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In Vivo Effect of Thiolutin on Cell Growth and Macromolecular Synthesis in *Escherichia coli*

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Thiolutin reversibly inhibits growth and ribonucleic acid synthesis in *Saccharomyces cerevisiae*. It is now demonstrated that, at 5 μg/ml, thiolutin rapidly inhibits all incorporation of radioactive precursors into ribonucleic acid and protein in *Escherichia coli*, although the incorporation of deoxythymidine into deoxyribonucleic acid continues for some time. Concentrations of thiolutin of 5 μg/ml and above are bacteriostatic and do not lead to unbalanced growth, so that cell size remains constant. The antibiotic and its inhibitory effects are easily removed by washing, whereupon macromolecular synthesis and cell division resume unimpeded. These data are consistent with reversible inhibition of ribonucleic acid synthesis being the primary mode of action of thiolutin in *E. coli*, and suggest that thiolutin may be a useful tool for studies where such reversible inhibition is required.

It has been demonstrated (5) that thiolutin, a sulfur-containing antibiotic produced by *Streptomyces luteoreticuli* (1), reversibly inhibits growth and ribonucleic acid (RNA) synthesis in *Saccharomyces cerevisiae*. Minimal inhibitory concentrations also inhibit deoxyribonucleic acid (DNA)-dependent RNA polymerases I, II, and III of yeast in vitro (12). It has also been reported that thiolutin is a potent inhibitor of growth of several gram-positive and negative bacteria (11). We have initiated studies on the mechanism of action of thiolutin on *Escherichia coli*, and this paper describes its effects on growth and macromolecular synthesis in this organism.

MATERIALS AND METHODS

Bacterial strains and growth media. Two of the bacterial strains used in this study were K-12 strains (GK 19 and GK 255) and the third was a 15 TAU strain (GK 117). GK 19 (F^-, prototroph, str^s, λ^-) was obtained from C. A. Saunders; GK 117 (*arg*$^-$, *ura*$^-$, *thy*$^-$) from P. C. Hanawalt; and GK 255 (Hfr, prototroph, str^s, λ^-) from R. Curtiss III.

The growth media consisted of nutrient broth (Difco Laboratories, Detroit, Mich.), minimal salts medium E (13), tryptone broth (medium A; 6), and a combination of media A and E (1:1, vol/vol), supplemented with thymidine (10 mM) and uracil or uridine (10 mM). The carbon source (glucose or glycerol) was added at 0.5% (wt/vol). Bacteria were grown at 37 C in a New Brunswick shaker (New Brunswick Scientific Co., New Brunswick, N.J.) with vigorous aeration. Plating media were the same as above with 1.5% agar (Difco) added.

Antibiotics and chemicals. Thiolutin (TL) was a gift from J. Davies, University of Wisconsin, Madison, Wisc. A stock of TL (2 mg/ml) in dimethyl sulfoxide was kept in the dark at −20 C. Immediately before use, it was thawed and used at final concentrations indicated in the text.

Total bacterial count methods. Culture samples (50 μliters) were diluted into 5-ml filtered (0.45 μm membrane filter, HA type; Millipore Corp., Bedford, Mass.) aqueous solutions of 0.85% NaCl to which 0.5% formaldehyde (vol/vol) was added. Cells were then counted by using a Coulter counter (Coulter Electronics, Hialeah, Fla.) equipped with a Spectrazoom Channelizer pulse height analyzer and integrator system (Packard Instruments). The counter had a 30-μm orifice tube and the settings were: aperture current of 2; amplification of 4 or 8; and gain of 300. The total counts were determined by integrating the areas under the accumulated size distribution from visual outputs and subtracting the background noise. The cells were diluted to obtain counts within a range of 10 to 40,000/0.1 ml.

Viable cell counts. Samples of 0.1 ml were withdrawn from cultures and appropriately diluted into a sterile solution of buffered saline gelatin (2). Portions were removed and spread on appropriate media. The plates were incubated for 24 to 48 h at 37 C. Colonies were counted on a colony counter model C-110 (New Brunswick Scientific Co.).

Bacterial turbidity measurements. Sample turbidity was monitored with a Klett-Summerson spectrophotometer using a red filter. Absorbances at 600 nm (A_{600}) were measured using a Zeiss PMQII spectrophotometer.

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Incorporation of radioactive precursors. DNA, RNA, and protein synthesis were monitored by determining the incorporation of ^{3}H-labeled thymidine (0.1 μCi/ml), uridine (0.1 μCi/ml) or uracil (0.5 μCi/ml), and leucine (2 μCi/ml), respectively, into cold trichloroacetic acid-insoluble materials. Samples of labeled cultures (100 μliters) were spotted on Whatman 3MM paper disks (W. & R. Balston, Ltd., England). The filters were immediately submerged in ice-cold 10% trichloroacetic acid, washed twice in 5% trichloroacetic acid and twice in 95% ethanol, air-dried, and counted.

Reversibility of TL action. A 5-ml sample of culture containing TL was filtered through a 0.45-μm membrane filter (Millipore Corp.) and washed with 125 ml of TL-free medium which had been prewarmed to 37 C. Filters containing the cells were placed in fresh medium at 37 C (5 ml) and vortexed vigorously to resuspend the cells. Cell samples were then transferred into new flasks. The operation lasted approximately 2 to 2.5 min. Exponential cultures not treated with TL served as controls. The lag in growth introduced by these manipulations did not exceed 2 to 3 min.

Measurement of DNA and stable RNA degradation. Cells growing exponentially in the presence of radioactive uridine or thymidine were washed free of the label by filtration. Cells were resuspended in cold medium and allowed to grow one-half a generation to eliminate labeled precursor pools. TL was added to the samples and, at timed intervals, 100-μliter samples of the culture were removed, spotted on 3MM filter disks, and submerged in cold 10% trichloroacetic acid. These samples were allowed to stand at 4 C for 3 h. The amount of radioactivity remaining on the filters was determined after two additional washes with trichloroacetic acid and ethanol.

Determination of radioactivity. After heat-drying, filters were placed into scintillation vials containing 5 ml of a toluene-base scintillation counting solution. Samples were counted in a Nuclear Chicago Isocap 300 scintillation counter (Nuclear Chicago, Des Plaines, Ill.).

RESULTS

Effect of TL on cell division and growth. The effect of TL on cell growth of exponential cutlures of *E. coli* K-12, 15 TAU, and B/r strains was tested. Figure 1 represents the typical results with GK 117 growing in rich medium with a doubling time of 25 min. TL, at 1 μg/ml, initially reduced the rate of cell division. After a 50% increase in cell number, cell division ceased for approximately 40 min, after which there was a resumption of cell division at a reduced rate (doubling time, 40 min). With higher concentrations of TL, there was a rapid cessation of cell division. However, again some cell division occurred with 5 μg of TL per ml, beginning 100-min post-treatment. At 20 μg/ml or higher, there was a complete inhibition of cell division. On extended incubation, the amount of residual

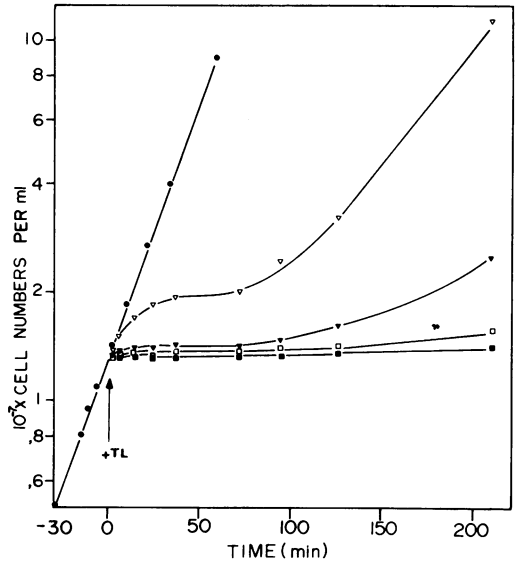

FIG. 1. *Effect of thiolutin on cell multiplication in E. coli 15 TAU. Exponentially growing cells of strain GK 117 in medium A + E + glucose were exposed to TL at concentrations of 1, 5, 20, and 50 μg/ml at time zero (arrow). The cell density at the time of addition of TL was 5 $\times$ 10^7 cells/ml. Cell counts are plotted versus incubation time.*

cell division occurring in the presence of TL at 20 μg/ml for exponential cultures was approximately 5% for minimal salts-glucose- and 15% for broth-grown cells.

By using the Coulter counter and pulse height analyzer, we next monitored changes in the cell volume distribution during TL treatment of exponentially growing cultures of *E. coli* (Fig. 2). In logarithmic growth phase, the population has a normal distribution (mode value at channel 25 to 28) with strong positive skewness, typical of exponential cultures of *E. coli* (4, 8). Treatment with a low (1 μg/ml) TL concentration did not affect the mode distribution, although initially there was an accumulation of some large cells (mode channel 34). This could be seen (Fig. 2A) as a shoulder in the bimodal size distribution. Between 75 to 125 min post-treatment, this shoulder began to disappear, coincident with initiation of renewed cell division (Fig. 1). The average cell size for cultures treated with 1 μg of TL per ml gradually diminished (mode 17 at 210 min). This result suggests that slower growth due to the presence of subinhibitory concentrations of TL affects the general physiology of cell growth and hence the mean cell volume. At 5 μg of TL per ml the size distribution after 150 min reflected the slow accumulation of a population of small cells like

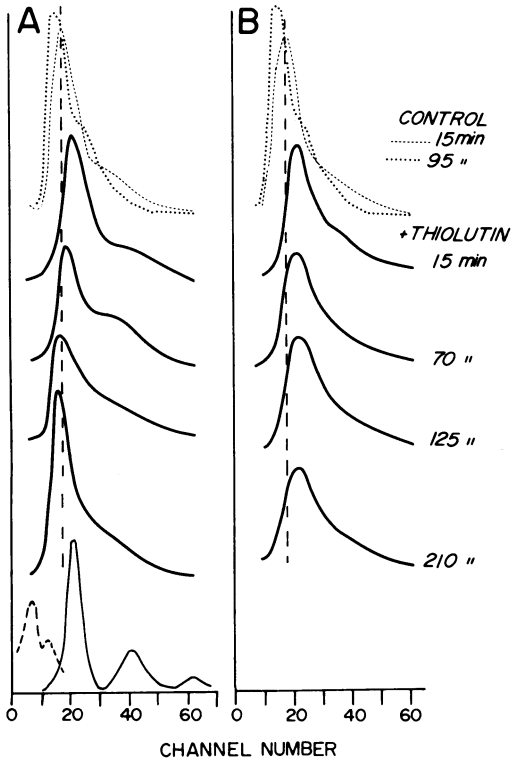

FIG. 2. *Effect of TL on cell growth. Profiles of cell volume distribution for GK 117 treated with TL at 1 (A) and 20 (B) μg/ml were obtained from plots of the pulse height analysis of cell samples taken after addition of drug at times indicated in the right-hand side. Untreated controls are shown at the top. The vertical dashed line in each panel indicates the mode at 0 min for cell size distributions. In each case, the total number of cells under the profile were kept at a constant cell number of 4.00 ± 0.02 × 10⁷ cells/ml. The ordinate represents the cell numbers per channel, and the abscissa represents the pulse height analyzer channel number (0 to 200). The lower left distribution (A) is calibration with polystyrene latex microspheres 0.8 μm in diameter (volume, 0.27 μm³) in broken lines and 1.099 μm in diameter (volume, 0.69 μm³) in solid line. The peaks represent the volumes of one, two, or three microspheres.*

those seen at 1 μg of TL per ml. Treatment with higher concentrations (20 and 50 μg/ml) inhibits changes in the cell volume distribution as well as inhibiting cell division.

The effect of TL on cell viability is shown in Fig. 3. There were varying degrees of reduction in cell viability in the drug-treated cultures which is dependent upon the concentration of drug as well as on the culture media used.

Inhibition of macromolecular synthesis in whole cells. RNA and protein synthesis in *E. coli* K-12, growing with a doubling time of 25 min, were blocked within a few minutes by using 5 to 50 μg of TL per ml (Fig. 4A). TL caused a more gradual inhibition of DNA synthesis under these conditions. After addition of 5, 20, or 50 μg of TL per ml, uptake of [³H]thymidine into the trichloroacetic acid-precipitable fraction ceased in 40, 30, and 20 min, respectively. This results in approximately a 70, 42, and 28% increase in net DNA synthesis for the whole culture. Whether the residual DNA synthesized in the presence of TL corresponds with completion of ongoing rounds of DNA replication remains to be investigated.

The kinetics of incorporation of these macromolecular precursors can be determined with more precision in the multiply auxotrophic 15 TAU strain GK 117. This strain also seemed to be more susceptible to TL (refer to Fig. 2) and when a similar experiment was performed with it, the discernible effects of TL on macromolecular synthesis were more dramatic (Fig. 4B). After labeling for 10 min and adding TL, net

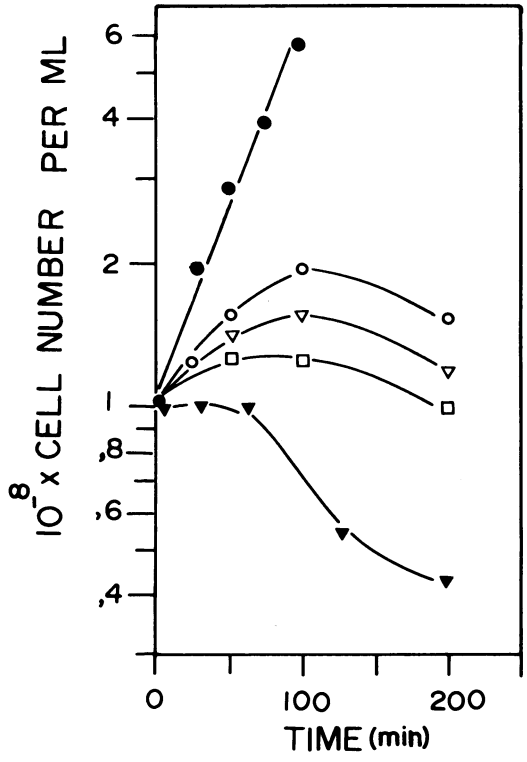

FIG. 3. *Effect of TL on cell viability. Exponentially growing cultures of E. coli 15 TAU (GK 117) growing in NB and E. coli K-12 (GK 19) growing in medium E + glycerol were exposed to TL at zero time at 5 (○), 20 (▽), and 50 (□) μg/ml. (▼) represents GK 19 treated with 20 μg of TL/ml. Closed circles (●) represent untreated control of strain GK 117.*

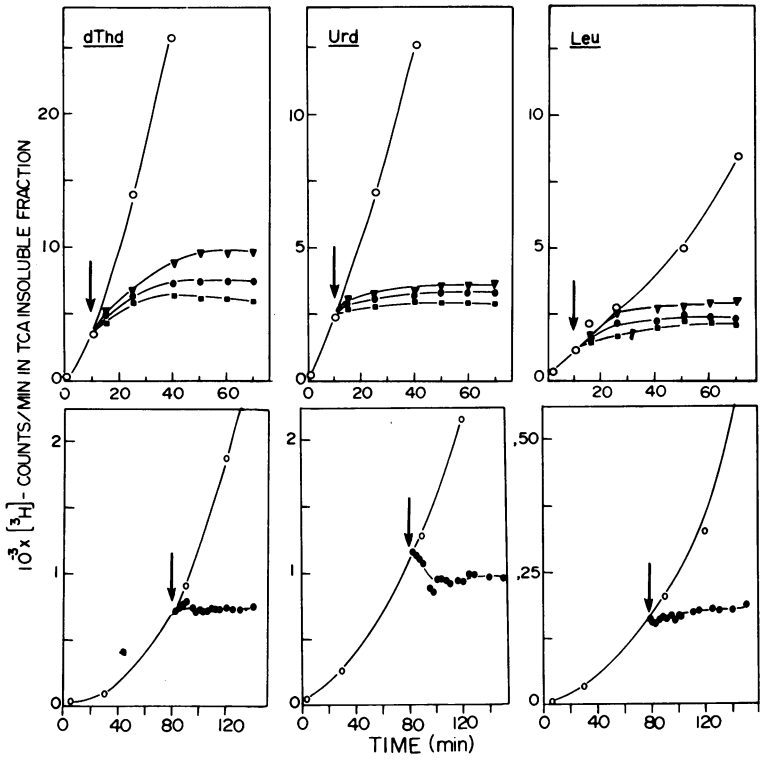

FIG. 4. *Effect of TL on macromolecular synthesis in E. coli. Incorporation of radioactive precursors of DNA, RNA, and protein synthesis, thymidine (10 mM), uridine (10 mM), and leucine (20 mM) into cells of GK 255 (upper panel) and GK 117 (lower panel) are shown. TL at concentrations of 5 (▼), 20 (●), or 50 (■) μg/ml was added as indicated by the arrow. Open circles represent control (dimethyl sulfoxide-treated) cells.*

uridine incorporation immediately decreased to a level of 80%. This probably indicated cessation of all new RNA synthesis and degradation of prelabeled, unstable messenger RNA. Leucine incorporation halted after a short lag consistent with known messenger RNA half-lives in *E. coli*.

Reversibility of TL inhibition. Cells of GK 19 were grown in medium E plus glucose in the presence or absence of Casamino Acids and were treated with 20 μg of TL per ml. At intervals, cells were washed free of TL and were resuspended in drug-free medium. Cell numbers were determined as a function of time (Fig. 5). Table 1 compares the relationship between length of inhibition of cell growth and lag before recovery of cell division after removal of TL. This lag was shorter when cells were growing in the rich medium and was independent of the time of exposure to TL over the periods tested. Furthermore, recovery of cell division in this medium rapidly approached the control rate.

In the minimal medium (Fig. 5A, Table 1), the lag time before resumption of cell division after exposure to TL was greater and was a function of the period of exposure to TL. Moreover, after exposure to TL for more than 0.5 generation time, the recovery was gradual. This may reflect the greater susceptibility to TL of cells grown in minimal medium (Fig. 2). The effects of treatment for the same period to differing concentrations of TL are consistent with this interpretation (see below, Fig. 6).

Cells of GK 117 growing in rich medium (doubling time, 25 min) were exposed to 5 or 20 μg of TL per ml. Treated cultures were then washed free of TL at intervals and recovery of cell division was followed (Fig. 6). Treatment with 5 μg of TL per ml (Fig. 6A) for a duration of 0.2, 0.4, 0.6, and 1.2 times the generation time resulted in a constant lag in recovery of about 8 to 10 min (0.4 generation time). Longer treatments, for example 60 and 100 min (2.4 and 4 generation time), increased the recovery lag to 18 and 22 min (0.8 to 1.0 generation time). Similar results with longer recovery lags (0.5 to 1.5 generation time) were obtained with 20 μg of TL per ml (Fig. 6B).

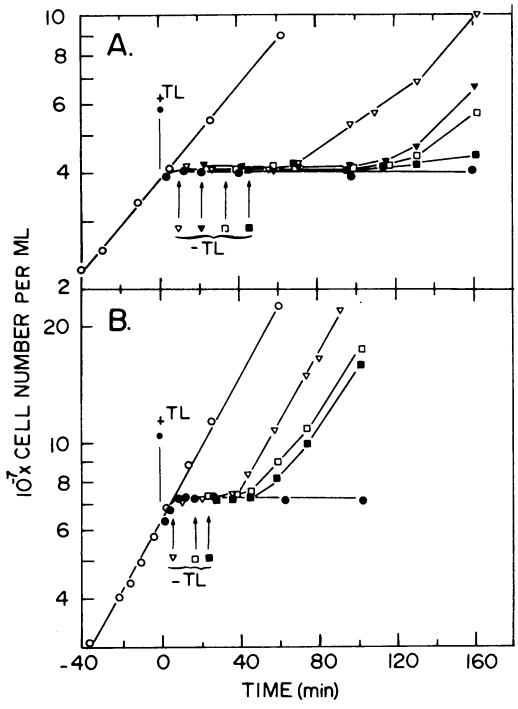

FIG. 5. *Effect of growth media on recovery of cell division. Cells of E. coli K-12 (GK 19) were grown in the absence (A) or presence (B) of Casamino Acids in medium E + glucose (○) and treated with 20 μg of TL per ml (●) at zero time. At times equivalent to the 0.25 (▽), 0.50 (▼), 0.75 (□), and 1.0 (■) generation time, samples of TL-treated cells were removed and washed free of the drug. Recovery of cell division was monitored with a Coulter counter.*

TABLE 1. *Relationship between length of inhibition of cell growth and the recovery cell division*[a]

Growth medium	Culture generation time (min)	TL pulse[b]		Recovery lag[c]/τ
		Min	Pulse/τ	
E + glucose	48	11	0.23	1.25
		22	0.46	1.85
		33	0.69	2.10
		45	0.94	2.40
E + glucose + Casamino Acids	32	6	0.18	0.875
		18	0.56	0.875
		25	0.78	0.875

[a] Data taken from Fig. 5.

[b] Time in the presence of TL presented in minutes and as a fraction of the culture generation time (τ).

[c] Time elapsed between resuspension in drug-free medium and initiation of cell division as a fraction of culture generation time.

The time course of macromolecular synthesis during recovery from treatment with 20 μg of TL per ml was studied (Fig. 7). Macromolecular synthesis was restored almost immediately upon removal of the drug. The rate of incorporation of uracil reverted to that in the untreated control in 5 min, and incorporation of thymidine in 9 min, irrespective of the duration of prior exposure to TL.

Stability of DNA and stable RNA in TL-treated cells. The effect of TL on stimulating the breakdown of long-term labeled DNA and RNA into trichloroacetic acid-soluble material was tested. Cells of GK 117 grown for several generations in [^{3}H]thymidine or [^{3}H]uridine were treated with 20 μg of TL per ml. Over a period of 1 h, the extent of breakdown into trichloroacetic acid-insoluble counts was negligible (less than 5%).

DISCUSSION

This study describes the effects of TL on cell growth and macromolecular synthesis in *E. coli*. At a concentration of 5 μg/ml or higher, TL inhibited cellular multiplication, whereas transient effects were seen at 1 μg of TL per ml (Fig. 1). At 5 μg of TL per ml, TL rapidly inhibited all incorporation of radioactive precursors into RNA and protein (Fig. 4), although the incorporation of thymidine into DNA continued for some time. Concentrations of TL which totally inhibited RNA and protein synthesis and cell division had relatively little bactericidal effect (Fig. 3), and unlike reversible inhibitors of DNA synthesis such as nalidixic acid, which cause unbalanced growth, the mean cell size of *E. coli* did not change during effective inhibition of cell division by TL (Fig. 2). These facts suggest that TL may become a very useful tool for physiological studies in *E. coli* where reversible inhibition of macromolecular synthesis is desired.

The effects of TL on *E. coli* could be due to a specific inhibition of RNA or protein synthesis or both, or to some general effect upon energy metabolism or essential transport functions. The kinetics of inhibition of macromolecular synthesis (Fig. 4) suggest the former possibility and that RNA synthesis is the primary target. The in vivo rate of RNA synthesis, determined by uptake of [^{3}H]uridine in 3-min pulses, is inhibited by more than 95% with 5 min of exposure of *E. coli* K-12 to 5 μg of TL per ml. This conclusion has been strengthened by studies of the effects of TL on the synthesis of β-galactosidase in *E. coli*, which indicate that protein synthesis is not affected by the drug (7). Also, this conclusion is consistent with previous studies of the effects of TL on yeast which indicated that it primarily inhibited RNA synthesis in vivo (5) and that it inhibited the RNA polymerases of yeast in vitro (12). The effect of

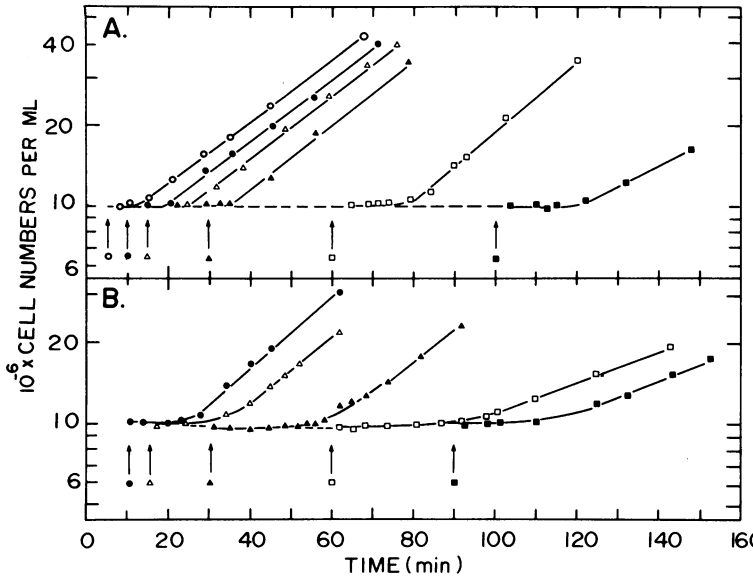

FIG. 6. *Recovery of cell division after treatment with different concentrations of TL. Exponentially growing cultures of GK 117 (in medium A + E) were treated with (A) 5 or (B) 20 μg of TL per ml at zero time. After a period of treatment indicated in each graph by the arrows, the cultures were washed free of TL and incubation was resumed in drug-free medium. Cell counts were monitored for each case and are plotted versus incubation time.*

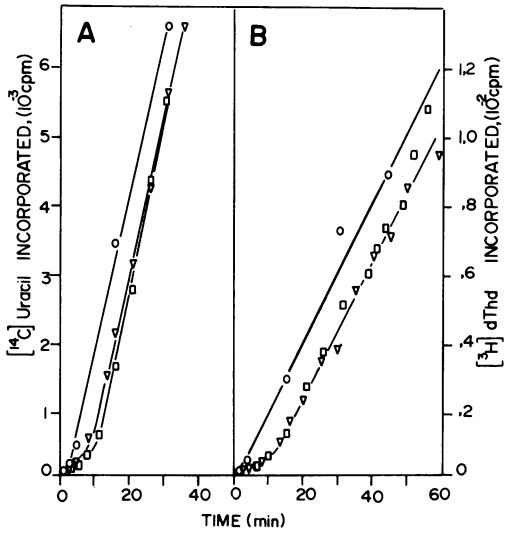

FIG. 7. *Recovery of macromolecular synthesis after treatment with TL. Cells of E. coli K-12 (GK 255) growing in medium A + glucose at 37 C were treated with 20 μg of TL per ml. Five (▽) and 40 min (□) after the treatment began, cells were washed free of the drug and incubated in drug-free medium. Incorporation of [^{3}H]thymidine (A) and [^{14}C]uracil (B) as shown as a function of time of incubation in drug-free medium. Unlabeled thymidine and uracil were each added at 10 μM/ml. Circles represent controls subjected to the same procedure but without TL.*

TL on DNA synthesis remains to be investigated, but is obviously not the primary site of action.

The influence of growth rate and/or media on the growth inhibitory effects of TL remains to be explained (Fig. 5). Thus, growth in different media results in differential residual cell division on exposure to TL and variation in the rate of recovery after removal of TL. This could result from differential permeability of the cells to TL under different conditions of growth, to differential susceptibility determined, for example, by the concentration of RNA polymerase in cells of different growth rates (3, 10), or to different effective concentrations of TL due to binding by medium components. It has been observed that TL diffuses poorly in solid media, which may support the latter suggestion. An effect of growth rate on in vivo action of streptomycin, which is known to bind to free 30S ribosomal subunits, has been demonstrated (8). We hope to identify the primary target for TL action by the analysis of resistant mutants, and it will then be possible to determine whether the concentration of this target is affected by the growth rate and media composition.

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