The Gross and Biochemical Effects of 1-β-D-Arabinofuranosyl Cytosine on an Actinobolin-Resistant Strain of *Streptococcus faecalis*[1] (37152)

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1-β-D-Arabinofuranosyl cytosine (ara-c) has been shown to be effective against various animal neoplasms (1), certain deoxyribonucleic acid (DNA) viruses (2, 3), and in the treatment of human leukemia (4). Ara-c has also been shown to inhibit the growth of various cell culture systems *in vitro* by interfering with the synthesis of DNA (5, 6) but with virtually no initial inhibitory effect on the synthesis of ribonucleic acid (RNA) or protein (7). Additional work has revealed that this compound inhibits DNA synthesis catalyzed by mammalian DNA polymerase in which ara-c appears to be competing with deoxycytidine triphosphate (dCTP) (8). A limited number of reports are available concerning the effect of ara-c in microbial systems because this agent is not strongly inhibitory for microorganisms (9), presumably because bacteria readily deaminate ara-c to 1-β-D-arabinofuranosyl uracil (10); however, at high concentrations (approx 1–2 mg/ml), ara-c has been reported to interfere with the synthesis of DNA in *Escherichia coli* (11) and to cause thymineless death in thymine-requiring strains of *E. coli* (12). Furth and Cohen have emphasized that since ara-c does not inhibit the growth of *E. coli* at concentrations comparable to those at which it inhibits animal cells, it should not be assumed *a priori* that the mechanism by which ara-c inhibits *E. coli* is comparable to the inhibition observed in animal cells (13). No reports have been found which concern the subcellular effect of ara-c on gram-positive bacteria. The availability of a unique strain of *Streptococcus faecalis* ATCC 8043, which is resistant to the cariostatic antibiotic actinobolin (14) and is sensitive to inhibition by as little as 3 μg ara-c/ml (9), allowed studies that revealed the subcellular mechanism by which ara-c inhibits the growth of this gram-positive organism at concentrations of ara-c that approximate those required to inhibit the growth of mammalian cells.

Materials and Methods. Bacteria. Streptococcus faecalis ATCC 8043 and a laboratory-derived strain of this organism designated *S. faecalis* Acb-r, >60-fold resistant to actinobolin, were used in this study. The parent culture was maintained in stab cultures of folic acid assay agar (Difco, Detroit, MI). The antibiotic resistant strain was maintained in stab cultures of folic acid assay agar containing 1 mg actinobolin/ml. Folic acid assay medium, with or without actinobolin as required by the experimental design, was used in all experiments.

Growth and preparation of inocula. For inocula, stationary cultures of *S. faecalis* and *S. faecalis* Acb-r were incubated for 18 hr at 37°. The cells from these cultures were collected by centrifuging, washed twice in 0.85% NaCl (saline), resuspended in saline and adjusted to 20% light transmittance (540 nm) in a Bausch and Lomb Spectronic 20 colorimeter. Such suspensions contain approximately 1.0×10^9 viable cells/ml.

Extraction and measurements of nucleic acids and protein. Ten milliliter volumes were withdrawn at hourly intervals from culture flasks which had received ara-c simultaneously with the bacterial inoculum and at 15 min intervals from cultures after they had re-

[1] Supported by U.S. Public Health Service Grant DE-02670. Portions of this report were presented at the 50th Meet. Int. Ass. Dent. Res., Las Vegas, NV, 1972.

ceived ara-c during their middle exponential phase of growth. Protein from these samples was extracted and measured by the method of Lowry *et al.* (15). Nucleic acids were extracted from duplicate samples by the method of Hatcher and Goldstein (16) and were assayed by the procedures reported by Schneider (17). In DNA determinations, however, acetaldehyde (1.6 mg/20 ml reagent) was used as described by Burton (18), and the colorimetric reaction was allowed to proceed at 37° for 5.5 hr. This gave the best and most consistent color response.

Radioactive tracer studies. Radiolabeled compounds used were: (6-^{3}H) uridine, (^{14}C) sodium formate, (^{14}C) adenine, (Schwartz Bioresearch, Inc., Orangeburg, NY) and thymidine-methyl-T (Calatomic, Los Angeles, CA). Broth cultures of *S. faecalis* Acb-r were incubated at 37° until the middle exponential phase of growth was reached, at which time ara-c and the appropriate radiolabeled compounds were added. Control flasks received the labeled tracers but no ara-c. After 20 min further incubation at 37°, the cells were centrifuged and washed twice in deionized water. The cold acid-soluble and ethanol-precipitable fractions were extracted by the method of Park and Hancock (19). Separation of DNA and RNA was accomplished by the procedures reported by Schneider (20) and Schmidt and Thannhauser (21). The DNA and RNA fractions were solubilized in Beckman "Bio-Solv" BBS-2 (Fullerton, CA), and aliquots were diluted in a 1:1 mixture of Fluoralloy toluene and methanol. These samples were counted in glass vials in a Beckman LS-150 liquid scintillation counter.

Reversal of ara-c inhibition. Immediately after inoculation, separate broth cultures of *S. faecalis* Acb-r received either ara-c, deoxycytidine (Calbiochem, Los Angeles, CA) or ara-c plus deoxycytidine (see Fig. 7 for concentrations). Growth in these cultures and in nontreated control cultures was monitored by hourly determinations of viable cells in the respective cultures.

Results. The comparative inhibitory effect of ara-c on the growth of *S. faecalis* Acb-r and the parent strain of *S. faecalis* is shown in Figs. 1 and 2. Concentrations of this agent as low as 1.0×10^{-5} *M* strongly inhibited

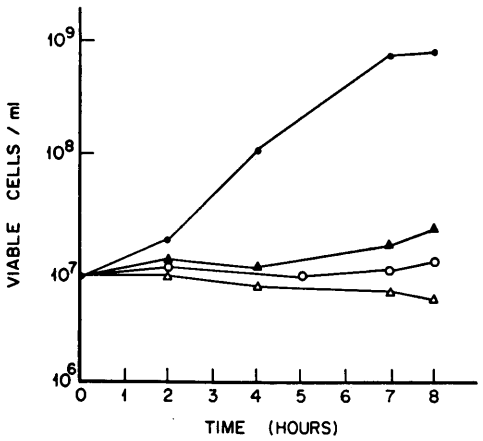

FIG. 1. Inhibition of *S. faecalis* Acb-r by ara-c. The indicated concentrations of inhibitor were added immediately after the cultures were inoculated. All cultures were incubated at 37°. (●—) Control; (△—) 1.0×10^{-4} *M* ara-c; (○—) 3.5×10^{-4} *M* ara-c; (▲—) 1.0×10^{-5} *M* ara-c.

the growth of *S. faecalis* Acb-r (Fig. 1) in contrast to *S. faecalis* which was inhibited only slightly by concentrations as high as 1.0×10^{-4} *M* ara-c (Fig. 2). When the inhibitor was added to cultures of *S. faecalis* Acb-r 3 hr postinoculation (Fig. 3), a marked decrease in the number of viable cells was noted in those cultures treated with 1.0×10^{-4} or 3.5×10^{-4} *M* ara-c. The effect of ara-c on the synthesis of protein, RNA, and DNA is presented in Figs. 4 and 5 which show

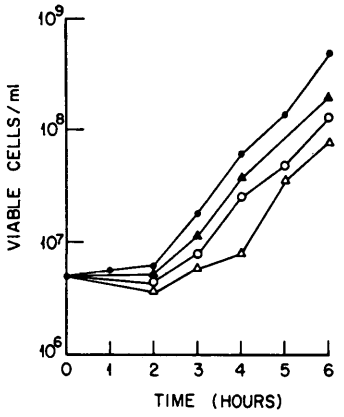

FIG. 2. Inhibition of *S. faecalis* by ara-c. Conditions were identical to those described in the legend for Fig. 1. (●—) Control; (△—) 1.0×10^{-4} *M* ara-c; (○—) 3.5×10^{-4} *M* ara-c; (▲) 1.0×10^{-5} *M* ara-c.

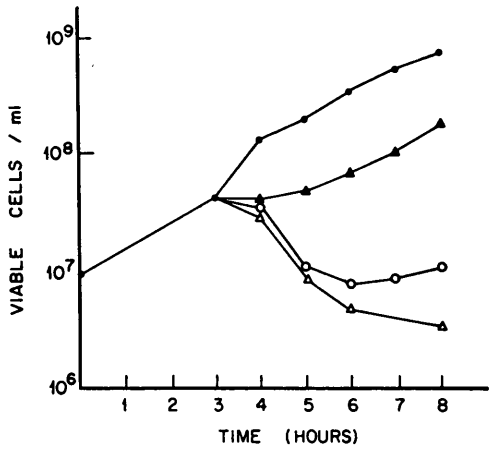

FIG. 3. Inhibition of exponentially dividing cultures of *S. faecalis* Acb-r by ara-c. Conditions were as described in the legend for Fig. 1 except the inhibitor was added 3 hr postinoculation of cultures. (●—) Control; (△—) 1.0 × 10^{-4} *M* ara-c; (○—) 3.5 × 10^{-4} *M* ara-c; (▲—) 1.0 × 10^{-5} *M* ara-c.

that 1.0 × 10^{-4} or 3.5 × 10^{-4} *M* ara-c completely inhibited the synthesis of DNA when this inhibitor was added to cultures at the time of inoculation or 3 hr postinoculation. These same concentrations of ara-c were substantially less inhibitory for the synthesis of RNA and protein. To evaluate the data in Fig. 5 more objectively, the percentage inhibition of RNA, DNA, and protein at 5 hr postaddition of inhibition was plotted on semilogarithmic probability paper as a function of the logarithm of the molar concentration of ara-c (Fig. 6). This procedure, which has been used to show the response of microbial growth to various concentrations of antibiotics (22), is a probit transformation of inhibitor dosage response curves to linearity. In Fig. 6, note that the response lines of RNA and protein synthesis to the two concentrations of ara-c were nearly identical and revealed by extrapolation, a 50% effective dose of approximately 2.0 × 10^{-5} *M* ara-c.

This concentration, by extrapolation of the response line of DNA synthesis to ara-c, would be expected to inhibit DNA synthesis by more than 99%. Since this further indicated that the primary inhibitory effect of ara-c was on DNA synthesis, various nucleosides and nucleotides were tested for their ability to prevent the inhibition of *S. faecalis* Acb-r by ara-c, and of those tested, only deoxycytidine prevented inhibition (Fig. 7). Additional data, summarized in Table I, revealed that ara-c inhibited the incorporation of various ^3H or ^{14}C labeled nucleic acid precursors into DNA by approximately 60–90%, while it had virtually no effect on the incorporation of these precursors into RNA with the exception of ^{14}C-adenine whose incorporation into RNA was inhibited by approximately 38%.

Discussion. This study has confirmed and extended other investigations which have shown that ara-c strongly inhibits the synthesis of DNA but not the synthesis of RNA or protein in gram negative bacteria (11) or

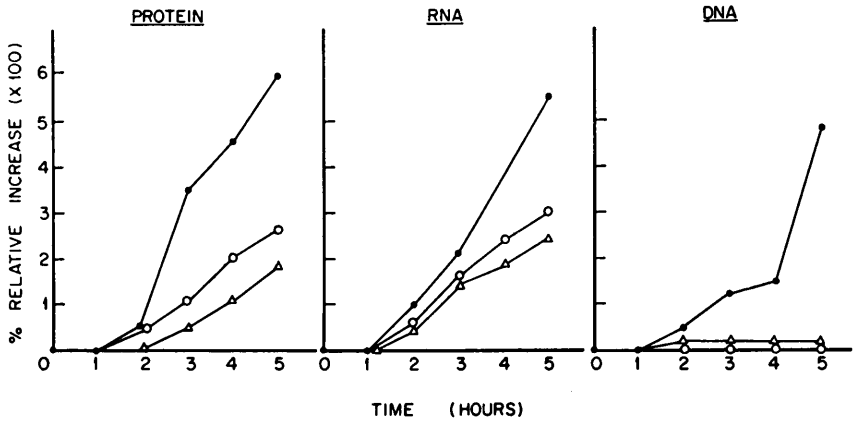

FIG. 4. The effect of ara-c on RNA, DNA, and protein synthesis in *S. faecalis* Acb-r. Conditions were identical to those described in the legend for Fig. 1. (●—) Control; (○—) 1.0 × 10^{-4} *M* ara-c; (△—) 3.5 × 10^{-4} *M* ara-c.

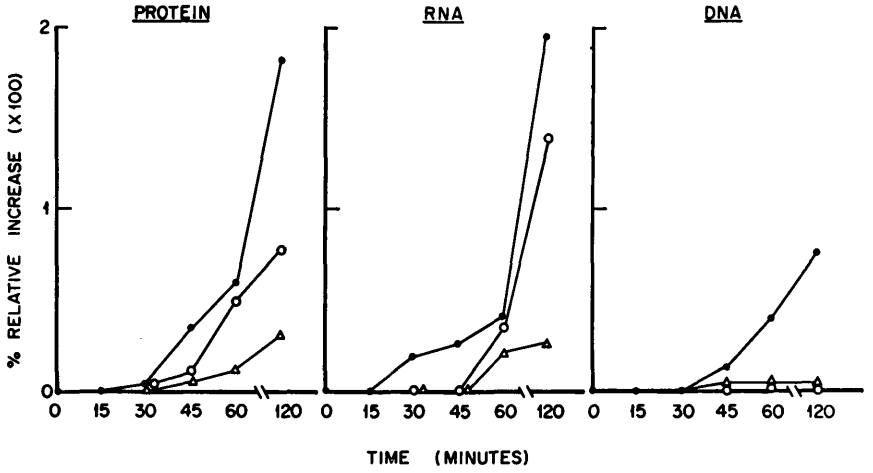

FIG. 5. The effect of ara-c on the synthesis of RNA, DNA, and protein in exponentially dividing cultures of *S. faecalis* Acb-r. The indicated concentrations of inhibitor were added 3 hr postinoculation (shown as zero time). (●—) Control; (△—) 1.0×10^{-4} *M* ara-c; (○—) 3.5×10^{-4} *M* ara-c.

in mammalian cells (7). The mechanism by which ara-c inhibits the growth of *S. faecalis* Acb-r also appears to be in the area of DNA synthesis. This is indicated by the variety of data presented in this communication. The addition of ara-c to rapidly dividing cultures of *S. faecalis* Acb-c (Fig. 3) produced a dose-related effect ranging from an inhibitory effect on growth at 1.0×10^{-5} *M* to an apparent bactericidal effect, indicated by a marked decrease in the number of viable cells, at higher concentrations. This bactericidal effect is consistent with the observation that inhibition of DNA synthesis may be lethal to bacteria (23). Concentrations of ara-c that strongly inhibited the synthesis of DNA in *S. faecalis* Acb-r did not greatly inhibit the synthesis of RNA and protein, and by probit analysis, extrapolated dose–response lines revealed that concentrations of ara-c sufficient to inhibit the synthesis of RNA and protein by approximately 50% would inhibit DNA synthesis by >99%. Ara-c inhibited by 60–90% the incorporation of various radiolabeled nucleic acid precursors into DNA but not to any marked extent the incorporation of these precursors into RNA. Of various nucleosides and nucleotides tested, only deoxycytidine prevented ara-c from inhibiting the growth of *S. faecalis* Acb-r. Three prevalent biochemical explanations for the inhibition of DNA synthesis by ara-c are: (a) ara-c, in animal cells and in *E. coli*, inhibits the reductase which is essential for the conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP), (b) ara-c is incorporated into DNA, or (c) ara-c inhibits DNA polymerase (13). At this time, there are insufficient data to sup-

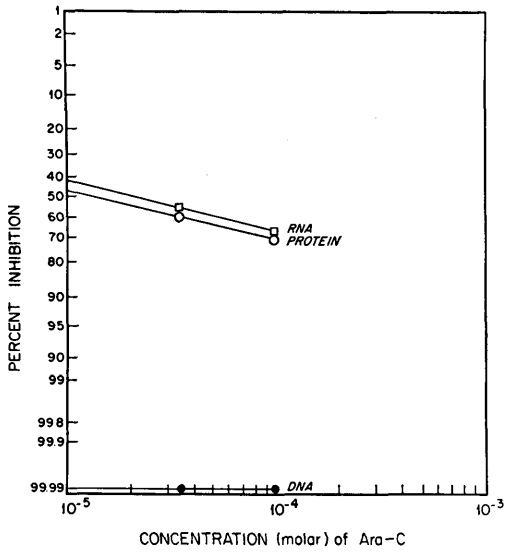

FIG. 6. Percentage inhibition of RNA, DNA, and protein synthesis in *S. faecalis* Acb-r as a function of the concentration of ara-c.

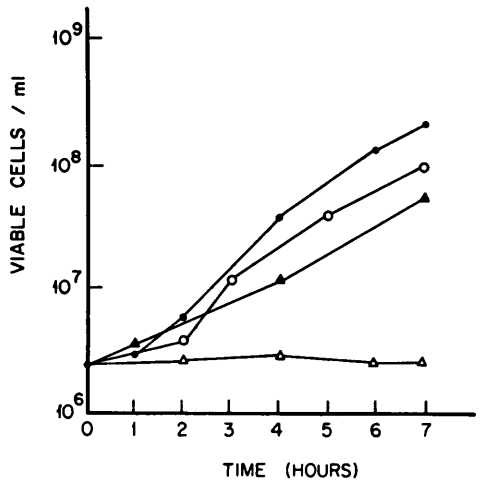

FIG. 7. The effect of deoxycytidine on the inhibition of *S. faecalis* Acb-r by ara-c. The inhibitor and nucleic acid precursor were added, at the indicated concentrations alone or in combination, immediately after the cultures were inoculated. All cultures were incubated at 37°. (●—) Control; (△—) 3.5 × 10^{-4} *M* ara-c; (○—) 3.5 × 10^{-4} *M* deoxycytidine; (▲—) 3.5 × 10^{-4} *M* ara-c + 3.5 × 10^{-4} *M* deoxycytidine.

port the hypothesis that ara-c inhibits dCPT reductase and no strong data are available to support the position that ara-c is incorporated into bacterial DNA (13). Conversely, considerable data are available which indicate that ara-c inhibits DNA polymerase, since leukemic cells are known to convert ara-c to ara-CTP (15), and this analog has been reported to inhibit DNA polymerase in mammalian cells (8). This is in accord with the observation that deoxycytidine will prevent the inhibitory action of ara-c (13). Ara-c may have a similar action in *S. faecalis* Acb-r since deoxycytidine specifically prevented ara-c from inhibiting the growth of this organism; however, this is tentative since ara-CTP does not inhibit DNA polymerase in *E. coli* (13). The data presented in this communication do not allow definite statements to be made concerning the precise biochemical mechanism of action of ara-c in *Streptococcus faecalis* Acb-r; however, the significance of this study is that ara-c strongly inhibits growth and the synthesis of DNA in this gram-positive organism at concentrations comparable to those required to inhibit the growth of animal cells. This makes available a new and sensitive system for additional studies with ara-c.

TABLE I. The Effect of Ara-c on the Incorporation of Nucleic Acid Precursors into RNA and DNA of *S. faecalis* Acb-r.[a]

Radioactive precursor	Concn of ara-c × 10^{-4} *M*	Incorporation RNA	DNA (cpm)
Adenine-^{14}C	0	52,194[b]	15,593
	3.5	32,633	1936
Formate-^{14}C	0	1366	1905
	3.5	1379	636
Thymidine-^{3}H	0	294	1250
	3.5	258	500
Uridine-^{3}H	0	19,506	8905
	3.5	19,626	2521

[a] Broth cultures were incubated 37° until the middle exponential phase of growth was reached at which time ara-c and the appropriate radiolabeled compounds were added. Control cultures received labeled compounds but no ara-c.

[b] Each value represents the incorporation (cpm) of the indicated radioactive metabolites into RNA and DNA exclusive of cold trichloroacetic acid- and ethanol-soluble materials.

Summary. The availability of a unique strain of *Streptococcus faecalis,* designated *S. faecalis* Acb-r, allowed studies which revealed the subcellular mechanism by which 1-β-D-arabinofuranosyl cytosine (ara-c) inhibits the growth of this organism. In *S. faecalis* Acb-r, concentrations of 1.0 × 10^{-4} or 3.5 × 10^{-4} *M* ara-c strongly inhibited the synthesis of DNA while the synthesis of RNA and protein was not greatly diminished by the same concentrations of this inhibitor. Ara-c inhibited by approximately 60–90% the incorporation of various radiolabeled nucleic acid precursors into DNA but not to any marked extent the incorporation of these precursors into RNA. Among various nucleosides and nucleotides tested, only deoxycytidine prevented inhibition of *S. faecalis* Acb-r by ara-c. These observations indicate that the primary effect of ara-c on *S. faecalis* Acb-r is the inhibition of DNA synthesis.

Actinobolin sulfate was generously supplied by Dr. H. W. Dion, Parke, Davis and Co., Detroit, MI. The assistance of Mrs. Nell McDonald and Mrs. Frances Hardy is greatly appreciated.

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Received Nov. 9, 1972. P.S.E.B.M., 1973, Vol. 142.