JOURNAL OF BACTERIOLOGY, Feb. 1969, p. 761-768

Vol. 97, No. 2
Printed in U.S.A.

Relationships Between Bactericidal Effect and Inhibition of Ribonucleic Acid Nucleotidyltransferase by Rifampicin in *Escherichia coli* K-12

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Received for publication 31 October 1968

The mechanism of action of rifampicin, an antibiotic which inhibits in vitro the polycondensation of ribonucleotides by ribonucleic acid (RNA) nucleotidyltransferase, was studied in vivo in *Escherichia coli*. It is argued that the inhibition of RNA nucleotidyltransferase represents the primary lesion and is responsible for the bactericidal effect. This conclusion is based on (i) the correlation between concentrations of the antibiotic which block in vivo incorporation of labeled uracil and the bactericidal concentrations, (ii) the evidence that the loss of viability of the cells immediately follows the block of RNA synthesis, and (iii) the observation that the reversal of the inhibition of RNA synthesis goes together with a reversal of the loss of viability.

Rifampicin, a broad-spectrum antibiotic belonging to the family of rifamycins (6), has been shown to inhibit in vitro the polycondensation of the four ribonucleotides by the deoxyribonucleic acid- (DNA) dependent ribonucleic acid (RNA) nucleotidyltransferase (EC 2.7.7.6) of *Escherichia coli* (3, 7-9).

The purpose of this study is to relate the effect discovered by the biochemists in a cell-free reaction mixture to the action of the antibiotic on the whole cell and to determine whether the inhibition of RNA nucleotidyltransferase is the causal event of the loss of cell viability in *E. coli*.

The discovery that a key enzyme is inhibited in vitro cannot be considered as sufficient evidence that this inhibition is the primary cell lesion (4). This evidence can be obtained only when it has been proved (i) that the antibiotic concentration effective in vivo on the enzyme is of the same order as the bactericidal concentration, (ii) that the loss of viability of the cells is contemporary or immediately successive to the inhibition of the enzyme, and (iii) that a reversal of the inhibition of the enzyme is promptly accompanied by a reversal of the loss of viability.

To study some of these problems, advantage has been taken of the existence of some strains of *E. coli* K-12, named RC^{rel} (relaxed control), which continue to synthesize RNA under conditions in which protein synthesis is blocked, ruling out any new enzyme synthesis and, for this reason, providing a simpler experimental model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A prototrophic strain of *E. coli* K-12, supplied by the Institute of Genetics of Pavia, was used in some experiments, together with a methionine auxotrophic derivative of *E. coli* K-12, strain Hfr Cavalli, containing the RC^{rel} allele, supplied by G. Tocchini-Valentini (LIGB, Naples). All the experiments were performed with cultures starting from a set of lyophilized tubes and prepared from a culture derived from a single cell.

Bacteria were cultivated in an aerated minimal medium (2). Viable counts were made in quadruplicate and surface plating was performed on MacConkey Agar (Difco), unless otherwise stated.

Radioisotopes assay. RNA synthesis was measured by the rate of incorporation of uracil-2-^{14}C into the cold trichloroacetic acid-insoluble fraction. The determination was performed by withdrawing 2-ml samples, at intervals, which were diluted with 3 ml of cold 8% trichloroacetic acid and filtered after at least 0.5 hr on membrane filters (GSWPO 2500; Millipore Corp., Bedford, Mass.). After drying, the membranes were fixed on stainless-steel planchets and counted in a thin-window counter. Comparison with a standard revealed a counting efficiency of about 30%. Throughout this investigation, uracil-2-^{14}C, purchased from the Radiochemical Center of Amersham, was diluted with cold uracil to give a final specific activity of 1.2

μc/μmole. This material was added to the cultures to obtain a final concentration of 5 μg/ml. Similar determinations were performed for measuring protein and DNA synthesis, adding ^{14}C-phenylalanine (specific activity 0.73 μc/μmole) or thymine-2-^{14}C (2.4 μc/μmole), respectively. The DNA synthesis was measured in a thymine-requiring *E. coli* K-12 strain, supplied by the Institute of Genetics of Pavia.

Optical density measurements were made with a Coleman spectrophotometer at 650 nm.

RESULTS

Short pulses of rifampicin are bactericidal. It has been shown by Arioli *et al.* (1) that rifampicin has a bactericidal effect. The aim of this experiment was to check how rapid the killing effect was.

E. coli K-12 cells, exponentially growing in minimal medium, were treated with various rifampicin concentrations. After 30 sec and at regular intervals, samples were withdrawn, diluted in saline, and immediately plated.

Under these conditions, rifampicin killed the cells on contact, showing that the adsorbed rifampicin is not easily washed out from the cells during manipulations. No lag period was observed before the onset of the loss of viability, showing that the penetration of rifampicin and its interference with the sensitive site are very rapid.

The rate of killing depended on the antibiotic concentrations (Fig. 1).

Complete block of RNA synthesis obtained with bactericidal concentrations. Figure 2 illustrates the inhibition of RNA synthesis under the same conditions in which the bactericidal effect was studied.

At the lowest antibiotic concentration (0.5 μg/ml), a decrease of the rate of incorporation of uracil was observed. Uracil uptake was completely blocked after a certain time with antibiotic (1 and 5 μg/ml) and immediately with 20 μg/ml or more.

The uracil uptake is in agreement with the data of the bactericidal effect because the number of viable cells in the presence of 0.5 μg of antibiotic per ml was reduced to about one-fourth within the first hour of contact; with a concentration of 5 μg/ml, the number of viable cells fell to one-tenth within the first 5 min and decreased to less than 1% in 20 min. With higher concentrations, the number of viable cells was immediately too low to produce a detectable variation in the radioactivity incorporated.

The effect of the antibiotic on protein and DNA synthesis was also studied by determining the incorporation into the trichloroacetic acid-insoluble fraction of ^{14}C-phenylalanine and of thymine-2-^{14}C.

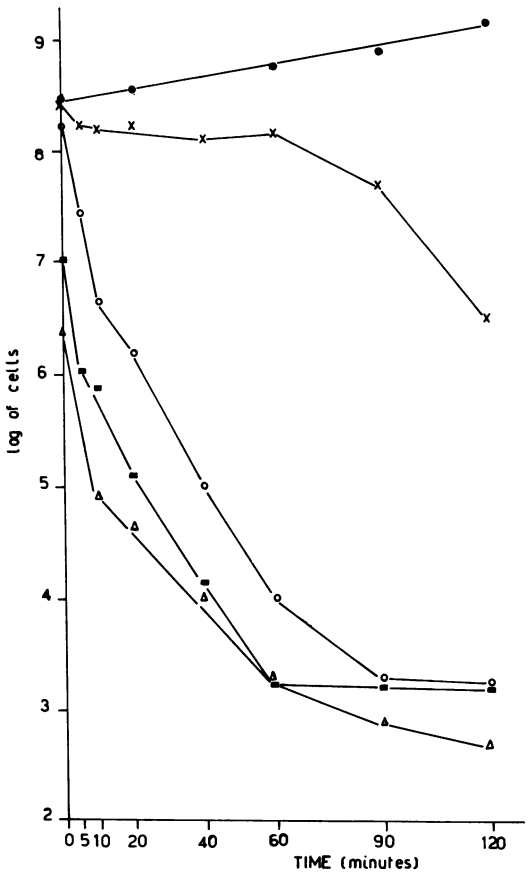

FIG. 1. *Bactericidal effect of rifampicin. E. coli K-12 cultures, growing exponentially in minimal medium, were treated with rifampicin. Samples were withdrawn at the time indicated and, after suitable dilutions, plated in quadruplicate on Mac Conkey Agar.* (●) *Control;* (×) *rifampicin, 0.5 μg/ml;* (○) *rifampicin, 5 μg/ml;* (■) *rifampicin, 40 μg/ml;* (△) *rifampicin, 80 μg/ml.*

At all the concentrations tested, the block of the phenylalanine uptake was observed about 10 min later than that of uracil. The thymine uptake decreased slowly, but continued for about 1 hr after the block of uracil incorporation.

Neither net protein nor RNA synthesis is required for the bactericidal effect. *E. coli* K-12 cells growing exponentially in minimal medium were washed by filtration on MF 50 membranes and suspended in the same medium depleted of the source of nitrogen (ammonium sulfate) to block protein and RNA synthesis. After 10 min, to allow complete starvation, rifampicin was added

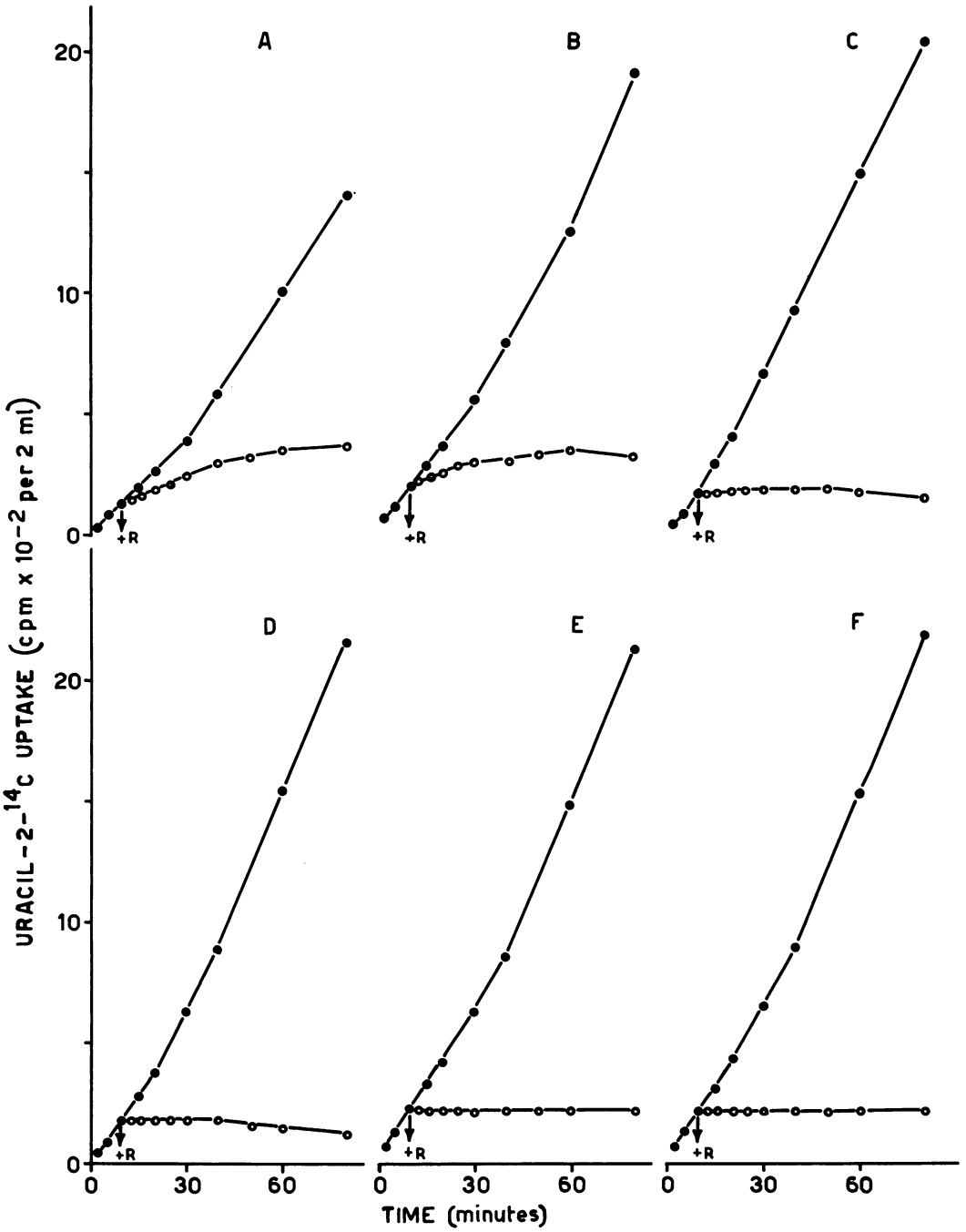

FIG. 2. *Inhibition of RNA synthesis in growing cells by different concentrations of rifampicin. To E. coli K-12 cultures, exponentially growing in minimal medium supplemented with 5 μg of uracil-2-^{14}C per ml, rifampicin was added at the final concentrations: A = 0.5 μg/ml; B = 1 μg/ml; C = 5 μg/ml; D = 20 μg/ml; E = 40 μg/ml; F = 80 μg/ml. At the times indicated, samples were withdrawn, and the insoluble fraction in cold trichloroacetic acid was counted. (●) Control cultures; (○) cultures treated with rifampicin (the arrows, +R, indicate the time of addition).*

and, after different intervals, samples were withdrawn and plated to count the survivors. The bactericidal effect was of the same order as that on growing cells.

Kinetics of the decline of the rate of uracil incorporation in RC^{rel} cells are of first order. The preceding experiment has shown that growth is not required for the bactericidal effect. However, under ammonium sulfate starvation conditions, both protein and RNA synthesis were interrupted, and it was impossible to correlate uracil incorporation with the bactericidal effect.

But an RC^{rel} methionine-requiring strain starved of methionine could continue to synthesize RNA also under conditions forbidding protein synthesis. Advantage of this fact has been taken to study the kinetics of the inhibition of the RNA-synthesizing machinery, considering that the instantaneous rate of uracil incorporation is a function of the residual activity of RNA nucleotidyltransferase.

Cells growing in minimal medium, supplemented with methionine, were washed by filtration on MF 50 membranes and resuspended in the same medium, devoid of methionine. Uracil-2-^{14}C was added, and, after 5 min of starvation, different concentrations of rifampicin were administered.

Incorporated uracil was counted in samples withdrawn at regular intervals until a plateau was reached. The instantaneous rate of incorporation of uracil-2-^{14}C could be derived from this cumulative curve of the uptake and plotted against time (Fig. 3). The decline of the rate of incorporation follows a first-order kinetics, with only a certain departure from the expected curve of the first point which is lower. The intercept with the rate axis corresponds with the incorporation rate observed in the controls. The departure of the first point from the expected curve indicates that during the first few minutes the rate of inhibition of RNA nucleotidyltransferase is higher.

Cells treated with rifampicin, when washed, resume incorporating uracil and forming colonies on plates. RC^{rel} cells, growing in minimal medium supplemented with methionine, were washed and resuspended in the same medium devoid of methionine, but containing uracil-2-^{14}C.

After 5 min of starvation, rifampicin was added in concentrations sufficient to completely block uracil incorporation.

After a certain period of time from the moment when a plateau of uracil incorporation was reached, cells were washed by filtration on FG 50 membranes and resuspended in the same medium, devoid both of methionine and rifampicin. After 30 min, a resumption of uracil incorporation was observed (Fig. 4, 5). The resumption was observed also when washing was performed after 180 min of complete uracil incorporation block.

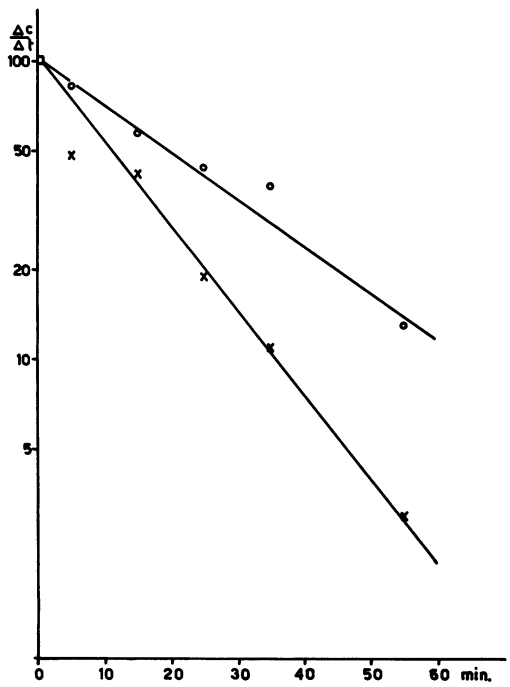

FIG. 3. *Kinetics of inhibition of RNA synthesis by different concentrations of rifampicin. Rifampicin was added to cultures of a methionine-requiring E. coli K-12 RC^{rel} strain starved of methionine, and the inhibition of RNA synthesis was determined. The residual rate of synthesis expressed as $\Delta c/\Delta t$ (increase of radioactivity in trichloroacetic acid-insoluble fraction per unit of time) was plotted against time.* (○) *Rifampicin added, 1 μg/ml;* (×) *rifampicin added, 5 μg/ml;* (□) *control culture. (Similar results were observed with 2 and 10 μg/ml.)*

A similar experiment was carried out under the same conditions to count the cells that were able to form colonies on plates. After a sufficient time of contact with rifampicin to completely block RNA-synthesis, cells were washed by filtration, resuspended in minimal medium devoid of methionine, and plated at regular intervals on MacConkey Agar plates. The number of cells that were able to form colonies increased during the period of incubation in minimal medium, reaching a plateau at last.

In other experiments instead of washing out rifampicin by filtration, the culture was simply diluted 10^{-3} or 10^{-2} times with the same medium, devoid of methionine; also, in this case, an increase of the number of survivors was observed

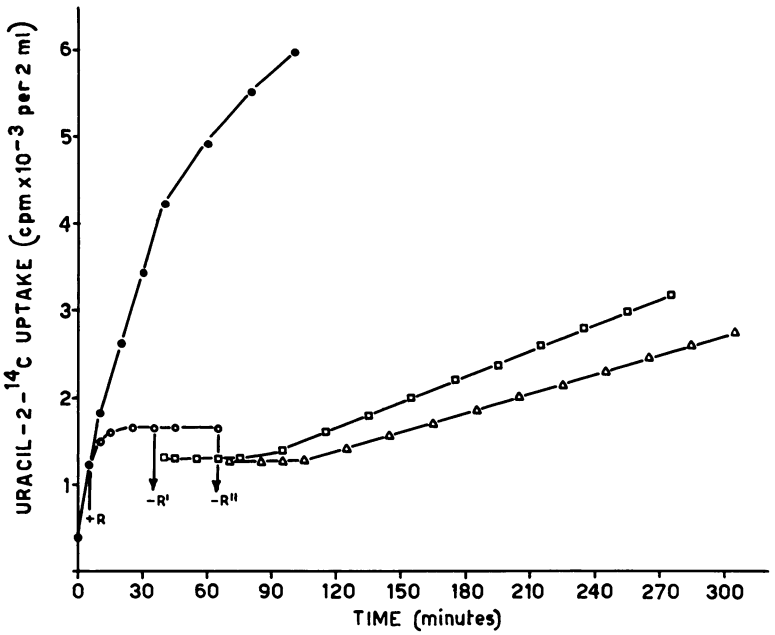

FIG. 4. *Resumption of uracil incorporation by E. coli cells treated with rifampicin after removal of the antibiotic. Rifampicin to a final concentration of 10 μg/ml was added (+R) to an E. coli K-12 RC^{rel} strain culture, resuspended in a medium containing uracil-2-^{14}C, and deprived of methionine. At the time indicated by −R′ and −R″ samples of the culture were filtered and resuspended in minimal medium supplemented with uracil-2-^{14}C. The uracil incorporation was determined.* (●) *Control;* (○) *added with rifampicin;* (□) *resuspended in fresh medium (−R′) after 30 min of contact with rifampicin;* (△) *resuspended in fresh medium (−R″) after 60 min of contact with rifampicin.*

with time of permanence in minimal medium (Fig. 6).

In separate experiments, washed cells were plated on minimal medium agar plates, supplemented with methionine instead of on MacConkey Agar plates, to determine whether the resumption of viability was due to the minimal medium. But also under these conditions, a similar resumption of viability was observed for cells plated after a certain period of time. Addition of Ca^{++} to the minimal liquid medium showed that this ion had no influence on the reversibility of the inhibition.

In additional experiments, wild-type cells treated with rifampicin were resuspended in saline instead of in minimal medium. Under these conditions, a resumption of viability was observed.

The increase in number of survivors starts almost immediately after washing out the rifampicin, but the resumption of uracil uptake was observed only after 30 min. This apparent discrepancy is due to the insensitivity of the counting method which can detect an incorporation only when its rate is not very much smaller than that of the control.

There is a good correlation between the observed rate of uracil-2-^{14}C incorporation and the number of survivors before and after washing out the rifampicin.

DISCUSSION

The results are compatible with the assumption that the inhibition of RNA synthesis is the primary lesion and the causal event of the bactericidal effect of rifampicin. This interpretation is supported by the following evidence: (i) the bactericidal effect is shown by the same concentrations that are effective in blocking the uracil-2-^{14}C incorporation; (ii) the bactericidal effect is just as early an effect as the block of RNA synthesis; (iii) when cells, under conditions forbidding net protein synthesis, resume RNA synthesis, they recover also their ability to form colonies.

The experiments show also the specificity of action of rifampicin. DNA synthesis continues for a period of time that corresponds to a duplication time. The residual synthesis of DNA is almost 50%, showing that cells unable to form

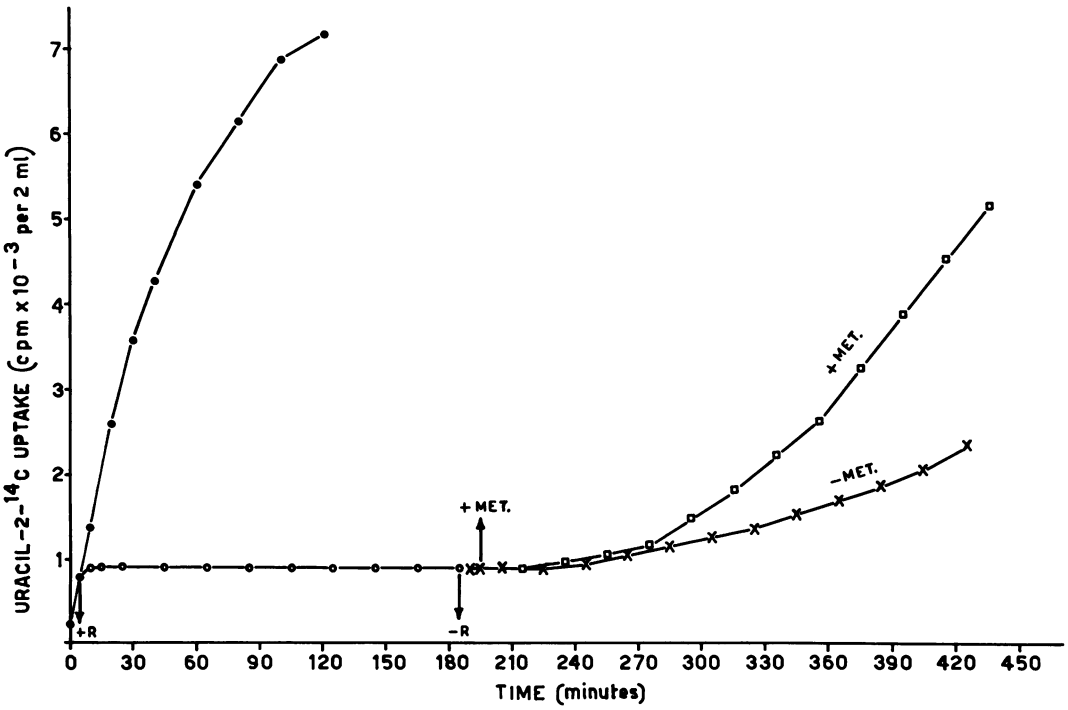

FIG. 5. *Resumption of uracil incorporation by E. coli cells treated with rifampicin after removal of the antibiotic. Strain and procedure are as described in Fig. 4; rifampicin was added (+R), 40 μg/ml. To a portion of the culture after removal of the antibiotic (−R), methionine was added (+Met) to a final concentration of 10 μg/ml. (●) Control; (○) added with rifampicin; (×) resuspended in minimal medium after 180 min of contact with rifampicin; (□) resuspended in minimal medium supplemented with 10 μg of methionine per ml, after 180 min of contact with rifampicin.*

colonies can complete a round of replication of their chromosomes.

Protein synthesis is completely blocked only 12 min after complete block of RNA synthesis; this is just the time necessary to dispose of all the available messenger RNA.

No appreciable difference was detected in the lethality of cells actively synthesizing RNA and of cells that do not synthesize RNA. This result requires a certain comment since the in vitro experiments (7, 8) have shown that rifampicin acts on RNA nucleotidyltransferase prior to the formation of the initiation complex, i.e., the complex formed by the enzyme, the DNA, and the purine ribosides. Because in the growing cells the enzyme molecules which are tested by the incorporation method are just those engaged in active synthesis, they should be protected from rifampicin. But this apparent paradox can be resolved; it requires only a very short time to make a 23*S* RNA molecule (5).

At higher concentrations of rifampicin, an immediate block of uracil incorporation without any apparent delay was observed. Also this fact depends upon the high rate of growth of single RNA molecules and upon the relatively short average time of completion of these molecules, compared to the interval between addition of rifampicin and first sampling.

The fact that protein synthesis is blocked 10 min after complete inhibition of uracil incorporation demonstrates that rifampicin is active on RNA nucleotidyltransferase-synthesizing messenger RNA. The fact that rifampicin inhibits RNA synthesis in RC^{rel} cells, under conditions forbidding protein synthesis, demonstrates that rifampicin is active also on RNA nucleotidyltransferase-synthesizing ribosomal RNA. Although these data do not prove the existence of only one type of RNA nucleotidyltransferase in bacterial cells, they are compatible with this hypothesis.

The resumption of RNA synthesis and the increase in the number of survivors in cultures of RC^{rel} cells with rifampicin washed out and resuspended in minimal medium, under conditions

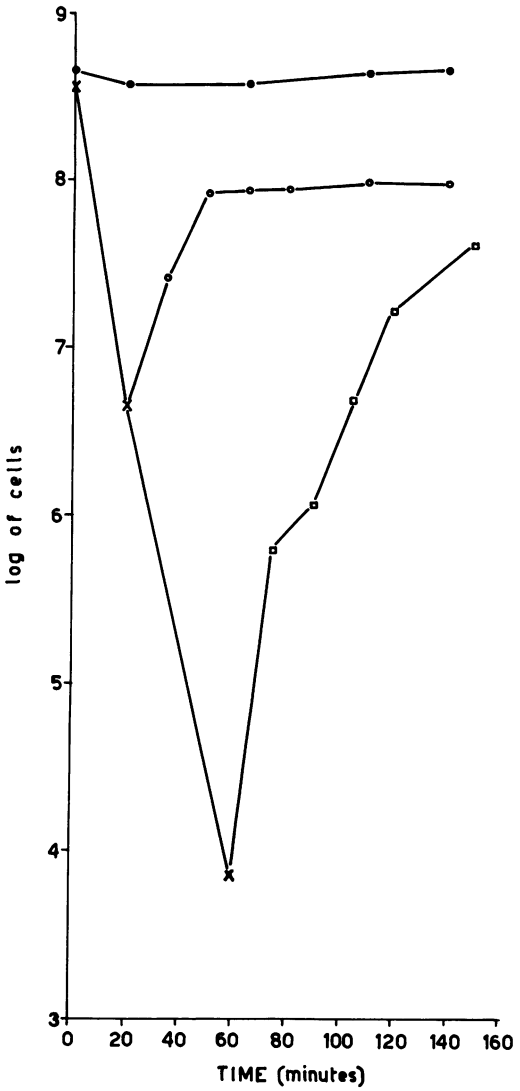

FIG. 6. *Recovery of cells treated with rifampicin after removal of the antibiotic. A methionine-requiring E. coli RC^{rel} culture, growing in minimal medium supplemented with methionine, was resuspended in minimal medium without methionine and treated with 40 μg of rifampicin per ml. After 20 and 60 min of contact, the culture was diluted in minimal medium without methionine, and at intervals samples from dilutions were withdrawn and plated on MacConkey Agar.* (●) *Control;* (X) *survivors in the presence of 40 μg of rifampicin per ml;* (○) *recovery of cells in absence of rifampicin after 20 min of contact with the antibiotic;* (□) *recovery of cells in absence of rifampicin after 60 min of contact with the antibiotic.*

forbidding new protein synthesis, could be interpreted as evidence that under these conditions the binding of rifampicin with the enzyme is slowly reversible. However, G. Hartmann (*personal communication*) has informed us that in vitro the complex of rifampicin with the enzyme is not destroyed by dialysis. This observation opens the question of whether the stability of the complex in vitro is comparable to that in vivo. At the present time, we are unable to clarify this point. In contrast, the inactivation of the enzyme seems to be more stable when the cells are plated on agar plates. It is not easy to find an explanation for this difference, but it can be ruled out that conditions allowing for resumption of growth may be responsible for the higher lethality. When methionine is added in minimal medium to RC^{rel} cells, with rifampicin washed out and thus conditions allowing for protein synthesis are restored, the rate of RNA synthesis is increased (Fig. 5) in comparison with that of starved cells. The interval between the washing out of rifampicin and resumption of RNA synthesis is the same under both conditions. The observed increased rate of RNA synthesis in cultures with methionine added can be explained as caused by both the reversal of the inhibition of the enzyme and by the further synthesis of new proteins; this rules out that resumption of growth is responsible for the irreversibility of the loss of viability. The reversal of the loss of viability can neither be explained as caused by the specific composition of the minimal medium, as it is observed when cells are plated on minimal agar instead of MacConkey agar, nor can it be attributed to metabolic activity, as it can be observed in cells resuspended in saline. Apparently, the only condition required for reversal of the loss of viability is suspension in a liquid medium, as if under these conditions, some inhibitory factor could diffuse more easily than on agar plates.

ACKNOWLEDGMENTS

We thank Giuseppe Sartori and Giuliana Bolzoni for excellent technical assistance.

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