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

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ABSTRACT: In vitro inhibitions by coumermycin A_1 of DNA and RNA synthesis in toluenized cells were studied. In a sensitive strain, 50% inhibitions of replication and transcription were observed at 0.035 and 0.600 μg/ml, respectively. DNA synthesis in a toluenized-resistant mutant was 50% inhibited at 0.140 μg/ml of coumermycin A_1, whereas RNA synthesis was unaffected at all concentrations tested. Studies with a mixture of toluenized-sensitive and -resistant bacteria ruled out the presence of a diffusable activator or inhibitor of coumermycin A_1 action. Density label studies with toluenized *pol* A^+ and *pol* A^- strains indicated that replicative DNA synthesis was specifically inhibited, in agreement with the in vivo studies in the preceding paper of this issue (Ryan, M. J. (1976), *Biochemistry 15*). Highly purified *Escherichia coli* DNA polymerase III and RNA polymerase both were inhibited by this antibiotic. However, the high concentrations necessary for these inhibitions suggest that they are not biologically relevant. No interaction between DNA and coumermycin A_1 was observed with the following analytical procedures: ultraviolet difference spectra, DNA absorbance—temperature transitions, equilibrium buoyant density centrifugation, and DNA cross-linking determinations.

The preceding paper in this issue (Ryan, 1976) showed that coumermycin A_1 preferentially inhibited chromosomal DNA replication in vivo. Although in vivo studies can show a primary site of action of an antibiotic, they reveal little concerning its detailed mode of action. In order to characterize further the effects of this antibiotic on *E. coli*, in vitro experiments were performed, testing coumermycin A_1 against DNA and RNA synthesis in toluenized cells. In addition, the sensitivity of purified nucleic acid polymerases to coumermycin A_1 was determined and physical studies were performed to test the possibility of a direct interaction between this antibiotic and DNA. A portion of these results was reported previously (Ryan and Wells, 1975).

Materials and Methods

Preparation of Toluenized Bacteria. Toluenization was carried out essentially as described (Moses and Richardson, 1970).

Enzymes. E. coli DNA polymerase III, prepared as described (Kornberg and Gefter, 1972), was a gift of T. Tamblyn and its characteristics were reported (Tamblyn and Wells, 1975). *E. coli* RNA polymerase was purified as described (Burgess, 1969), including the additional glycerol gradient centrifugation. This fraction was a gift of J. B. Dodgson.

Assay Conditions. DNA synthesis in toluenized cells was assayed as described (Moses and Richardson, 1970). Reactions were run at 36 °C for 45 min, and the entire assay was precipitated according to procedure B (Ryan, 1976).

Assays for RNA synthesis in toluenized bacteria were as above except that the dNTP's were replaced by 500 μM each of ATP[1] and GTP, 100 μM each of UTP and CTP (with [^{3}H]UTP labeled at a specific activity of 40 to 70 cpm/pmol). Assays were performed for 20 min at 36 °C and radioactive product was determined as described above.

In almost all cases, assays were run in duplicate and the average values are reported. For DNA and RNA synthesis, each assay normally was within 5 or 10% of the average, respectively. *E. coli* RNA polymerase assays were as described (Burgess, 1969) except that 17 μM φX174 replicative form DNA was used as template. Assays were performed at 30 °C with aliquots analyzed using procedure A (Ryan, 1976).

E. coli DNA polymerase III was assayed at 26 °C essentially as described (Tamblyn and Wells, 1975) using as template 300 μM salmon sperm DNA that had been activated as described (Gass and Cozzarelli, 1973). Aliquots were analyzed using procedure A (Ryan, 1976).

Drugs. Rifampicin was purchased from Sigma and streptolydigin was a gift to Julian Davies (this department) from the Upjohn Company. The latter two antibiotics were dissolved in Me_2SO and stored at −20 °C.

DNAs. λplac$_5$S$_7$CI$_{857}$, *E. coli*, and φX174 replicative form DNAs were prepared and characterized as described (Chan and Wells, 1974; Wartell et al., 1974; Dodgson et al., 1976) and were gifts of I. Nes, J. E. Larson, and J. B. Dodgson, respectively.

Reversal of Coumermycin A_1 Inhibition of E. coli RNA Polymerase. Two 0.1-ml reactions, one containing 50 μg/ml of coumermycin A_1 and the second an equivalent amount of the drug diluent, Me_2SO, were incubated at 30 °C. At regular intervals, three 0.02-ml aliquots were removed and, after 14 min, 0.02 ml of the reaction was diluted tenfold into an identical, prewarmed drug-free reaction mixture. Thereafter, 0.045-ml aliquots were taken at regular intervals.

Other Materials and Methods. Other materials and techniques including drugs, enzymes, labeled and nonlabeled nu-

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[1] Abbreviations used are: ATP, GTP, UTP, CTP, TTP, adenosine, guanosine, uridine, cytidine, and thymidine triphosphates; uv, ultraviolet.

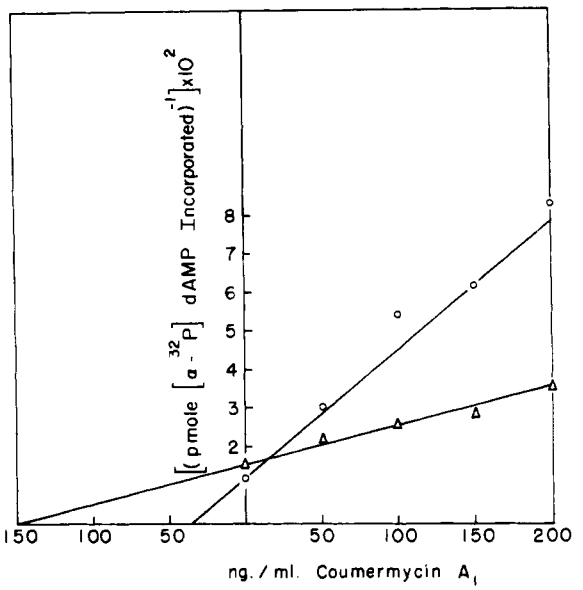

FIGURE 1: Comparative inhibition of DNA synthesis by coumermycin A_1. Assays were performed as under Methods. Each 1.0-ml reaction contained 2×10^8 toluenized bacteria (○) D110; (△) CRBD110.

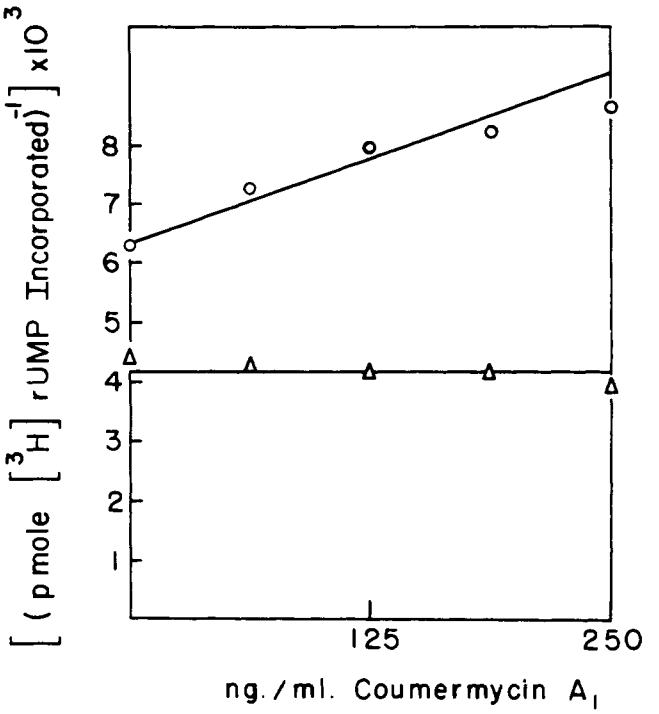

FIGURE 2: Comparative inhibition of RNA synthesis by coumermycin A_1. Assays were performed as under Methods. The 0.4-ml reactions contained 8×10^7 toluenized CRBD110 (△) or 1×10^8 toluenized D110 (○).

cleoside triphosphates, dBrUTP, thermal denaturation, and analytical sedimentation studies were as described (Ryan, 1976; Harwood et al., 1970; Grant et al., 1972; Burd et al., 1975; Wartell et al., 1975).

Results

Inhibition of Nucleic Acid Synthesis in Toluenized Bacteria. E. coli treated with toluene carry out ATP-dependent replicative DNA synthesis (Moses and Richardson, 1970) that continues from the same point at which the replicating fork stopped in vivo (Burger, 1971). When this system was tested using toluenized D110, it was found (Figure 1) that DNA synthesis was very sensitive to coumermycin A_1. For the purpose of making quantitative comparisons, this reciprocal plot was useful, since there was a linear relationship between (product synthesized)$^{-1}$ and the level of antibiotic in the assay. The amount of drug needed for a 50% inhibition of synthesis (K_{50}) was the absolute value of the intercept on the abscissa.

There are a number of significant conclusions to be drawn from this experiment. First, since coumermycin A_1-sensitive synthesis is a continuation of the in vivo process, it is clear that this antibiotic inhibits the elongation of chromosomal DNA. Second, since the necessary deoxyribonucleoside triphosphates were supplied exogenously and inhibitions were still obtained, it can be concluded that this antibiotic did not function by inhibiting the synthesis of precursors. Third, assuming that there is a free exchange of both coumermycin A_1 and Mg^{2+} between the assay medium and the interior of the cell, it is unlikely that this antibiotic acted simply by depletion of Mg^{2+}, as suggested by other workers (Brock, 1967), since the concentration of Mg^{2+} is six orders of magnitude higher than the levels of drug used in these experiments. Finally, DNA synthesis in these cells was almost three orders of magnitude more sensitive than in vivo (Ryan, 1976) indicating a marked increase in cellular permeability.

When a resistant derivative of this strain (CRBD110) was toluenized and compared with its sensitive parent (D110) it was found (Figure 1) that it required 140 ng/ml of coumermycin A_1 to inhibit its DNA synthesis 50%, a fourfold higher level than required for D110.

When these two strains were toluenized and tested for sensitivity of their RNA synthesis (Figure 2) to coumermycin A_1, it was found that approximately 600 ng/ml of coumermycin A_1 would have been required to give a 50% inhibition of this synthesis in D110, whereas its resistant derivative, CRBD110, showed no detectable sensitivity even at the highest concentration tested (0.25 μg/ml).

These results are in good agreement with the data obtained in vivo (Ryan, 1976), since (a) DNA synthesis was more sensitive to this antibiotic than RNA synthesis and (b) DNA synthesis in CRBD110 still showed some sensitivity to this drug but its RNA synthesis was not affected by the presence of coumermycin A_1. These data suggest that DNA synthesis is the primary site of action of coumermycin A_1 especially since the growth rate of CRBD110 is inhibited by this drug (Ryan, 1976) in the absence of any effects on transcription. Although toluenized D110 and CRBD110 demonstrated a differential sensitivity to coumermycin A_1, they both were equally sensitive to nalidixic acid and rifampicin, specific inhibitors of DNA and RNA synthesis, respectively (Corcoran and Hahn, 1975). Nalidixic acid gave a 50% inhibition of DNA synthesis in both strains at 47 μg/ml, and rifampicin gave an equivalent inhibition of RNA synthesis at 38 ng/ml.

Differential Inhibition of Replicative and Repair Synthesis of DNA by Coumermycin A_1. In vivo studies indicate that coumermycin A_1 inhibited replicative DNA synthesis more strongly than repair synthesis (Ryan, 1976). A similar experiment was performed using toluenized 1100 (*pol*A$^+$ *end*I$^-$) and D110 (*pol*A$^-$ *end*I$^-$). DNA synthesis in the latter strain was completely (95%) dependent on the presence of ATP, whereas it was not in the former strain. Each strain was incubated in reaction mixtures in which 5-bromodeoxyuridine triphosphate replaced dTTP, in the presence or absence of 100

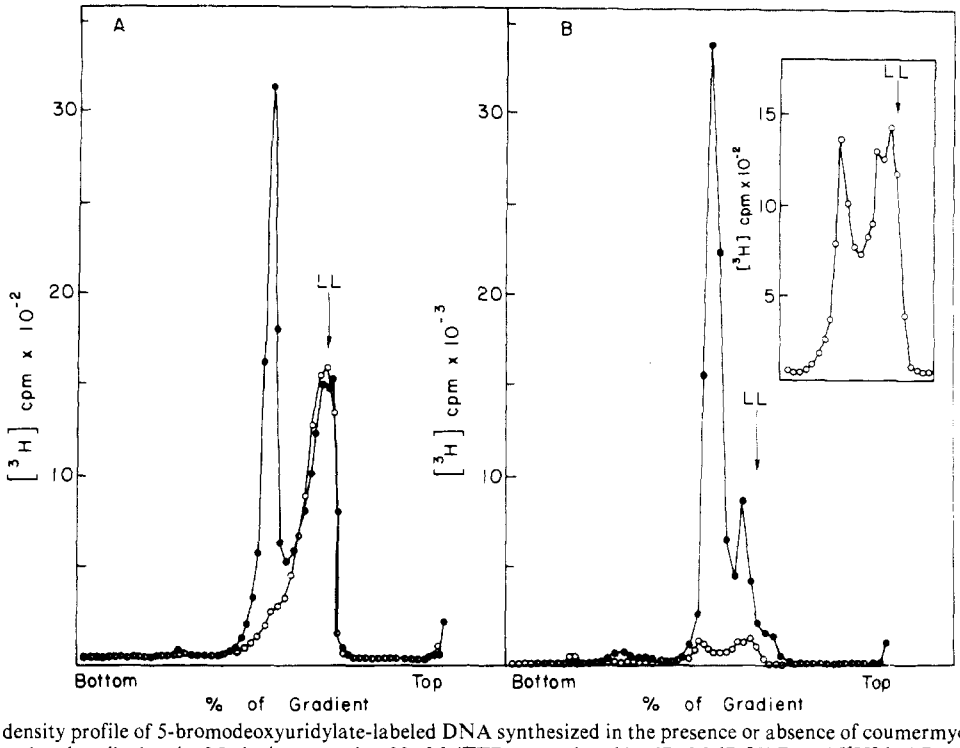

FIGURE 3: Buoyant density profile of 5-bromodeoxyuridylate-labeled DNA synthesized in the presence or absence of coumermycin A_1. Reactions (2 ml each) were performed as described under Methods except that 30 μM dTTP was replaced by 67 μM dBrUTP, and [^{3}H]dATP was added to a specific activity of 100 cpm/pmol. The assays were performed with two strains of toluenized *E. coli:* 1100 (*pol*A$^+$) and D110 (*pol*A$^-$). Cell lysis and CsCl density gradient centrifugation were performed as described under Methods (Ryan, 1976). The arrow indicates the position of fully light ^{32}P λ DNA marker. Panel A is the profile obtained with toluenized 1100, panel B is that obtained with toluenized D110. (●) No drug, (○) 100 ng/ml of coumermycin A_1.

ng/ml of coumermycin A_1. Buoyant density profiles of the DNA synthesized in each case are presented in Figure 3.

Panel A shows that in the *pol*A$^+$ strain, 1100, the DNA had two distinct peaks that corresponded to replicative and repair synthesis as defined previously (Ryan, 1976). After addition of coumermycin A_1, the repair peak was unchanged, whereas the hybrid density peak of replicative DNA synthesis was virtually abolished, indicating that this antibiotic had a clear specificity for replicative DNA synthesis in this system. The DNA product isolated from the *pol*A$^-$ strain, D110, also had two peaks in the CsCl gradient (panel B). In this case, however, only about 20% of the products was repair synthesis. Addition of coumermycin A_1 to this reaction reduced the replicative DNA synthesis by approximately 95%. In this case (inset to panel B), there was more repair synthesis than replicative synthesis. This data indicates that repair synthesis of DNA was less inhibited than replicative synthesis; however, both were strongly inhibited in D110 even if to a somewhat varying degree.

The repair synthesis seen in the *pol*A$^+$ strain, 1100, was likely the result of endogenous nucleases-generating primers that then were extended by DNA polymerase I. D110, the *pol*A$^-$ strain, lacks this polymerase and, therefore, the repair synthesis seen with this strain may employ factors that were shared with a replicative DNA synthetic complex, e.g., DNA polymerases II and III (Tait et al., 1974). If this were the case, it might explain why this repair synthesis was inhibited by coumermycin A_1.

Thus, it is concluded that replicative synthesis was specifically inhibited by coumermycin A_1, whereas repair synthesis, at least that mediated by the *E. coli* DNA polymerase I, was not affected.

Irreversibility of Inhibition of DNA Synthesis by Coumermycin A_1 in Toluenized Cells. Attempts were made to reverse the inhibition of DNA synthesis in toluenized D110 by diluting the reaction tenfold into a drug-free assay mixture. Alternatively, the cells were centrifuged after a short incubation with the drug and were resuspended in an equal volume of drug-free reaction mixture (data not shown). Both experiments indicated that, within the time periods examined, coumermycin A_1 inhibition of DNA synthesis was not reversed. This could indicate the formation of a drug–receptor complex that was very stable, once formed under these conditions.

Absence of a Diffusable Activator or Inhibitor of Drug Action. The observed differential sensitivity of D110 and CRBD110 to coumermycin A_1 might be explained by the presence of an activator or inhibitor of drug action present in the toluenized bacteria. Testing this possibility involved performing reactions with each strain alone in the presence and absence of coumermycin A_1. This data was then compared with that obtained with a mixture of both strains of toluenized bacteria. Table I shows that the DNA synthesis, both in the presence and absence of coumermycin A_1, of each strain appeared to be independent of the other, since the incorporation for the mixture of strains was equal to the sum obtained with each assayed separately. The close agreement between the sum of 1 and 2 and the actual incorporations (3) in each case indicated the absence of diffusable factor that either potentiated or diminished the inhibitory action of coumermycin A_1 in this system.

Absence of a Detectable Interaction between Coumermycin A_1 and DNA. Since most of the data obtained could be explained by the presence of a drug–DNA interaction, studies were performed to test for such a complex. Two determinations of the ability of the drug to perturb DNA properties and one determination of the ability of DNA to perturb the drug properties were performed. Thermal denaturation studies (data not shown) were carried out with *E. coli* DNA. When added,

TABLE I: Additivity of DNA Synthesis in Toluenized D110 and CRBD110 in the Absence and Presence of Coumermycin A_1.[a]

Toluenized Strain	pmol of [α-^{32}P]dGMP Incorporated		
	Control (+ diluent)	Coumermycin A_1 (+55 ng/ml)	Coumermycin A_1 (+110 ng/ml)
(1) CRBD110	129	76	55
(2) D110	325	85	56
Sum of (1) + (2) performed separately	454	161	111
(3) Mixture of CRBD110 and D110	457	173	119

[a] Assays were performed as described under Methods. Three sets of 0.9-ml reactions were carried out with the first containing 1×10^8 toluenized CRBD110, the second containing 3×10^8 toluenized D110, and the third containing both 1×10^8 toluenized CRBD110 and 3×10^8 toluenized D110. Each set was made up of three reactions, the first containing no drug, and the second and third containing 55 and 110 ng/ml of coumermycin A_1, respectively.

coumermycin A_1 was present in the ratio of 1 drug molecule/base pair of DNA. The T_m's were identical in the presence and absence of coumermycin A_1 and were 72 °C (14 mM Na^+) and 86 °C (0.1 M Na^+).

The analytical buoyant density of *E. coli* DNA in the presence and absence of coumermycin A_1 was also tested as described (Wartell et al., 1974); no change was detected at the ratio of 2 drug molecules/DNA base pair.

Coumermycin A_1 absorbs strongly in the uv region with a ϵ_M of approximately 6×10^4 at 282–284 nm. Since a drug-macromolecule interaction might be expected to alter this spectrum, difference spectra were studied. No effect of λ DNA on the spectrum of coumermycin A_1 was observed at the ratio of 1 drug molecule/1.6 base pairs. However, bovine serum albumin, present at 69 μg/ml, had a considerable influence on the spectrum of coumermycin A_1, inducing a hypochromic shift below approximately 318 nm (11% hypochromism at 285 nm) and a slight hyperchromic shift at longer wavelengths (data not shown). From this experiment it is suggested that there is an interaction, probably nonspecific, between coumermycin A_1 and bovine serum albumin. However, there is no indication of an interaction between this antibiotic and DNA.

Inhibition of Purified Polymerases by Coumermycin A_1. *E. coli* DNA polymerase III and RNA polymerase are essential for the in vivo processes of replication and transcription, respectively, and, therefore, are possible target sites for coumermycin A_1. When tested, both were 50% inhibited by coumermyin A_1 at levels between 10 and 20 μg/ml (Figure 4). These values are approximately 30- and 200-fold higher than the levels required for inhibition of RNA and DNA synthesis, respectively, in toluenized D110. In contrast, streptolydigin gave a 50% inhibition of RNA synthesis in toluenized cells at 5–10 μg/ml (data not shown), and approximately an 80% inhibition of the purified RNA polymerase at 10 μg/ml. Despite the apparent sensitivity of these purified enzymes to coumermycin A_1, it is not likely that they are specific inhibitions. For example, in contrast with the results obtained in toluenized cells, a tenfold dilution of an RNA polymerase assay immediately and completely reversed a total inhibition of this enzyme by coumermycin A_1 (Figure 5). There is also a considerable amount of evidence that this antibiotic can interact

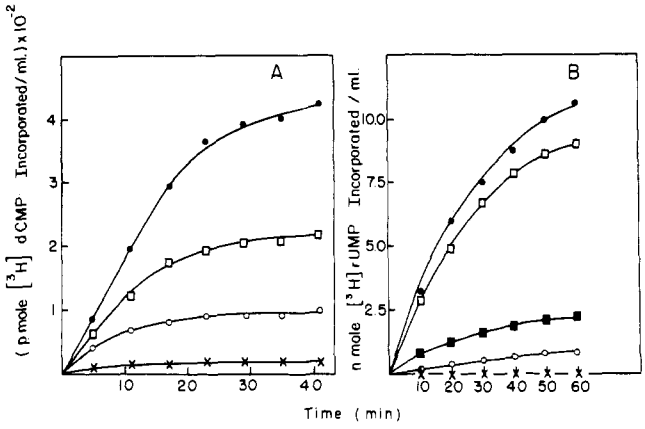

FIGURE 4: Inhibition of *E. coli* DNA polymerase III and *E. coli* RNA polymerase by coumermycin A_1. Assay conditions were as described under Methods. Inhibition of DNA polymerase III and RNA polymerase are shown in panels A and B, respectively. (●) No drug, (□) 10 μg/ml of coumermycin A_1, (○) 20 μg/ml of coumermycin A_1, (X) 50 μg/ml of coumermycin A_1, (■) 10 μg/ml of streptolydigin.

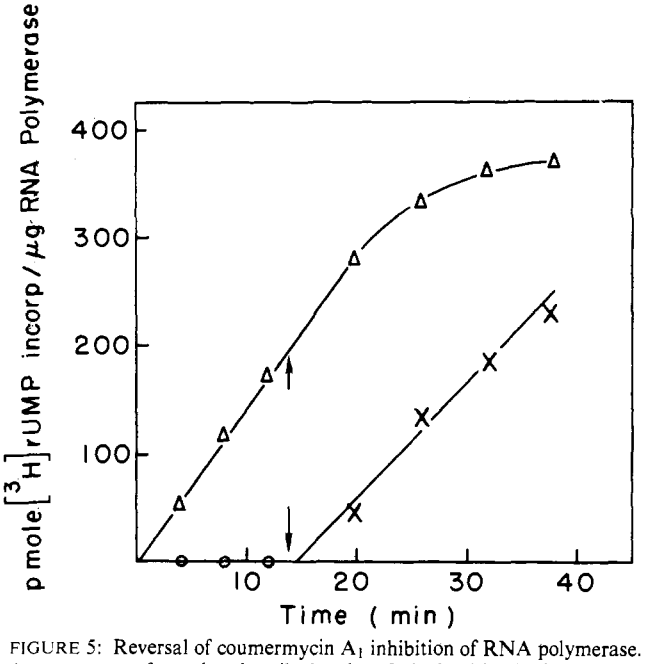

FIGURE 5: Reversal of coumermycin A_1 inhibition of RNA polymerase. Assays were performed as described under Methods, either in the presence (○) or absence (△) of 50 μg/ml of coumermycin A_1. After 14 min of incubation (arrow), each reaction was diluted tenfold, with the concentration of coumermycin A_1 reduced to 5 μg/ml (x) in one assay.

nonspecifically with proteins including: (1) the difference spectrum obtained above with bovine serum albumin; (2) the observation (Fedorko et al., 1969) that the presence of serum proteins raises the minimal inhibitory concentration for coumermycin A_1 from 0.004 μg/ml to 6.25 μg/ml, a 1500-fold increase, when tested against strains of *S. aureus;* (3) the large number of inhibitions of unrelated enzymatic activities reported for novobiocin (Brock, 1967), an antibiotic closely related to coumermycin A_1. In summary, it is concluded that the inhibitions of *E. coli* DNA polymerase III and RNA polymerase are probably nonspecific and unrelated to the in vivo mechanism of inhibition by this antibiotic.

Discussion

Coumermycin A_1 at very low concentrations preferentially inhibits DNA replication in toluenized *E. coli.* However, it is

somewhat perplexing that, in vitro, transcription is so much less sensitive than replication to coumermycin A_1 compared to the results obtained in vivo (Ryan, 1976). These observations are in agreement with effects seen with novobiocin in which DNA synthesis was inhibited to a greater degree than RNA synthesis in vivo (Smith and Davis, 1967). However, in vitro studies with toluenized cells (Staudenbauer, 1975) demonstrated an 80% inhibition of replication at 2 μg/ml of novobiocin with no apparent affect on transcription. Thus, in vitro coumermycin A_1 and novobiocin are specific inhibitors of DNA replication, whereas in vivo the differential effects on replication and transcription are much less pronounced. These observations have led us to refer to coumermycin A_1 as a "preferential" as opposed to a "specific" inhibitor.

One hypothesis to explain this is that, in vivo, there is some control mechanism that represses transcription when replication is inhibited and this mechanism is not functional in toluenized cells. However, there is no evidence for this and, in fact, it is contrary to observations with nalidixic acid (Goss et al., 1965) and 6-(*p*-hydroxyphenylaza)uracil (Brown and Handschumacher, 1966). In both cases, almost total inhibition of DNA synthesis was obtained with negligible effects on RNA synthesis. Furthermore, there was no inhibition of uv-induced repair synthesis of DNA by 6-(*p*-hydroxyphenylaza)uracil despite a complete inhibition of DNA replication (Brown, 1971).

Another possibility is that RNA synthesis in toluenized cells is not representative of in vivo transcription. However, this is not likely, since this system was tested by others and found to correspond well with known properties of transcription (Peterson et al., 1971).

A third possibility is an extension of a speculative model (Ryan, 1976) in which coumermycin A_1 inhibits transcription and replication by forming a highly stable complex with a target protein and the chromosomal DNA. The synthesis of RNA in vivo could be less sensitive to this complex because it would have a greater ability to bypass or overcome this block than does DNA synthesis. This was proposed since, in drug resistant mutants, RNA synthesis was unaffected by coumermycin A_1, whereas DNA synthesis still showed a reduced sensitivity to this antibiotic. Since the internal environment of the cell after toluenization is probably different from that in vivo, it could be hypothesized that there is a slight reduction of the stability of the inhibitory complex that is sufficient to allow transcription to proceed while replication is still inhibited.

The observation that there is an apparent interaction between coumermycin A_1 and bovine serum albumin, in addition to other studies (Brand and Toribara, 1975) with the closely related novobiocin, suggests that these antibiotics are capable of nonspecific interactions with proteins, perhaps by a hydrophobic interaction. The possibility of such interactions is important in evaluating the inhibitions of in vitro polymerase reactions, since the concentration of drug used is critical. Our results with toluenized *E. coli*, combined with the minimal inhibitory concentrations for other organisms (Fedorko et al., 1969), indicate that potent inhibitions can be found at levels of 0.04 to 0.004 μg/ml. Thus, inhibitions of purified DNA and RNA polymerases by 10–20 μg/ml of coumermycin A_1 are probably nonspecific. The mode of action of coumermycin A_1 will be established with certainty only when it can be shown that a purported target site, isolated from a drug-resistant bacterium, confers resistance on an in vitro, reconstituted system for the replicative synthesis of DNA.

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