecker and R. Greene, *Biochemistry* **21**, 1707 (1982).