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Coumermycin A_1: A Preferential Inhibitor of Replicative DNA Synthesis in *Escherichia coli.* I. In Vivo Characterization†

Michael J. Ryan‡

ABSTRACT: Coumermycin A_1, an antibiotic related to novobiocin, inhibited nucleic acid synthesis in intact *Escherichia coli* with replication being slightly more sensitive to this drug than transcription. The ultraviolet-induced repair synthesis of DNA was only partially inhibited under conditions where replication was eliminated by coumermycin A_1. Inhibition of protein synthesis was a secondary effect. Coumermycin A_1-resistant *E. coli* were isolated and the mutation was mapped near *dna*A. Chromatography of crude protein extracts of sensitive and resistant bacteria on drug affinity columns implicated a soluble protein of approximately 37,000 molecular weight as the target site for coumermycin A_1. Depending on the medium used, this antibiotic had either a bacteriocidal or a bacteriostatic effect on *E. coli.* Results showed that the effect of coumermycin A_1 cannot be explained by the degradation of DNA under bacteriocidal growth conditions.

Although a relatively large number of antibiotics inhibit chromosomal replication by interacting with the DNA template or by inhibiting the synthesis of precursors (Corcoran and Hahn, 1975; Kersten and Kersten, 1974; Gale et al., 1972), few interfere with the actual polymerization reactions. Only one compound, the chemically synthesized 6-(*p*-hydroxyphenylazo)uracil, specifically inhibits a DNA polymerase from *Bacillus subtilis* (Mackenzie et al., 1973; Gass et al., 1973).

This is surprising in view of the fact that a substantial segment of contemporary cancer research is aimed at the biochemistry and pharmacology of antitumor agents, many of which interfere with some aspect of nucleic acid metabolism. One reason for our meager understanding lies in the nature of the mechanism of DNA replication, which is still unclear relative to those for transcription and translation. However, this position has improved considerably with the recent classification of thermosensitive mutants in DNA replication (Wechsler and Gross, 1971) and the reconstitution of an in vitro system for DNA replication (Wickner and Hurwitz, 1974; Schekman et al., 1975).

Coumermycin A_1 and novobiocin (Kawaguchi et al., 1965a; Berger et al., 1966) are related coumarin and carbohydrate-containing antibiotics (Figure 1) produced by *Streptomyces.* Coumermycin A_1 is particularly active against *Staphylococcus aureus,* inhibiting the growth of many strains at a very low concentration (0.004 μg/ml) (Kawaguchi et al., 1965b; Grunberg and Bennett, 1966; Fedorko et al., 1969). The antimicrobial spectrum of both drugs was qualitatively very similar, but coumermycin A_1 was up to 50-fold more active than novobiocin (on a weight basis). The mode of action of novobiocin has been widely studied in a variety of systems but its elucidation has been complicated by a number of pleiotropic, secondary effects (Brock, 1967). However, one study with *E. coli* indicated that this antibiotic inhibited the in vivo synthesis of DNA (Smith and Davis, 1967). Recent in vitro studies

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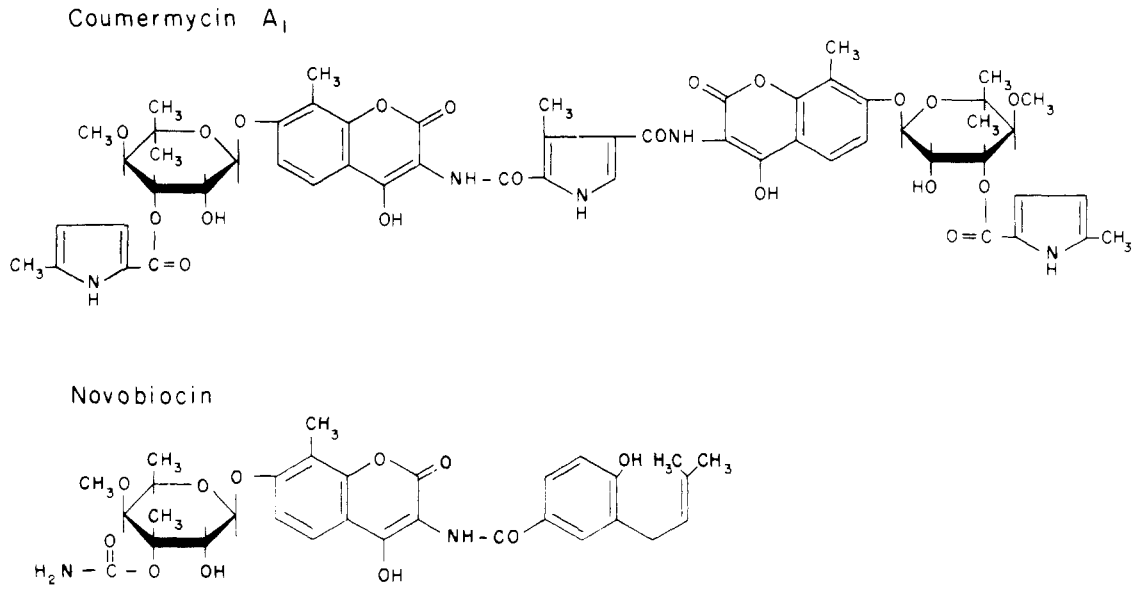

FIGURE 1: Structures of coumermycin A_1 and novobiocin.

(Staudenbauer, 1975) have extended these findings with novobiocin. Also, coumermycin A_1 was shown to inhibit the in vivo synthesis of nucleic acids in *S. aureus* (Michaeli et al., 1971).

Studies were performed with coumermycin A_1 with intact *E. coli* in order to determine its primary site of action on macromolecular synthesis. A drug-resistant derivative was constructed, the mutation was mapped, and the existence of an altered protein in the mutant was established. In addition, the influence of the drug on various features of DNA synthesis were investigated.

The following paper in this issue (Ryan and Wells, 1976) describes further studies on the effect of coumermycin A_1 on permeabilized *E. coli* and on highly purified nucleic acid polymerases. A preliminary report (Ryan and Wells, 1975) of a portion of these findings was published.

Materials and Methods

Drugs. Coumermycin A_1 (Na^+), novobiocin (Albamycin), and mitomycin C were gifts to Julian Davies (this department) from Bristol Laboratories, the Upjohn Company, and Kyoma Kogyo Co., respectively. Coumermycin A_1 (H^+) was provided by Martin J. Cron, Bristol Laboratories. Nalidixic acid and streptomycin sulfate were purchased from Sigma.

Coumermycin A_1 was made up as a concentrated stock solution (40 mg/ml) in dimethyl sulfoxide and stored at −20 °C without loss of activity. Mitomycin C was dissolved in M9 salts at 0.25 mg/ml and used as a fresh solution. Nalidixic acid was usually dissolved in 0.1 N NaOH at 15 mg/ml and used immediately.

Bacteriological Procedures. Growth media, plates, uninterrupted matings, growth of phage P_1, transduction experiments, and determination of viable cell counts were essentially as described (Miller, 1972).

Radioactive DNA Markers. For buoyant density analyses, a [^{32}P]λplac$_5$S$_7$CI$_{857}$ DNA marker was prepared and characterized as described (Chan and Wells, 1974). [^{3}H]TdR-labelled colicin E_1 DNA was used as a marker for alkaline sucrose gradient sedimentation. Since this DNA preparation was a mixture of supercoiled and nicked circular DNAs, it actually provided two markers, 17.5S for the separated single strands and 54S for the denatured supercoil. The DNA was prepared essentially as described (Clewell and Helinski, 1969). The final sample had a specific activity of 710 cpm/nmol.

Precipitation and Counting of Radioactively Labelled Samples. Aliquots were precipitated by one of two procedures: (A) a previously described (Nishimura et al., 1964) filter-paper assay or, for larger samples, (B) the aliquots were added to 10% Cl_3CCOOH–0.1 M sodium pyrophosphate and the precipitate was collected and washed on glass-fiber filters (Whatman GF/C).

Replicative and Repair Synthesis of DNA. E. coli were grown at 37 °C to an OD_{590}[1] of 0.4 in 400 ml of M9 medium supplemented with 0.5% glucose and 50 μg/ml of thymidine. The cells were centrifuged and resuspended with 200 ml of M9 medium and half were then irradiated in a 10-in. Pyrex pie plate for 2 min with gentle swirling at a distance of 50 cm from a uv lamp (Champion Model G15T8 Germicidal). Twenty milliliters of M9 medium, supplemented with 1% glucose, 4 μg/ml of thiamine, 2 mM $MgSO_4$, and 100 μg/ml of 5-bromodeoxyuridine, was added to each of four red Erlenmeyer flasks. To two of these was added a 20-ml aliquot of the uv-irradiated bacteria, and to the remaining two a 20-ml aliquot of the nonirradiated bacteria. In addition, one of each pair contained coumermycin A_1 at 100 μg/ml, whereas the other was given an equivalent level of the diluent, Me_2SO. After a 10-min incubation at 37 °C, [^{3}H]thymidine (50 Ci/mol) was added to 10 μCi/ml and the incubation was continued for another 60 min. The cultures were then centrifuged, washed, and resuspended in 2 ml of 50 mM Tris-HCl (pH 7.9)–20 mM EDTA. The bacteria were converted to spheroplasts by lysozyme treatment and lysed by adding sodium dodecyl sulfate to 0.6% and heating at 56 °C for 45 min. Denatured protein and sodium dodecyl sulfate were removed by adding KCl to 0.5 M, chilling the samples on ice, and centrifuging them. The supernatant was then used for buoyant density analysis.

Stability of Parental DNA in the Presence of Coumermycin. To prepare DNA for sedimentation analyses, D110 was grown at 37 °C in LB to an OD_{590} of 0.25 and a 20-ml aliquot was diluted ten times into the same medium containing 10 μCi/ml

[1] Abbreviations used are: OD, optical density; uv, ultraviolet; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Bacterial and Phage Strains.[a]

Strain Designation	Genotype
D110[b]	*pol*A, *thy*, *end*I
CRBD110[c]	*pol*A, *thy*, *end*I, *cou*^{r}
CRKL16[c]	Hfr, *thi*, *cou*^{r}
CRJC12[c]	Hfr, *met*, *pur*C, *thi*, *xyl*, *mtl*, *lac*, *cou*^{r}
CRT46[b]	*thr*, *leu*, *thi*, *thy*, *mal*, *ilv*, *lac*, *dna*A
D_2TT[d]	*thy*A, *str*^{r}
KL16[e]	Hfr, *thi*
KL25[e]	Hfr, *sup*
KL99[e]	Hfr, *thi*
ATR2444[f]	HfrH, *thi*, *met*E, *rif*^{r}

[a] Genetic markers are as described (Taylor and Trotter, 1972). The generalized transducing phage, P_1 *clr* was a gift of J. E. Davies. [b] J. E. Davies. [c] This paper [d] P. Kuempel. [e] W. S. Reznikoff. [f] P. F. Schendel.

of [^{3}H]TdR and incubation was continued for three generations. The cells were centrifuged, resuspended in 200 ml of LB supplemented with 200 μg/ml of thymidine, recentrifuged, and resuspended in 400 ml of LB supplemented with 200 μg/ml of thymidine. Aliquots (100 ml) were incubated for 33 min at 37 °C and then coumermycin A_1 was added to the first flask to 30 μg/ml, an equivalent amount of Me_2SO to the second, and nalidixic acid to 100 μg/ml to the third. At 66 min after the addition of drugs, 10-ml aliquots were removed, added to an equal volume of "ethanol-phenol" (75 ml of 95% ethanol-21 ml of 0.1 M sodium acetate (pH 5.1)-2 ml of 0.1 M EDTA-2 ml of water-saturated phenol) (Manor et al., 1969), and centrifuged.

The pellets were resuspended with 0.4 ml of 0.3 N NaOH-20 mM EDTA-0.5% sodium dodecyl sulfate, heated at 37 °C for 20 min, cooled on ice, and centrifuged. The supernatant was layered directly onto 15-ml linear 5-20% sucrose gradients containing 0.3 N NaOH, 0.7 M NaCl, and 20 mM EDTA over a 1.5-ml shelf of 82% sucrose in the same solution. The gradients were overlaid with paraffin oil and centrifuged for 19 h at 22 500 rpm and 4 °C in an SW 27.1 rotor. Fractions of 0.3 ml were collected from the top using an ISCO Model 640 density gradient fractionator with Fluorinert as the extruding liquid.

CsCl Buoyant Density Centrifugation. Aliquots, 0.5 ml, of the DNA-containing samples were combined with 0.02 ml of the ^{32}P marker λ DNA and 2.9 ml of a CsCl solution (sp gr 1.857) was made up with 0.1 M Tris-HCl (pH 7.9)-20 mM EDTA. Each sample was layered over 0.3 ml of Fluorinert, a dense (sp gr = 1.93) water-immiscible liquid (3 M Co.). The remaining volume was filled with paraffin oil. Centrifugation was for 30 h at 36 000 rpm and 20 °C in an SW 50.1 rotor. Fractions were collected through a hole in the bottom of the tube onto filter papers and prepared according to procedure A.

To plot the data, the fractions were converted to percent of gradient and the appropriate gradients were aligned so that their internal [^{32}P]λDNA markers would coincide. The overall recoveries for these samples (total cpm recovered from the CsCl gradient relative to cpm incorporated in the original culture) were: (1) −uv, −drug = 112%; (2) −uv, +coumermycin A_1 = 85%; (3) +uv −drug = 95%; (4) +uv +coumermycin A_1 = 92%.

A portion of each DNA sample was denatured by the addition of NaOH to 0.3 M and then reneutralized with HCl prior to centrifugation as described above. Recoveries were as follows, numbers corresponding to the native samples described above: (1) 80, (2) 81, (3) 75, and (4) 58%.

For the experiment in which coumermycin A_1 was varied (Figure 10), the overall recoveries were 72, 57, 44, and 39%, respectively, for the cultures labeled, as above, in the presence of 0, 5, 10, and 20 μg/ml of coumermycin A_1. The repair peak was defined as the total radioactivity from 70% gradient to three fractions beyond the fully light peak, with the remainder defined as representing the replicative synthesis of DNA.

Novobiocin Affinity Column Chromatography of Crude Extracts. Novobiocin was coupled to cyanogen bromide-activated Sepharose 4B using a previously described procedure (Parikh et al., 1974). Crude extracts of D110 and CRBD110 (Table I) were prepared as described (Shizuya and Richardson, 1974) from 3-l. stationary-phase cultures of each strain grown in 2XLB medium (Miller, 1972).

The novobiocin-Sepharose columns (10-ml bed volume) were prepared for chromatography by washing with 50 ml of 5 mg/ml of bovine serum albumin in 50 mM Tris-HCl (pH 7.5)-10% sucrose (Tris-sucrose), and then with 120 ml of Tris-sucrose. The crude extract from each strain was applied to the column, the flow-through (fraction I) was collected, and then the column was washed with 60 ml of Tris-sucrose (II), 60 ml of Tris-sucrose containing 20 μg/ml of coumermycin A_1 (III), and 60 ml of 1.0 M sodium acetate (pH 4.5) (IV).

The samples were dialyzed extensively against 50 mM $(NH_4)_2CO_3$ and then 10 mM $(NH_4)_2CO_3$ and then lyophilized. After being dissolved in water, they were dialyzed against 5 mM Na_2HPO_4 and analyzed by sodium dodecyl sulfate gel electrophoresis essentially as described (Laemmli, 1970). The gels were stained with Coomassie brilliant blue (Mann Research Laboratories). Scanning of each gel at 600 nm was performed as described (Burd and Wells, 1974).

Determination of Cross-Links in DNA. Cross-linked DNA from drug-treated bacteria was defined as that material that renatured rapidly after thermal denaturation. The proportion of this material was determined using published techniques (Iyer and Szybalski, 1963). From this fraction and the weight-average molecular weight of the DNA, determined as described (Wells and Larson, 1972), the number of cross-links per genome could be calculated. Only in the case of mitomycin C treatment was rapidly renaturing material observed. Under the conditions used, 8 cross-links/genome were detectable.

Centrifugation. Unless otherwise specified, all centrifugation was at 10 000 rpm for 15 min in a SS34 rotor in a Sorvall RC2-B refrigerated centrifuge.

Results

Effect of Coumermycin A_1 on DNA, RNA, and Protein Synthesis. The influence of coumermycin A_1 on DNA, RNA, and protein synthesis in *E. coli* is shown in Figures 2-4. The A panels, in Figures 2-4, show that both DNA and RNA synthesis were affected soon after addition of coumermycin A_1 to 30 μg/ml with the sensitive parent strain, D110 (Table I). The considerable lag (approximately 30 min), observed before protein synthesis was inhibited, indicates that this was probably a secondary effect of the drug. The inhibitions, based on the total amount of radioactivity incorporated in the first hour after addition of coumermycin, were 80% for DNA synthesis and 68% for RNA synthesis. In contrast, the B panels, in Figures 2-4, show DNA synthesis in CRBD110, a spontaneous coumermycin-resistant mutant (described below), was 37% inhibited, whereas RNA and protein synthesis were unaffected. Similar results were obtained when these strains were

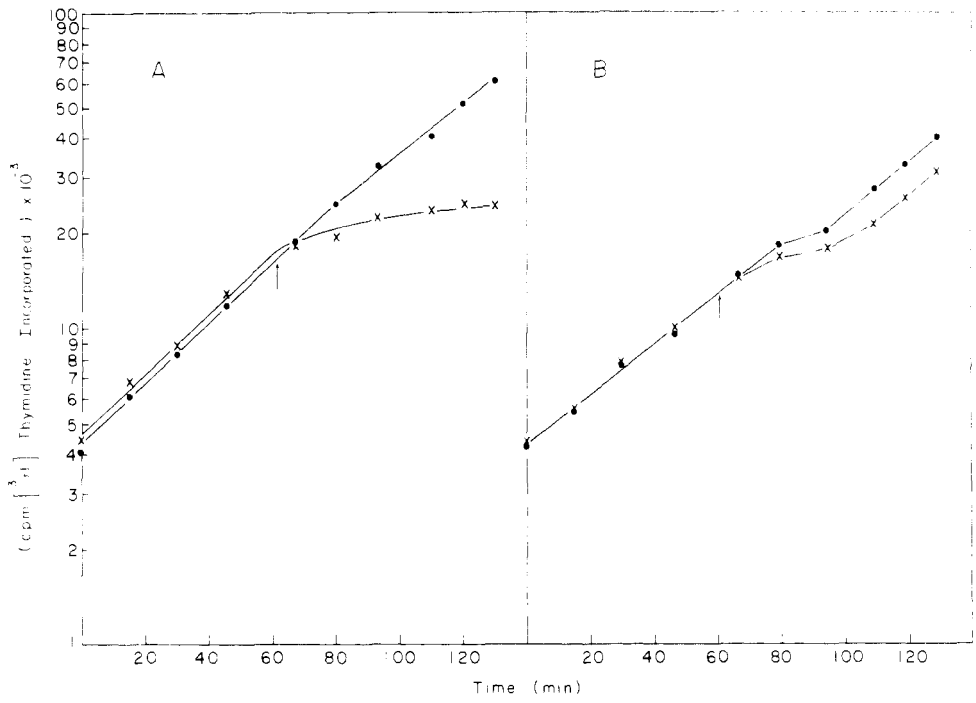

FIGURE 2: Inhibition of DNA synthesis by coumermycin A_1. *E. coli* D110 (panel A) and CRBD110 (panel B) were grown at 37 °C in LB medium supplemented with 20 μg/ml of thymidine, 10 μg/ml of uracil, and 10 μg/ml of leucine, with [^{3}H]thymidine added to 75 μCi/ml. The arrows mark the points of addition of coumermycin A_1 (30 μg/ml) or its diluent (50 mM NaOH). At each time point, an aliquot (0.050 ml) was precipitated and counted by procedure A (Methods). (x) Coumermycin A_1; (●) diluent.

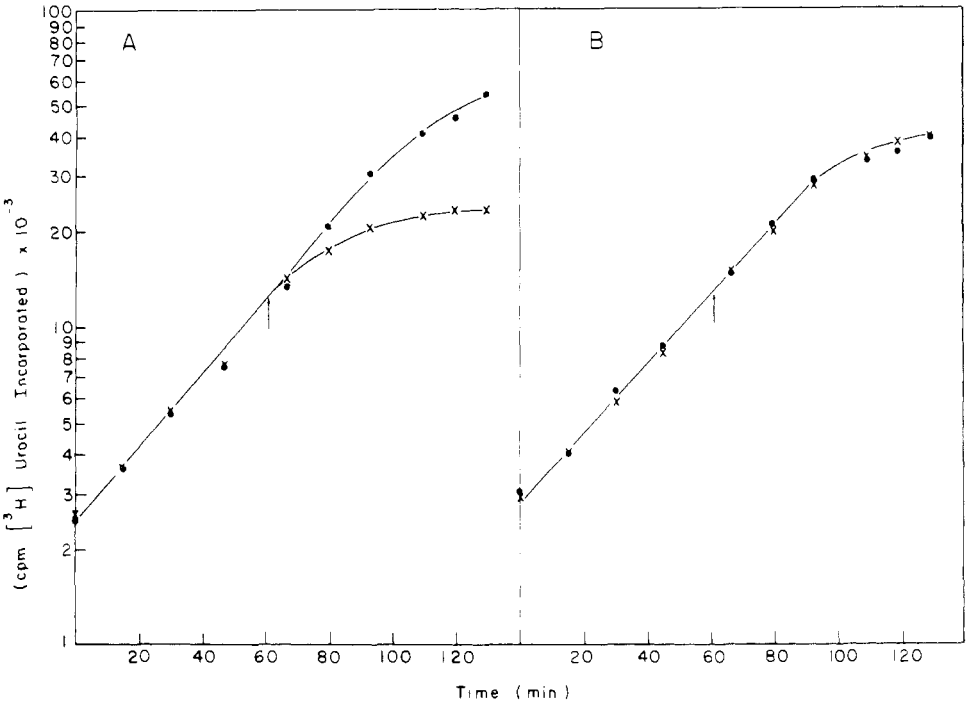

FIGURE 3: Inhibition of RNA synthesis by coumermycin A_1. Conditions and procedures were as in Figure 2 except that [^{3}H]thymidine was replaced by 75 μCi/ml of [^{3}H]uracil. The strains tested were D110 (panel A) and CRBD110 (panel B). (x) 30 μg/ml of coumermycin A_1; (●) diluent.

converted to spheroplasts. In both cases, DNA synthesis was somewhat more sensitive than RNA synthesis. However, in other experiments in which toluenized D110 and CRBD110 were studied, replication was clearly more sensitive than transcription to coumermycin A_1 (Ryan and Wells, 1976).

Genetic Mapping of the Mutation to Coumermycin A_1 Resistance (cou^r). *E. coli* strains normally do not grow in the presence of 15 μg/ml of coumermycin A_1. The spontaneous drug-resistant mutant CRBD110 was selected as a derivative of D110 capable of growing in the presence of 50 μg/ml of this antibiotic. After preliminary uninterupted mating experiments localized the position of this mutation, P_1 transduction was used to further define its location. Resistance to coumermycin A_1 was found to be closely linked to *dna*A, near 73 min on the *E. coli* genetic map (Taylor and Trotter, 1972). The position of *cou*^{r} relative to *dna*A and *ilv* was established by three-factor

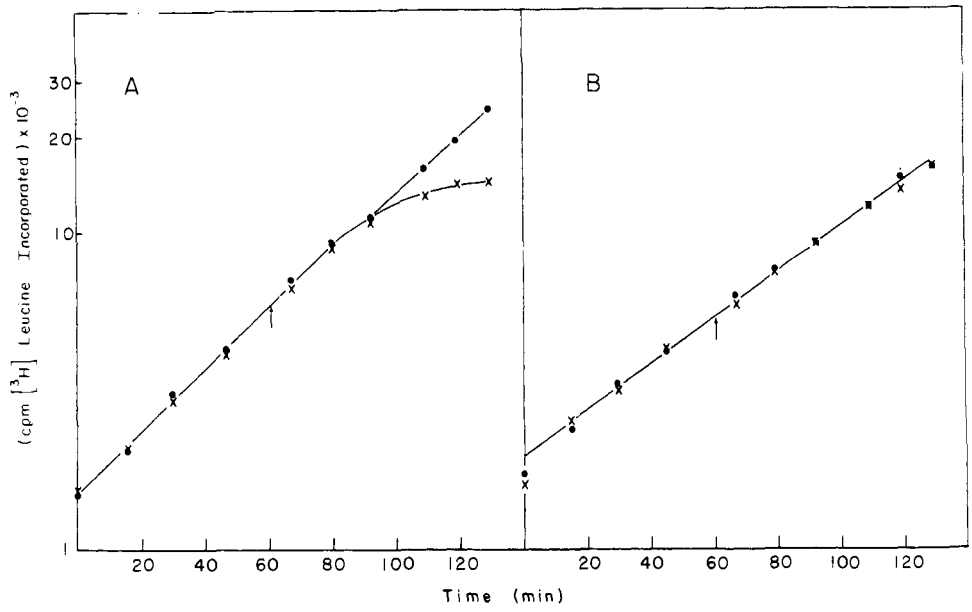

FIGURE 4: Inhibition of protein synthesis by coumermycin A_1. Conditions and procedures were as in Figure 2 except that [^{3}H]thymidine was replaced by [^{3}H]leucine at 75 μCi/ml. The strains tested were D110 (panel A) and CRBD110 (panel B). (x) 30 μg/ml of coumermycin A_1; (●) diluent.

TABLE II: Ordering of *cou*^{r}, *dna*A, and *ilv* by P_1 Transduction.[a]

Donor Genotype *cou*^{r} *dna*$^+$ *ilv*$^+$	Recipient Genotype *cou*^{s} *dna*A *ilv*	Selected Markers *ilv*$^+$ *dna*$^+$	Linked Marker *cou*^{r}	% Segregation of linked with selected Marker
CRBD110		93	78	84
CRJC12	CRT46	304	269	88
CRKL16		111	94	85
		ilv$^+$ *cou*^{r}	*dna*$^+$	
CRBD110		78	77	99
CRJC12	CRT46	67	67	100
CRKL16		94	93	99

[a] *dna*$^+$ recombinants were scored at 43 °C on TYE plates; *ilv*$^+$ were scored at 30 °C on M9 minimal plates lacking valine and isoleucine; *cou*^{r} was determined at 30 °C or 37 °C on TYE plates in the presence of 50 μg/ml of coumermycin A_1 when the P_1 stock was prepared from CRBD110 and 25 μg/ml when the phage host had been CRJC12 or CRKL16.

crosses (Table II). It was found that when P_1 phage were grown on coumermycin A_1-resistant strains (*cou*^{r} *dna*$^+$ *ilv*$^+$) and used to infect a drug-sensitive recipient (*cou*^{s} *dna*A *ilv*) almost all (237/239) of the *ilv*$^+$ *cou*^{r} recombinants were also *dna*$^+$, whereas only 87% (441/508) of the *ilv*$^+$ *dna*$^+$ transductants were also *cou*^{r}. This indicated that the order of these three mutations was *cou*^{r}, *dna*A, *ilv*.

Although it was not proven directly, it is likely that this mutation to coumermycin A_1 resistance was identical with that to novobiocin resistance. An *E. coli*-permeability mutant CF1 (Aline and Reznikoff, 1975) was used to select for novobiocin resistance. When tested, it was also found to be coumermycin A_1 resistant. This phenomenon of cross-resistance between novobiocin and coumermycin A_1 has been documented for *Staphylococcus aureus* mutants (Fedorko et al., 1969).

Identification of an Altered Protein in a cou^r *Strain.* CRBD110 was less sensitive to coumermycin A_1 than D110 even after toluene treatment that was expected to allow a free diffusion of low-molecular-weight compounds (Ryan and Wells, 1976). However, it could still be argued that the mutation in CRBD110 resulted in drug resistance only in an indirect way, for example, by increasing the permeability barrier against this antibiotic. To differentiate this possibility from a direct alteration in the target site, a crude extract of each strain was applied to a novobiocin affinity column that was developed as described (Methods). When samples of each fraction were analyzed on polyacrylamide gels under denaturing conditions, it was found that the flow-through and buffer wash both contained too many bands for any meaningful analysis. The final wash with 1.0 M sodium acetate (pH 4.5) did not contain a significant amount of protein. Analysis of the eluate obtained with 20 μg/ml of coumermycin A_1 contained approximately 20 proteins (Figure 5). However, there is one major band having a molecular weight of approximately 37 000 (R_f = 0.58) that is present in the eluate of the sensitive parent strain D110 that is considerably reduced in intensity in the resistant derivative CRBD110.

The results shown in Figure 5 were those expected if a target protein interacted strongly with the antibiotic and, therefore, was bound to the affinity column until eluted with coumermycin A_1. However, the analagous protein, in CRBD110, either did not bind to the column or was easily eluted with the buffer alone. These data suggest, but do not prove, that mutation to coumermycin A_1 resistance resulted from an alteration in the target site, a soluble protein of 37 000 molecular weight.

Influence of Coumermycin A_1 on Cell Viability. The effect of coumermycin A_1 on cell viability was tested under conditions

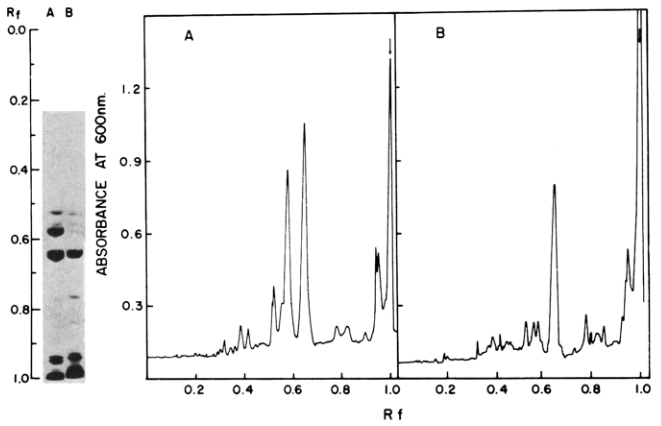

FIGURE 5: Gel electrophoresis of novobiocin affinity column eluates. Gel A and its corresponding absorbance profile represent those proteins in a crude extract of D110 that adhered to a novobiocin affinity column and were eluted with 20 μg/ml of coumermycin in 50 mM Tris-HCl (pH 7.5)–10% sucrose. Gel B and its absorbance profile represent the corresponding fraction of crude extract protein in CRBD110. Crude extract preparation, column construction and development, and sodium dodecyl sulfate gel electrophoresis were as described under Methods. The arrow (R_f = 1.0) indicates the position of the bromophenol blue marker.

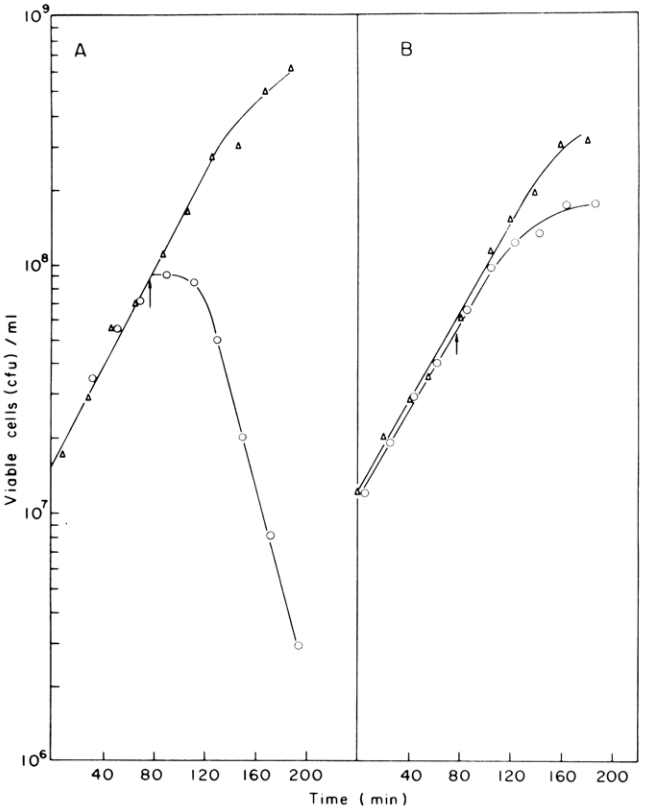

FIGURE 6: Bacteriocidal effect of coumermycin A_1. Two cultures each of D110 (panel A) and CRBD110 (panel B) were grown at 37 °C in LB medium supplemented with 20 μg/ml of thymidine and 10 μg/ml each of uracil and leucine. At the time indicated by the arrow, coumermycin was added to 30 μg/ml to one culture of each strain (○) and an equivalent amount of the drug diluent, Me_2SO, to the other (△). Viable cells were determined as described under Methods.

similar to those used for studying DNA, RNA, and protein synthesis. Figure 6 (panel A) shows that addition of this antibiotic to 30 μg/ml immediately arrests the viable cell count of D110 and, within approximately 40 min, it enters a logarithmic killing curve with a half-time of 15 min. The growth rate of the coumermycin A_1-resistant derivative CRBD110 is also affected (panel B) but without any evidence of cell killing.

A similar study was also performed in LB medium using four other strains (KL16, KL25, KL99, and ATR2444) in addition to D110 and CRBD110. The results (not shown) indicated that all five sensitive strains were killed in the presence of 30 μg/ml of coumermycin A_1. It was also observed that the optical density increased at least twofold during this incubation, whereas the viable cell count decreased at least 90%. One of these strains KL16 was subsequently tested in an M9 minimal medium where it was found that coumermycin A_1 exhibited a bacteriostatic effect on this strain. However, nucleic acid synthesis was still inhibited under these growth conditions indicating that the observed loss of viability in LB medium is not a primary effect of coumermycin A_1.

Stability and Integrity of the DNA Template in the Presence of Coumermycin A_1. A mechanism that could explain an inhibition of both DNA and RNA synthesis, as well as a bacteriocidal action under certain conditions, would be degradation of the bacterial chromosomal DNA. To test for this, D110 was labeled for three to four generations in the presence of [^{3}H]thymidine and then the radioactive label was removed from the medium. The maintenance of acid-precipitable radioactivity was monitored as an indication of the stability of parental DNA in the presence of 30 μg/ml of coumermycin A_1, the same concentration used in Figures 2–4 and 6. The results (not shown) indicate that the level of Cl_3CCOOH-insoluble radioactivity remained constant over a 3.5-h period of incubation in the presence of coumermycin A_1 even though a 100-fold reduction in viable cells occurred (Figure 6). In a control experiment, almost 50% was rendered acid soluble in the presence of 100 μg/ml of nalidixic acid.

To evaluate further the potential role of nucleases in explaining the effects of coumermycin A_1, the molecular weight distribution of the DNA, labeled as above and isolated from D110 after incubation in the presence of these drugs, was investigated. Figure 7 shows that DNA from coumermycin A_1-treated bacteria had a molecular weight distribution identical to that of the drug-free control. These profiles are in marked contrast to that obtained with the nalidixic acid-treated bacteria in which the average molecular weight of the DNA has decreased considerably. Since these analyses were done on alkaline sucrose gradients, extensive single-strand nicking would have been detectable.

These two experiments suggested that the observed inhibitions of nucleic acid synthesis and killing of bacteria were not the result of template degredation. They do not, however, rule out the possibility that the DNA strands were cross-linked by the drug or a metabolite of it. This possibility was examined to testing DNA isolated from coumermycin A_1-treated bacteria for the presence of a fraction that would renature rapidly after thermal denaturation. As a control, mitomycin C-treated bacteria were studied also. Results from these experiments indicated that if such cross-links were present, there were fewer than 10/chromosome (Methods). Other studies (Ryan and Wells, 1976) also have failed to demonstrate any interaction between coumermycin A_1 and DNA.

Preferential Inhibition of Replicative DNA Synthesis. A number of systems for DNA synthesis exist in *E. coli,* since there are different requirements for bacterial, phage, and plasmid replication, as well as for repair (Wells and Inman, 1973; Kornberg, 1974). Coumermycin A_1 inhibits DNA synthesis in *E. coli* (Figure 2) that is, presumably, almost entirely chromosomal replication. To determine the specificity, if any, of coumermycin A_1, the relative inhibition of replicative

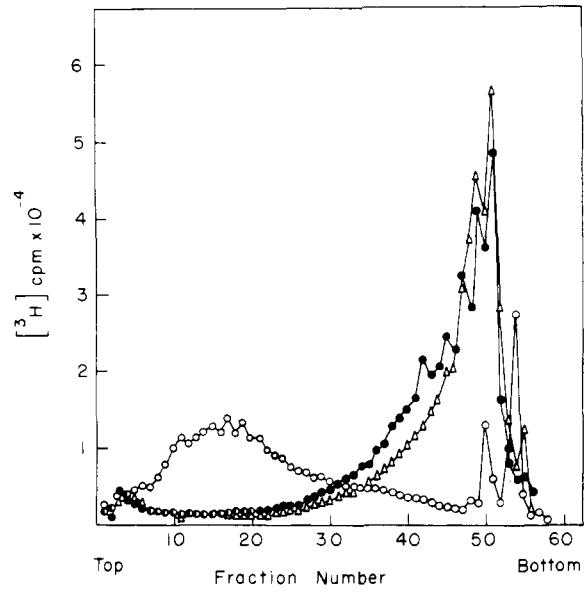

FIGURE 7: Molecular weight of parental DNA in the presence of coumermycin A_1. D110 was grown and its DNA was labeled as described under Methods. After 1 h in the presence of 30 μg/ml of coumermycin A_1 (△), its diluent (Me_2SO) (●), or 100 μg/ml of nalidixic acid (○), the bacteria were lysed and subjected to alkaline sucrose sedimentation as described under Methods. The expected positions of 17.5S and 45S colicin E_1 markers were at fraction 15 and 45, respectively.

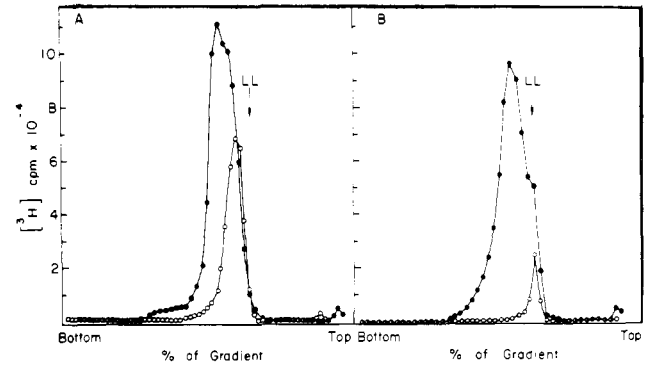

FIGURE 8: Density labeling of progeny DNA in the presence of coumermycin A_1. The DNA of *E. coli* D_2TT was density labeled with 5-bromodeoxyuridine in the presence of 100 μg/ml of coumermycin A_1 (○) or an equivalent amount of its diluent, Me_2SO (●), either with (panel B) or without (panel A) prior exposure to uv light. The arrow marks the position of the fully light ^{32}P λ DNA internal marker. Conditions and procedures were as described under Methods. Fully heavy DNA (4.3-h labeling period) was found 33% of the gradient from the λ DNA marker.

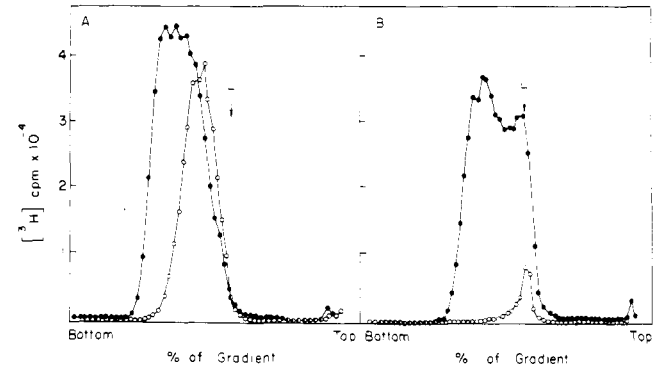

FIGURE 9: Buoyant density of denatured progeny DNA labeled in the presence of coumermycin A_1. DNA from *E. coli* D_2TT, density labeled in the presence (○) or absence (●) of 100 μg/ml of coumermycin A_1, with (panel B) or without (panel A) exposure to uv light, was denatured with NaOH, reneutralized, and analyzed on CsCl density gradients as described under Methods.

as opposed to uv-induced repair synthesis of DNA was studied.

Repair synthesis can be differentiated from replicative DNA synthesis primarily by the length of the product made in each case. After one complete round of DNA replication, the entire length of one strand of the chromosome is newly made. However, repair processes result in only a relatively short "patch" of nascent DNA. Substitution of 5-bromodeoxyuridine for thymidine in growing *thy*$^-$ bacteria will raise the buoyant density of a sheared DNA fragment in proportion to the relative amounts of newly made (5-bromodeoxyuridine labeled) to parental (thymidine labeled) DNA in an individual fragment. Consequently, nascent DNA that has not increased in density above a fully light (thymidine labeled) internal marker is defined as repair, whereas any DNA product of greater density was considered to represent DNA replication (Hanawalt and Cooper, 1971).

The effects of coumermycin A_1 on replicative and the uv-induced repair synthesis of DNA are shown in Figure 8. In the absence of drug, the 5-bromodeoxyuridine-labeled DNA was clearly of greater density than the fully light marker. Since the 1-h labeling period was equal to the generation time of these bacteria, the small heavy peak should represent DNA-containing 5-bromodeoxyuridine in both strands as a result of reinitiation of a round of chromosomal replication.

After addition of coumermycin A_1 (resulting in a 40% inhibition of DNA synthesis), the product was still of greater density than the internal fully light marker and, therefore, was considered due to replication. However, the peak fractions of these profiles for the two samples do not coincide, neither in their native (Figure 8, panel A) nor in their denatured states (Figure 9, panel A). In addition, the profile of the untreated control, after denaturation, was very broad, indicating a varying degree of substitution of the dBrU density label for dT. This would be the expected result if there were some endogenous dT present that is known (Hackett and Hanawalt, 1966; Kanner and Hanawalt, 1968) to be incorporated preferentially to dBrU. The source of this dT pool is unknown but, if this were the case, the density of the product would gradually increase as this dT pool was exhausted, resulting in a spectrum of DNA products with varying degrees of 5-bromodeoxyuridine substitution. Under these conditions, the difference between the mean buoyant density distribution of DNA from the drug-treated culture and the control would be greatest in shorter incubations and then decrease with time. In fact, when the incubation was increased to 4.3 h, almost 90% of the control DNA was in a position expected for fully heavy DNA. The coumermycin A_1-treated culture (55% inhibition) yielded DNA that was primarily of hybrid density with approximately one-fourth in a position indicating 5-bromodeoxyuridine substitution in both strands. These results suggest that DNA replication was inhibited by coumermycin A_1. When parallel experiments were performed using toluenized bacteria and exogenously supplied dBrUTP, there was no significant dilution of the density label. In this case, the well-separated peak representing DNA replication was completely inhibited by coumermycin A_1 (Ryan and Wells, 1976).

After treatment of samples of bacteria with uv irradiation (Figure 8, panel B), the effect of coumermycin A_1 was more pronounced. With the drug-free control there was some replicative DNA synthesis with a light shoulder that represented uv-induced repair synthesis. The fractions were not as well

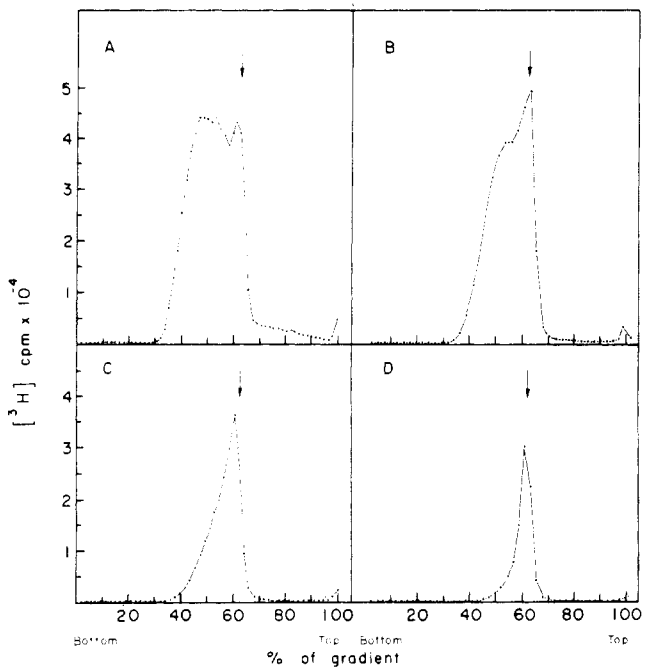

FIGURE 10: Buoyant density of progeny DNA synthesized by uv-irradiated D_2TT in the presence of low levels of coumermycin A_1. All procedures, including the alkali denaturation and reneutralization of samples, were as described (legend to Figure 9 and Methods) except that the levels of coumermycin A_1 tested were 0, 5, 10, and 20 μg/ml, respectively, in panels A, B, C, and D. The arrow indicates the position of the denatured ^{32}P λ DNA marker.

separated from the light marker as the control in panel A, probably the result of dilution of the density label by endogenous thymidine released by degradation of the uv-damaged DNA (Hanawalt and Cooper, 1971). Denaturation of this sample (Figure 9, panel B) clearly shows the presence of uv-induced repair synthesis. Addition of coumermycin A_1 reduced this light shoulder by roughly 80%, whereas replicative synthesis is completely inhibited. In a similar experiment (data not shown) employing identical conditions for uv irradiation but with the incubation period increased to 4.3 h, essentially the same result was obtained.

The observation that there was still some residual uv-induced repair synthesis, despite the total abolition of replicative DNA synthesis, indicated that coumermycin A_1 preferentially inhibited replicative synthesis. However, the uv irradiation of these bacteria has clearly increased their sensitivity to this antibiotic. Therefore, in order to demonstrate this preference, D_2TT was exposed to uv light and density labeled as above but lower concentrations of coumermycin A_1 were tested. In the presence of 10 μg/ml of this antibiotic, there was a 75% decrease in the peak of replicative DNA synthesis (Figure 10). Even at 5 μg/ml of coumermycin A_1 there was a significant shift in the buoyant density profile, indicating that replicative DNA synthesis was preferentially inhibited by this antibiotic.

Discussion

Coumermycin A_1 inhibited nucleic acid synthesis in *E. coli*. For D110, there was a slightly greater effect on replication than on transcription in vivo, while in vitro DNA synthesis was clearly more sensitive to this antibiotic than was RNA synthesis (Ryan and Wells, 1976). The drug-resistant strain tested, CRBD110, was a nonmutagenized spontaneous derivative of D110 and, therefore, it was very unlikely that these two strains differed in more than this one allele. However, as a result of this mutation, the coumermycin A_1 sensitivity of both replication and transcription was decreased, suggesting that a single target site had a role in or effect on both processes, at least in the presence of this antibiotic. Although CRBD110 survived in the presence of coumermycin A_1, its growth rate was still inhibited even though there was no inhibition of transcription. This indicated that the partial coumermycin sensitivity of DNA replication observed in this strain both in vivo as well as in vitro (Ryan and Wells, 1976) was probably responsible for the inhibition of growth. Because of this correlation and the greater sensitivity of DNA synthesis to coumermycin A_1 mentioned above, it is concluded that this antibiotic is a preferential inhibitor of relication.

Although less sensitive than replication, the uv-induced repair synthesis of DNA also was inhibited by coumermycin A_1. However, a fraction of this repair synthesis was detected even in the presence of 200 μg/ml of coumermycin A_1 (not shown). This could be significant, since it was reported that the "patch" size of repaired DNA is quite heterogeneous, with some repaired stretches being at least 3000 nucleotides in length (Cooper and Hanawalt, 1972a). Also, it was suggested that there was at least two separate and distinct systems for DNA repair in *E. coli*, with the *rec* system responsible for large patch synthesis and the *uvr* system carrying out efficient short-patch repair mediated by the *E. coli* DNA polymerase I (Cooper and Hanawalt, 1972b). Since this enzyme apparently is responsible for a repair-like synthesis that is not inhibited by coumermycin A_1 in toluenized bacteria (Ryan and Wells, 1976), it is possible that the fraction of repair synthesis observed in the presence of high concentrations of coumermycin A_1 could represent short-patch repair carried out by DNA polymerase I.

Coumermycin A_1 exerts either bacteriocidal or bacteriostatic effects on *E. coli* depending on the growth conditions. The lack of killing in the M9 medium may be the result of the presence of magnesium ions, since it was shown that this divalent metal ion antagonized both the leakage of cellular metabolites and the bacteriocidal effects of novobiocin on *E. coli* (Morris and Russel, 1969, 1970). It was suggested that novobiocin acted by inducing a magnesium deficiency (Brock, 1967). The mode of action of coumermycin A_1, however, is not related to the magnesium level, since inhibitions of nucleic acid synthesis were observed both in M9 medium (data not shown) as well as in spheroplasts tested in a medium containing 0.2% $MgSO_4$.

There are several general mechanisms by which coumermycin A_1 could hinder DNA replication. Two unlikely mechanisms are the inhibition of DNA precursor synthesis or a direct interaction of coumermycin A_1 with the DNA template; these are discussed in the accompanying paper (Ryan and Wells, 1976). Thirdly, a drug induced degradation of the chromosomal DNA does not agree with the data presented herein.

Instead, the data suggests that a protein, perhaps the 37 000 molecular-weight protein observed by drug-affinity chromatography, is the target site for coumermycin A_1. One speculative mechanism is that the coumermycin A_1 sensitive factor, which may be essential for replication, normally interacts reversibly with the chromosomal DNA. However, in the presence of this antibiotic, multiple copies of a drug–protein complex form bind to the DNA and dissociate more slowly than in the absence of drug. In this event, the degree to which a polynucleotide synthetic reaction is inhibited might reflect its rate of elongation relative to the dissociation rate of the protein-

drug-DNA complex, the length of the product made, and the ability of the synthetic process to displace such a block. This would explain the stronger inhibition of the faster and more extensive process of chromosomal replication relative to transcription and long-patch repair synthesis of DNA, while small patches of DNA repair would not be sensitive to this antibiotic. Mutation to coumermycin A_1 resistance could result from a decrease in the stability of the drug–protein interaction allowing, for example, transcription and, therefore, protein synthesis to proceed without hindrance, whereas DNA replication would still be inhibited to some degree. Further studies with the purified coumermycin A_1 sensitive protein are necessary to validate or disprove this model. Hopefully, these studies also will provide new information on chromosomal replication.

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