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6-Diazo-5-Oxo-L-Norleucine Inhibition of *Escherichia coli*

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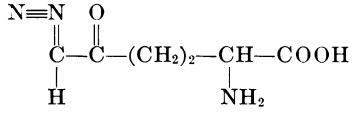
ABSTRACT

COGGIN, J. H., JR. (The University of Chicago, Chicago, Ill.), AND W. R. MARTIN. 6-Diazo-5-oxo-L-norleucine inhibition of *Escherichia coli*. J. Bacteriol. **89**:1348–1353. 1965.—The glutamine analogue 6-diazo-5-oxo-L-norleucine (DON) induced filaments and spheroplasts in *Escherichia coli* during the transition of sensitive populations to a state of resistance. Resistance developed at a frequency suggesting mutant selection. The morphology of cells resistant to 100 μg of DON per ml was indistinguishable from that of sensitive cells. DON-resistant cells exhibited an extended growth lag when cultured in the absence of the drug. This extended lag could be reduced to the lag time of parent sensitive cells by a combination of D-glucosamine and inosine or by DON. Viable counts during the lag period of resistant cells indicate that this lag results from a decrease in the number of cells during the first 2 hr of incubation. A combination of D-glucosamine and inosine was required for complete prevention of the DON inhibition of sensitive cells. The results indicate that DON not only inhibits de novo purine biosynthesis but that it also prevents hexosamine synthesis and, ultimately, cell-wall synthesis in *E. coli*.

The role of 6-diazo-5-oxo-L-norleucine (DON) as a glutamine antagonist has been clarified by numerous studies with both whole cells and isolated enzyme preparations (*see* Brockman, 1963). Particular attention has been given to the inhibitory role DON plays in de novo purine biosynthesis; this appears to be a major area of DON activity, because the addition of purine bases or ribonucleosides is moderately effective in preventing DON inhibition in bacteria. L-Glutamine effectively prevents DON inhibition of a cell-free enzyme system (Chakraborty and Hurlbert, 1961; Hurlbert, 1962) but has not proven to be effective in preventing DON inhibition in whole cells. The structure of DON is:



Martin and Moo-Penn (1963) demonstrated an extended lag when ethionine-resistant *Escherichia coli* were grown in a glucose-salts medium in the absence of ethionine. They further demonstrated that this lag could be prevented by including ethionine or the natural metabolite, methionine, in the growth medium. A strain of DON-resistant *E. coli* exhibited a similar lag in the absence of DON, and a study of the prevention of this lag proved to be a unique means of investigating both alternate sites of inhibition and possible mechanisms of resistance to DON in bacteria.

MATERIALS AND METHODS

The microorganism used in this study was *E. coli* ATCC 9637. The DON-resistant strain was obtained by serially passing the parent sensitive strain on glucose-minimal salts-agar (Anderson, 1946) containing increasing concentrations of drug. The resistant strain obtained by this method grew on 100 μg/ml of DON and was maintained on minimal agar slants containing this concentration of DON. For all media used in this investigation, glucose was sterilized separately and added to the sterile salts solution prior to inoculation.

The inhibitor, DON (Parke Davis lot no. BO-90961, Parke, Davis & Co., Detroit, Mich.), was stored at −15 C in the dark. Solutions of DON were prepared just prior to use by dissolving in sterile deionized water.

Inoculum preparation. Standardized inocula were prepared by growing cells in 25 ml of minimal broth, and incubating at 37 C on a rotary shaker (200 rev/min). Resistant cells were cultured under identical conditions in minimal broth containing 10 μg/ml of DON. Cells were harvested by centrifugation after 20 hr of incubation, suspended, and washed three times in 10 ml of 0.85% saline; they were then standardized in saline to an optical density of 0.66 at 660 mμ in a Bausch & Lomb Spectronic-20 colorimeter. There were approximately 10^9 viable cells per milliliter for DON-resistant and DON-sensitive cells at this

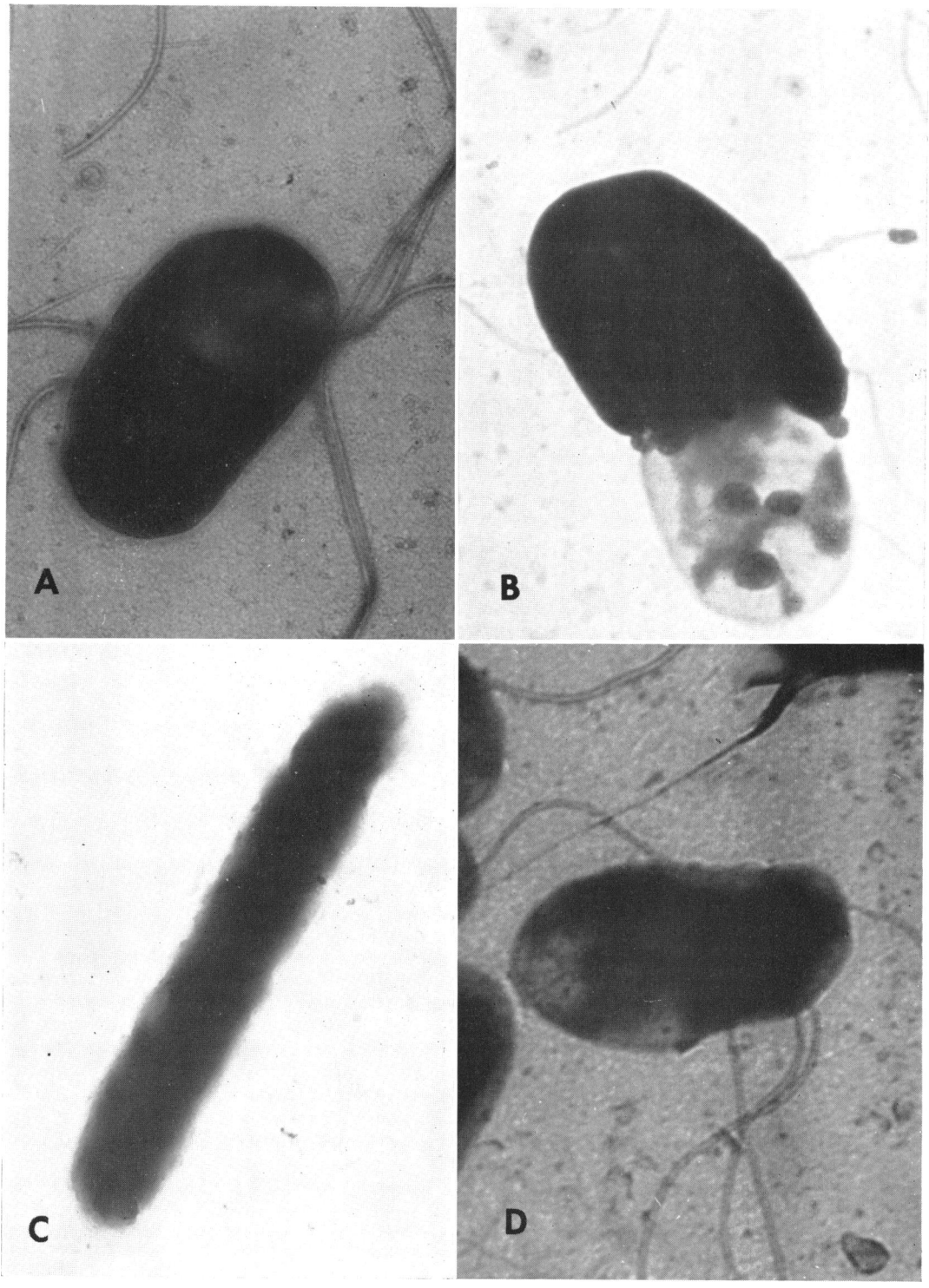


FIG. 1. *Electron micrograph whole mounts of (A) sensitive Escherichia coli, (B) sensitive E. coli inhibited by DON (10 μg/ml), (C) E. coli growing in presence of DON (1 μg/ml), and (D) resistant E. coli growing in presence of DON (100 μg/ml).* × *6,000.*

optical density. In all experiments, 0.5 ml of the standardized cell preparation was used to inoculate 25 ml of minimal broth in 300-ml nephelometry flasks (Bellco Glass, Inc., Vineland, N.J.) equipped with side arms (14 by 130 mm) to facilitate direct optical-density readings. Growth was measured as an increase in optical density as a function of time.

Viability determinations. Viability counts were made by spreading 0.1-ml samples of cold saline dilutions over the surface of minimal agar plates with a glass spreader. The plates were incubated at 37 C for 48 hr. Viable-cell counts for each dilution were determined in triplicate. Platings were also made on minimal agar containing 10 μg/ml of DON and on nutrient agar plates.

RESULTS

Mutant selection. The minimal inhibitory concentration of DON for *E. coli* was 1.0 μg/ml in spread plates and 0.1 μg/ml in broth when 10^7 viable cells per milliliter were tested.

When 10^7 viable sensitive cells of *E. coli* were spread on minimal agar containing 10 μg/ml of DON, resistance in a single-step experiment occurred with a frequency of 4.5×10^{-5} per viable cell. The loss of resistance when resistant cells were cultured in broth in the absence of DON for 24 hr, washed, and plated on minimal agar containing 10 μg/ml of DON was 8×10^{-3} per viable cell. Resistance was maintained, with this frequency of loss, even after cells were cultured in broth without DON through four subcultures.

Altered morphology. Filament formation was observed in cultures during the transition from DON sensitivity to full resistance. The changes in morphology are shown in Fig. 1. It is significant that these changes occurred only during the development of resistance; the morphology of the fully resistant cell appears identical to that of the sensitive cell. When sensitive cells were treated with DON, numerous spheroplast-like forms could be observed by phase microscopy in the presence of 20% sucrose.

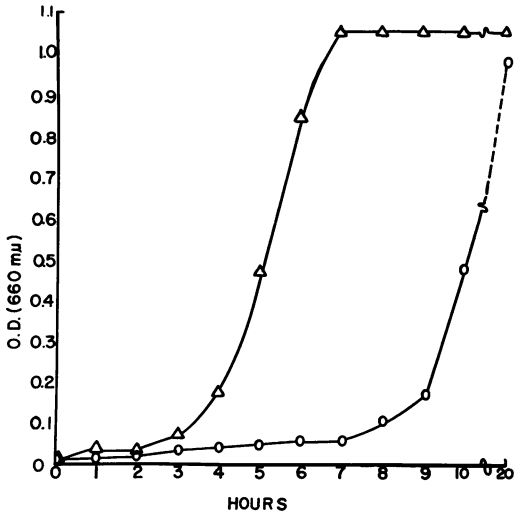


FIG. 2. *Growth of DON-sensitive and -resistant Escherichia coli in the absence of DON. Symbols:* △ = *sensitive cells;* ○ = *resistant cells.*

Growth characteristics of resistant cells. Resistant cells grown in the absence of DON had an extended lag some 5 hr longer than that of the sensitive strain (Fig. 2). This extended lag was observed only when the resistant inoculum was prepared from cells cultured in the presence of drug. Inocula prepared from resistant cells grown in the absence of DON, although still fully resistant, did not show a significant lag.

Prevention of the resistant cell lag phase. When minimal broth was supplemented with DON (1.0 μg/ml), the growth curve of the resistant culture approximated that of the drug-sensitive culture (Fig. 3). Since morphological observations had indicated that DON might interfere with cell-wall synthesis, several basic units of cell-wall structure were tested to determine whether their addition would prevent the lag in growth. In addition to DON, D-glucosamine or inosine individually decreased the apparent lag phase of resistant cells, but, when both were present in the minimal broth, the growth curve of resistant cells was almost identical to that of DON-sensitive cells (Fig. 3). L-Lysine, D-alanine, DL-diaminopimelic acid, L-glutamine, uridine diphosphate, and *N*-acetyl-D-glucosamine were without significant effect even when tested at a concentration of 300 μg/ml. Adenine (100 μg/ml) and guanine (50 μg/ml) were tested and were somewhat less effective than inosine. These purine bases are readily converted to adenosine and guanosine, respectively, in *E. coli* when supplied exogenously (Britten et al., 1963).

Viability studies on resistant cells during extended lag period. Viability studies were conducted on resistant cells during the extended lag phase. The results (Fig. 4) show that a decrease in the number of viable cells occurs during the first 2 hr, and is followed by recovery and the subsequent development of the culture. The loss in viability was prevented by the addition of DON (1 to 10 μg/ml) to the broth culture. Inosine or glucosamine reduced the viability loss to some extent, but, when both metabolites were present, viable counts did not decrease and normal growth ensued. When platings were made on nutrient agar for these test conditions, viability counts were lower than counts on minimal agar. If DON was present in the minimal agar, the

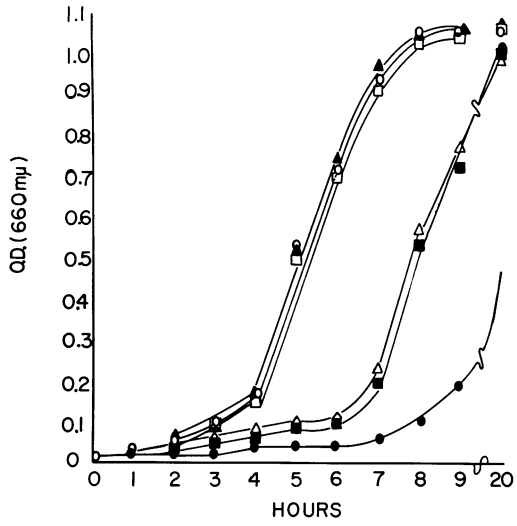


FIG. 3. *Effect of glucosamine (100 μg/ml), inosine (100 μg/ml), or DON (1 μg/ml) on growth of DON-resistant Escherichia coli. Symbols:* ● = *no addition;* △ = *glucosamine;* ■ = *inosine;* ▲ = *inosine and glucosamine;* □ = *DON;* ○ = *sensitive cell control.*

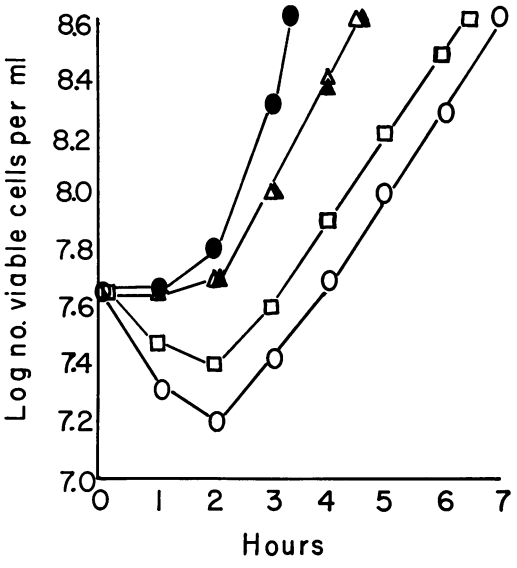


FIG. 4. *Viability of sensitive and resistant Escherichia coli in broth plated on minimal agar. Symbols:* ○ = *DON-resistant cells, no broth additions;* ● = *DON-resistant cells in broth supplemented with glucosamine (100 μg/ml) and inosine (100 μg/ml);* ▲ = *DON-resistant cells in broth supplemented with DON (1 μg/ml);* △ = *sensitive E. coli control;* □ = *DON-resistant cells, no broth additions, and plated on minimal agar containing 10 μg/ml of DON.*

viable counts fell by a value approximating one-half that observed when DON was not present in the plating agar (Fig. 4). Growth conditions were required for the observed loss in viable cells. No decrease in the number of drug-resistant viable cells was observed through 5 hr in minimal broth without glucose.

The resistance of cells developing in the culture broth in the absence of DON was examined in the following manner. Colonies appearing on minimal agar plates representing cells from the 5- and 6-hr samples in the viability study were replica plated onto minimal agar containing 10 μg/ml of DON. All colonies tested (265) were still resistant to DON.

When resistant isolates were picked from minimal plates, cultured in broth without DON, and tested for viability in aerated broth without additions as previously described, no decrease in the number of viable cells occurred. If the same colonies were cultured in broth with DON and these cells used as inocula in unsupplemented broth, a loss of about one-half the initial population occurred.

Reversal of DON-inhibited cells. The effect of inosine and glucosamine as preventative agents of DON inhibition of sensitive cells was next investigated (Fig. 5). When sensitive cells were inoculated into broth containing DON (10 μg/ml) and inosine (100 μg/ml), an initial phase of growth occurred for 3.5 hr, followed by rapid clearing of the culture. Secondary growth was observed between 6 and 8 hr, again followed by a loss in turbidity. In broth containing glucosamine and DON, no growth was observed through

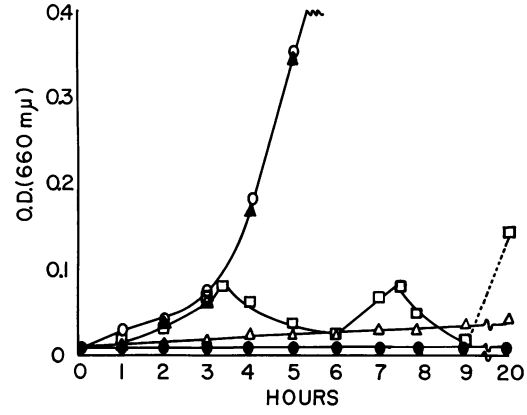


FIG. 5. *Inhibition of Escherichia coli by DON (10 μg/ml) and its prevention by a combination of inosine and glucosamine. Symbols:* ○ = *no additions;* ● = *DON;* △ = *DON* + *glucosamine (10 μg/ml);* □ = *DON* + *inosine (100 μg/ml);* ▲ = *DON* + *inosine* + *glucosamine.*

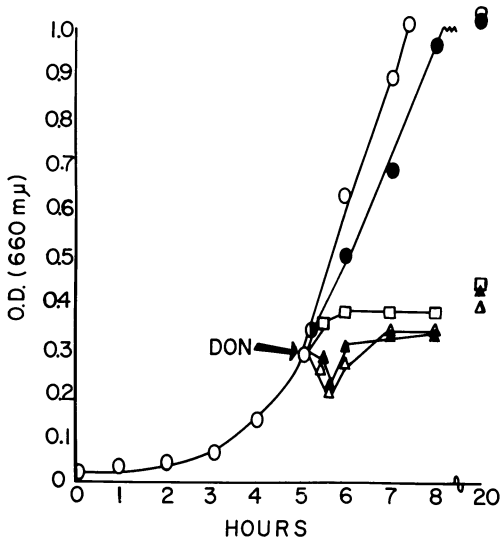


FIG. 6. *Effect of DON (10 μg/ml) added at mid-log phase to Escherichia coli growing in broth containing inosine (100 μg/ml), glucosamine (100 μg/ml), or a combination of inosine and glucosamine. Symbols:* ○ = *control,* △ = *DON;* □ = *DON + glucosamine;* ▲ = *DON + inosine;* ● = *DON + inosine + glucosamine.*

20 hr. A combination of glucosamine and inosine prevented the inhibition of growth by DON.

When the inhibitor was added to sensitive cells in mid-log phase of growth, an initial loss of turbidity of the culture was observed followed by some growth and then growth cessation (Fig. 6). The same effect was observed with cells grown in the presence of inosine. In broth containing glucosamine, further growth was prevented, but a decrease in turbidity was not observed. Growth of cultures containing both inosine and glucosamine was not inhibited by the addition of DON at mid-log phase, although there appeared to be a change in the growth rate. The addition of DON at mid-log phase to resistant cells growing in the absence of inhibitor (following an extended lag) resulted in only a very slight alteration in the rate of growth.

Effect of L*-glutamine.* L-Glutamine (100 μg/ml) did not prevent DON inhibition of sensitive cells nor did it prevent a loss in viability when resistant cells were cultured in the absence of DON. When L-glutamine was tested in combination with either inosine or glucosamine, no detectable difference was found from results obtained when these metabolites were used alone.

DISCUSSION

Our results indicate that the glutamine antagonist, DON, not only inhibits de novo purine biosynthesis but also blocks hexosamine synthesis in *E. coli*. DON inhibition could be completely prevented only by a combination of inosine and glucosamine. Results obtained when DON was added initially to broth culture suggest that inosine prevents DON inhibition leading to purine biosynthesis (nucleic acid synthesis) and growth ensues, but a limiting factor subsequently develops at the site of cell-wall synthesis. The role of inosine is evident, because the biosynthesis of this pivotal purine intermediate results from two transamidase reactions, both highly sensitive to inhibition by DON. Previous studies with *E. coli* and isolated enzyme systems from these cells indicate that resistance to DON is a result of altered sensitivity to this drug at susceptible enzyme sites in purine biosynthesis (Tomisek and Reid, 1962). The second period of growth and lysis in the presence of inosine cannot be explained by the available data.

The separate role of glucosamine as a reversing metabolite was more clearly demonstrated when DON was added to actively dividing cells in the mid-log phase of growth, resulting in a loss of turbidity. Lysis induced by DON under these conditions could only be prevented by D-glucosamine, a cell-wall precursor, although further growth was prevented. These data indicate that one additional site of DON inhibition involves cell-wall synthesis.

Pathways leading to the synthesis of both glucosamine and inosinic acid utilize amination reactions involving the transfer of the amide nitrogen from glutamine to an acceptor molecule. DON has been shown to be a strong inhibitor of the glutamine transamidase reactions converting fructose-6-phosphate to glucosamine-6-phosphate (Ghosh et al., 1960), and the binding of DON to transamidases appears to be irreversible (Hurlbert and Kammen, 1960).

Glucosamine can be directly phosphorylated by adenosine triphosphate to form glucosamine-6-phosphate, thus by-passing the DON-sensitive transamidase reaction in the biosynthesis of glucosamine-6-phosphate (Kornfeld et al., 1964), a precursor of the basic cell-wall constituent *N*-acetyl-glucosamine. The direct phosphorylation of *N*-acetyl-glucosamine has been demonstrated for *E. coli* (Ansensio, 1960), but may not occur under these conditions of test, possibly explaining the failure of this metabolite to prevent DON inhibition.

Maxwell and Nickel (1954) and Kilgore and Greenberg (1961) reported that azaserine, another glutamine antagonist, induced filament formation in *E. coli*, but they observed no filaments in DON-resistant cultures. Our observations show that filament forms occur in *E. coli* populations only when a drug-sensitive population is cultured

in the presence of increasing concentrations of DON; however, fully resistant cells exhibit morphology identical to that of parent sensitive cells, and would explain the failure of previous workers to observe this morphological change. Filament formation occurs in bacteria under conditions detrimental to nucleic acid synthesis, and spheroplast forms are indicative of impaired cell-wall synthesis. The observed changes in cell morphology reported here correlate with the dual requirement of inosine and glucosamine to prevent the inhibition of *E. coli* by DON.

The extended lag observed turbidimetrically when resistant cells were grown in minimal broth results from an initial loss in viable cells rather than the expression of a physiological growth lag. Vigorous aeration in minimal broth under rapid growth conditions in the absence of DON seems to be responsible for the initial viability loss, because death may be averted by removal of the cells to minimal agar plates or by withholding glucose from the minimal broth. It would appear that unbalanced synthesis occurs under rapid growth conditions; this synthesis is prevented by DON, a combination of inosine and glucosamine, or by less vigorous growth. The unusual behavior of drug-resistant cells under conditions used here cannot be explained by information available in the literature or by our data. An understanding of the effect of DON on resistant cells must await further study.

The expression of an extended growth lag by *E. coli* cells resistant to a number of antitumor agents has been observed in the laboratory. In addition to the study reported here, investigations with 6-mercaptopurine (*in preparation*) and L-ethionine demonstrated that a growth lag occurs in the absence of antimetabolite under conditions of rapid growth, and, in each case, the lag may be prevented either by the specific drug involved or by metabolites related to the site of drug inhibition.

ACKNOWLEDGMENTS

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