

# Effect of Thymine Starvation on Messenger Ribonucleic Acid Synthesis in *Escherichia coli*

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## ABSTRACT

LUZZATI, DENISE (Institut de Biologie Physico-Chimique, Paris, France). Effect of thymine starvation on messenger ribonucleic acid synthesis in *Escherichia coli*. *J. Bacteriol.* 92:1435-1446. 1966.—During the course of thymine starvation, the rate of synthesis of messenger ribonucleic acid (mRNA, the rapidly labeled fraction of the RNA which decays in the presence of dinitrophenol or which hybridizes with deoxyribonucleic acid) decreases exponentially, in parallel with the viability of the thymine-starved bacteria. The ability of cell-free extracts of starved bacteria to incorporate ribonucleoside triphosphates into RNA was determined; it was found to be inferior to that of extracts from control cells. The analysis of the properties of cell-free extracts of starved cells shows that their decreased RNA polymerase activity is the consequence of a modification of their deoxyribonucleic acid, the ability of which to serve as a template for RNA polymerase decreases during starvation.

The physiological disturbances caused by thymine deprivation in bacterial cells, which are unable to synthesize this pyrimidine, have been known since their first description in 1954, by Cohen and Barner (1, 2, 7). These authors demonstrated the lethal effect of thymine starvation on *Escherichia coli* 15 T<sup>-</sup> and showed that, whereas deoxyribonucleic acid (DNA) synthesis is immediately stopped upon thymine removal, protein and ribonucleic acid (RNA) synthesis still proceed for a while. The lethal effect of thymine starvation has since been a standard observation for every strain of *E. coli* or *Bacillus subtilis* examined (13, 17, 19, 28, 35, 36), and the first observations on its effects on the cell's biosynthetic capacities have been extended (20, 22, 23; C. Revel, Ph.D. Thesis, Strasbourg, France, 1963). Many other effects of thymine starvation have since been described, most of which seem to appear without lag. Among these are the induction of different prophages (17, 26) or of colicinogenic factors (19), mutagenicity (3, 9), the intracellular accumulation of deoxyadenosine-5'-triphosphate (29), and the induction of the synthesis of a new initiator of replication of the bacterial chromosome (34). The presence of two replication sites per chromosome does not, however, seem to bear pathological consequences for the bacterial cell (38), but it does account for the

increased rate of synthesis of DNA, observed after addition of thymine to the starved cultures (3, 34; Revel, Ph.D. Thesis, 1963).

The effects of thymine starvation on bacterial viability, as well as on protein and RNA synthesis, appear only after a certain lag. Immediately after thymine withdrawal, the synthesis of these macromolecules proceeds, first at the same or at a slightly increased rate in comparison with unstarved cells (21, 22; Revel, Ph.D. Thesis, 1963). After a while, however, their rates of synthesis decrease progressively, and, for *E. coli* 15 T<sup>-</sup> at least, RNA and protein synthesis stops after 120 min of starvation at 37 C (Revel, Ph.D. Thesis) or 4 hr at 20 C (23). The addition of thymine does not restore the cell's capacity to synthesize RNA or proteins (23; Revel, Ph.D. Thesis), even though the bacteria are still able to synthesize DNA (1, 30; Revel, Ph.D. Thesis, 1963).

Many hypotheses have been proposed to account for the major effects of thymine starvation, i.e., thymineless death and progressive inhibition of protein and RNA synthesis. Some investigators (7) have suggested that these consequences derive from the imbalanced growth which proceeds during starvation, whereas others have thought that stopping DNA synthesis while engaged in a replication cycle causes some modifications of this essential cell constituent (18, 20). However,

no change has yet been detected in the DNA extracted from thymine-starved *E. coli* which could account for the known effects of thymine starvation. All of the physicochemical properties of DNA from thymine-starved cells, such as viscosity, molecular weight, density in a cesium chloride gradient, melting curve, and sedimentation constant of native and denatured DNA, have been found identical to those of the DNA extracted from control cells (18, 37; Revel, Ph.D. Thesis, 1963).

It has been suggested (23; D. Luzzati, Abstr. Intern. Congr. Biochem., 6th, I-126, 1964) that the decrease of the starved cell's ability to synthesize RNA and proteins could depend on a decreased ability to synthesize messenger RNA (mRNA). This possibility has led us to examine the rate of synthesis of mRNA during thymine starvation. The results reported below confirm and enlarge these observations, and show that during thymine starvation the rate of synthesis of mRNA decreases at the same pace as cell viability. In addition, we demonstrate that the decrease in the rate of synthesis of mRNA comes from a decreased ability of the DNA of starved cells to serve as template for RNA polymerase.

#### MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains employed were *E. coli* 15 *T*<sup>-</sup> (*thy*<sup>-</sup>), obtained from S. S. Cohen, *E. coli* B<sub>3</sub> (*thy*<sup>-</sup>, *Sm*<sup>r</sup>), originally isolated by S. Brenner, and *E. coli* CR 34 (*thy*<sup>-</sup>), *threo*<sup>-</sup>, *leuc*<sup>-</sup>, *lac*<sup>-</sup>, *B*<sub>1</sub><sup>-</sup>, isolated by Okada (32).

**Media and growth conditions.** Minimal medium A (10) was supplemented, as needed, with thymine (10<sup>-4</sup> M) threonine (50 μg/ml), and leucine (50 μg/ml), or with Vitamin Free Casamino Acids (1%, Difco) and vitamin B<sub>1</sub> (1 μg/ml). Glucose at a final concentration of 1% was used as carbon source.

Bacterial growth was followed by absorbancy measurements at 630 mμ in a Zeiss spectrophotometer with cells having a 1-cm optical path.

For thymine starvation, the cultures were incubated at 37 C, with good aeration. The cells were collected during the logarithmic phase (optical density, 0.5 to 0.8) by centrifuging the cultures, after they had been cooled to 0 C. The cells were then washed twice with 1 volume of 0.15 M NaCl, resuspended in 1 volume of the same medium without thymine, and reincubated under the same conditions.

**Assay of rapidly labeled "dinitrophenol (DNP) decayable" RNA.** Samples (10 ml) from control and thymine-starved cultures were transferred at different times after the beginning of the experiments to vessels containing 1.1 μc of C<sup>14</sup>-uracil (Commissariat à l'Énergie Atomique, Saclay, France), 25 μc/μmole, and were shaken in a water bath at 37 C. Incorporation of the tracer was allowed to proceed for 15 sec, and was stopped by the addition of 1 ml per vessel of a 5 × 10<sup>-2</sup> M solution of DNP. Samples of 1 ml

were removed into cooled test tubes at 0.5, 2.5, 5, 10, 15, 20, 30, 45, and 60 min after DNP addition. The samples were immediately precipitated by adding 1 volume of 10% trichloroacetic acid. The precipitates were collected on a membrane filter (Millipore Filter Corp., Bedford, Mass.), washed, dried, and counted with a thin-window gas-flow counter (Tracerlab, Richmond, Calif.). The fraction of the pulse-labeled RNA which decays after addition of DNA was then determined graphically.

**Assay for rate of RNA synthesis.** Samples (10 ml) from control and starved cultures were transferred at different times after the beginning of the experiment to vessels containing 0.1 μc/ml of C<sup>14</sup>-uracil (25 μc/μmole), and were shaken in a water bath at 37 C. Samples of 1 ml were withdrawn at 1, 2, 5, 10, 15, and 25 min after transfer, and were immediately precipitated by addition of 1 ml of cold 10% trichloroacetic acid. The precipitates were collected on membrane filters, washed, dried, and counted as previously described. Under these experimental conditions, C<sup>14</sup>-uracil incorporation into RNA was linear for 15 to 20 min.

**Determination of the fraction of pulse-labeled RNA hybridizable with homologous DNA.** Control or starved bacteria were labeled as previously for 15 sec with either 25 μc/ml of C<sup>14</sup>-uracil (Commissariat à l'Énergie Atomique) or 0.55 μc/ml of H<sup>3</sup>-uridine (Radiochemical Centre, Amersham, England), 2.5 mc/μmole. The labeling period was ended by rapidly pouring the culture on crushed ice in the presence of sodium nitrite (5 × 10<sup>-3</sup> M). The bacteria were then centrifuged at 0 C and washed once with 0.1 volume of cold TM buffer [0.01 M tris(hydroxymethyl)amino-methane (Tris), pH 7.2; 0.002 M MgCl<sub>2</sub>]; the pellet was frozen.

The frozen pellets were resuspended in 3 volumes of TM buffer, and lysozyme (100 μg/ml) was added. The cell suspension was thawed and frozen (at -80 C) three times. Deoxyribonuclease (25 μg/ml) was added and allowed to react for 10 min at 0 C. Lysis was completed by the addition of sodium dodecyl sulfate to a final concentration of 0.5%.

RNA was extracted by the phenol method. One volume of water-saturated phenol (65% w/w; Merck & Co., Inc., Rahway, N.J.) was added to the lysate. After being shaken vigorously for 30 min at 4 C, the emulsion was centrifuged and the supernatant aqueous phase was decanted. The phenolic phase was washed once with 0.1 volume of the same buffer which was added to the previous supernatant fraction. The aqueous phase was treated twice more with phenol, as before, and the RNA was precipitated in the presence of 0.2 M NaCl, with 2 volumes of ethyl alcohol. The precipitate obtained after the solution had stood for 30 min at -15 C was collected by centrifugation and dissolved in 0.5 volume of 0.1 M sodium acetate buffer (pH 4.5). The RNA was again precipitated as before. The solubilization and precipitation cycle was repeated twice, and the purified RNA was finally dissolved in 0.25 volume of SSC (standard saline-citrate, 0.15 M NaCl and 0.015 M sodium citrate) diluted 10-fold. The concentration of RNA was

determined either by the orcinol method or by measuring the optical density of the solution at 260  $m\mu$  (40  $\mu\text{g/ml}$  RNA having an absorption of 10).

The technique used for hybridization was essentially that described by Nygaard and Hall (31). RNA solutions were heated for 5 min at 75 C and were immediately cooled to 0 C. DNA from *E. coli* 15 T<sup>-</sup>, prepared by a method similar to Marmur's (25), as described below, was thermally denatured. From 50 to 200  $\mu\text{g}$  of this DNA was mixed with variable amounts (10 to 100  $\mu\text{g}$ ) of RNA in a total volume of 0.3 ml of twice concentrated SSC. Hybridization was then carried out for 4 hr at 63 C. After slow cooling, 0.7 ml of a ribonuclease solution in twice concentrated SSC (final concentration 4  $\mu\text{g/ml}$ ) was added, and the mixture was incubated at 30 C for 20 min; 2 ml of twice concentrated SSC was then added to each tube, and the mixture was filtered on a coarse HA filter (Schleicher and Schuell Co., Keene, N.H.). The filters were washed with 60 ml of twice concentrated SSC, air-dried, and immersed in 10 ml of scintillation fluid containing 0.3 g of 1,4-bis-2(4-methyl-5) phenyloxazolyl benzene (POPOP), 0.5 g of diphenyloxazole (PPO), and 1 liter of toluene. The radioactivity retained by the filters was counted in a scintillation counter (Tricarb). Background counts, obtained by incubating RNA with or without native DNA, amounted to 0.1 to 0.5% of input counts.

**Determination of RNA polymerase activity of bacterial extracts.** For the preparation of cell-free extracts, the bacteria were harvested by centrifugation and were washed with 0.01 M Tris buffer (pH 7.2) equal to one-tenth of the culture volume. The pellet was frozen and ground in a precooled mortar with 2 volumes of precooled alumina. The ground bacteria were resuspended in 2 to 3 volumes of buffer A (8) containing 0.01 M mercaptoethanol. Alumina and bacterial debris were removed by two 10-min centrifugations at 15,000  $\times$  g.

Portions of the cell-free extracts (5 to 50  $\mu\text{liters}$ ) were added to 0.25 ml of the following reaction mixture: Tris buffer (pH 7.9),  $2.3 \times 10^{-2}$  M;  $\text{MgCl}_2$ ,  $2.3 \times 10^{-3}$  M;  $\text{MnCl}_2$ ,  $2.3 \times 10^{-3}$  M; spermine,  $1.4 \times 10^{-4}$  M; mercaptoethanol,  $1.6 \times 10^{-2}$  M; ATP, GTP, UTP, or CTP,  $5 \times 10^{-4}$  M;  $\text{C}^{14}$ -UTP or -CTP,  $2.5 \times 10^{-4}$  M. The incorporation of nucleotides into an acid-insoluble product was allowed to proceed for 15 min at 35 C. The reaction was stopped by rapidly cooling the mixtures to 0 C and adding 3 ml of cold 5% trichloroacetic acid. The precipitates were filtered, washed, dried, and counted as described before.

Under the above experimental conditions, the incorporation of nucleotides was linear for 20 to 30 min, and was completely stopped by the addition of 10  $\mu\text{g}$  of actinomycin D.

**DNA preparations.** The thymus DNA used was a gift from M. J. Pouyet, Commissariat à l'Énergie Atomique.

T4 DNA was extracted with water-saturated phenol from phage purified by differential centrifugation.

Bacterial DNA from control *E. coli* 15 T<sup>-</sup> and from cells thymine-starved for 120 min was purified by two different methods: (i) by a modification of Marmur's

method (25) and (ii) by density gradient sedimentation of portions of cell-free extracts.

For the first type of purification, frozen washed bacterial cells were thawed and resuspended in 0.15 M NaCl-0.015 M ethylenediaminetetraacetate (EDTA), 10 ml per g of wet packed cells. A neutralized solution of 20% sodium dodecyl sulfate (SDS) was added to a final concentration of 2%, and the bacterial suspensions were vigorously shaken for 30 min at 4 C. The NaCl concentration of the lysates was adjusted to 1 M, and a first cycle of chloroform-isoamyl alcohol (10:1, v/v) deproteinization was performed, followed by precipitation of DNA fibers with 2 volumes of ethyl alcohol. The DNA was dissolved in 0.15 M NaCl and the chloroform-isoamyl deproteinization was repeated six or seven times. The DNA preparations were freed from RNA and polysaccharides by incubation with ribonuclease (50  $\mu\text{g/ml}$ ) and lysozyme (10  $\mu\text{g/ml}$ ) for 2 hr at 37 C. After precipitation with ethyl alcohol as above, and dissolution in 0.15 M NaCl, the DNA was further deproteinized by three cycles of chloroform-isoamyl alcohol treatment, and was finally precipitated with ethyl alcohol. The DNA fibers were moderately dried, stored at 4 C, and dissolved in the proper buffer when needed. Before these DNA solutions were used for hybridization experiments or as template for RNA polymerase, they were subjected to three extractions with water-saturated phenol (to remove any remaining ribonuclease) followed by extensive dialysis against 10-fold diluted SSC or Tris buffer (0.01 M, pH 7.4).

The physical parameters of these purified DNA preparations were as follows. For DNA from the control cells, the molecular weight was  $10 \times 10^6$ , intrinsic viscosity was 143 dl/g, the midpoint of the absorbance temperature profile was  $91 \text{ C} \pm 0.5$ , and the buoyant density in a CsCl gradient (see reference 18) was 1.714. Physical characteristics of the DNA from the starved cells were the same except that the intrinsic viscosity was 171 dl/g.

For purification of bacterial DNA by the density gradient method, portions of cell-free extracts of control and thymine-starved *E. coli* 15 T<sup>-</sup>, prepared as described above, were freed from ribosomes by 2-hr centrifugation at 100,000  $\times$  g; 1 to 2 ml of the supernatant fluid was diluted with buffer A (8) to 3.8 ml, and enough solid CsCl was added to give a density of 1.71. After centrifugation at 36,000 rev/min in a SW 39 rotor on a Spinco model L centrifuge for 62 hr, a needle was inserted into the bottom of the tubes. About 20 fractions of 0.2 ml were collected and diluted to 1 ml with Tris buffer (0.01 M, pH 7.4). The DNA-containing fractions, detected by absorbance profile of the gradient at 260  $m\mu$ , were pooled and dialyzed against the same buffer. The exact DNA content was determined by diphenylamine reaction (6), as no attempt was made to remove residual RNA.

**Preparation of purified *E. coli* RNA polymerase.** The method used was essentially that of Chamberlin and Berg (8), with the following variation: the streptomycin sulfate step was omitted, and the DNA-RNA polymerase complex was precipitated with protamine sulfate. The precipitate thus obtained was

washed with  $\beta,\beta$ -dimethylglutarate buffer (0.5 M, pH 7), before the polymerase was eluted with 0.5 M succinate buffer (pH 6).

### RESULTS

*In vivo study of the effect of thymine starvation on mRNA synthesis.* We have measured, at various times after the beginning of thymine starvation, the rate of synthesis of mRNA, defined as the fraction of pulse-labeled RNA which decays after addition of dinitrophenol (16). After a slight increase, which occurred during the lag period, the fraction of the pulse-labeled DNP decayable RNA decreased exponentially in parallel with the viability of the starved bacteria, reaching a plateau at about 10% of its rate of synthesis in the control (Fig. 1a). The rate of decay of pulse-labeled RNA was the same at the beginning and at later times during thymine starvation. What was observed, then, was a decrease in the rate of synthesis of pulse-labeled RNA and not an increase in its lability. The rate of synthesis of the bulk of the RNA (Fig. 1b) increased slightly during the lag period and then decreased, but less rapidly than that of the pulse-labeled RNA.

Thymine starvation had a greater effect on the rate of synthesis of a given class of RNA, namely pulse-labeled DNP decayable RNA. To ascertain whether this class of RNA really corresponded to mRNA rather than to some ribosomal precursor, we measured the amount of rapidly labeled RNA which hybridized with *E. coli* 15 T<sup>-</sup> DNA. For starvation times longer than 40 min, the fraction of pulse-labeled RNA which hybridized with DNA decreased progressively, concomitantly with bacterial viability (Fig. 2, Table 1). As shown in Table 1, the decrease in rate of mRNA synthesis, as determined by pulse-labeled RNA decay after addition of DNP, agrees well with the decrease as determined by its ability to hybridize with DNA. This decrease can thus be ascribed to a diminution of the capacity of thymine-starved bacteria to synthesize mRNA.

*In vitro study of the effects of thymine starvation on mRNA synthesis.* The experiments reported above showed the decrease in the rate of mRNA synthesis during thymine starvation without allowing us to ascertain the cause. We have further investigated this effect by analyzing RNA synthesis in extracts of control and starved bacteria.

We will define the "RNA polymerase" activity of bacterial extracts as that activity which catalyzes the incorporation of a labeled ribonucleoside triphosphate in the presence of the other three nucleoside triphosphates into an acid-precipitable product.

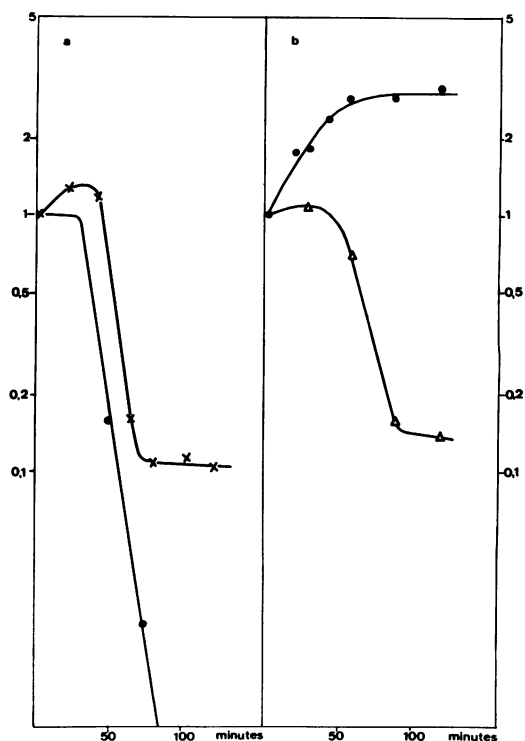


FIG. 1. Rate of RNA synthesis during thymine starvation of *Escherichia coli* 15 T<sup>-</sup>. At time zero, washed *E. coli* 15 T<sup>-</sup> cells, previously grown in complete medium, were resuspended in prewarmed thymine-free medium. Samples were withdrawn at different times after the beginning of thymine starvation to measure simultaneously the optical density, the number of viable cells per milliliter, the amount of 15-sec pulse-labeled RNA which is DNP decayable, and the rate of RNA synthesis. The variation of these different parameters, relative to their value at time zero, is plotted, on a logarithmic scale, as a function of the time of incubation in the thymine-free medium. (a) Symbols: X, amount of DNP decayable radioactivity after a 15-sec pulse labeling with C<sup>14</sup>-uracil, 25  $\mu\text{C}/\mu\text{mole}$ , 1  $\mu\text{C}/\text{ml}$ ; ●, number of viable cells per milliliter. (b) Symbols: Δ, rate of RNA synthesis; ●, optical density of the culture at 660 m $\mu$ .

The results obtained *in vitro*, described below, reflect the situation observed *in vivo*: the cell-free extracts of starved *E. coli* 15 T<sup>-</sup> showed a reduced capacity to incorporate nucleoside triphosphates into RNA as compared with control cell extracts. As shown in Fig. 3, incorporation of C<sup>14</sup>-uridine triphosphate (UTP) catalyzed by a given amount of starved cell extract, although linear for the same length of time (30 to 60 min), was always slower than incorporation catalyzed by an equivalent amount of control extract, and the maximal amount incorporated is lower. In-

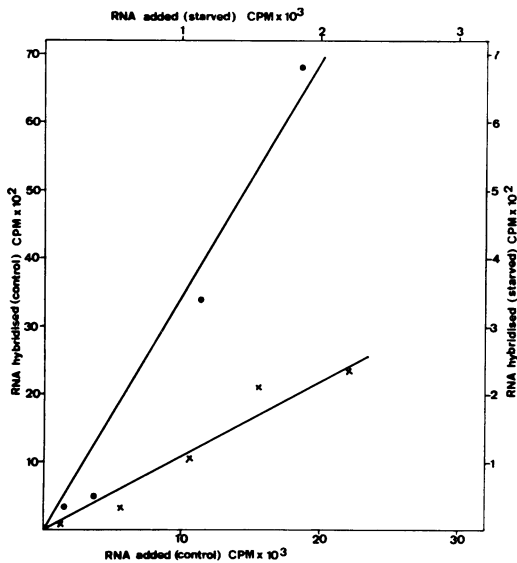


FIG. 2. Hybridization of pulse-labeled RNA from control and thymine-starved *Escherichia coli* 15  $T^-$  with control DNA. Hybridization of pulse-labeled RNA (15-sec pulse with  $5 \mu\text{C}/\text{ml}$  of  $C^{14}$ -uracil,  $25 \mu\text{C}/\mu\text{mole}$ ) with  $200 \mu\text{g}$  of purified DNA from exponential *E. coli* 15  $T^-$ , as a function of the quantity of RNA (10 to  $100 \mu\text{g}$ ) added to reaction media. Symbols: ●, control RNA (specific activity,  $7.7 \times 10^4$  counts per min per mg); ×, RNA from cells thymine-starved for 60 min (specific activity,  $1.1 \times 10^4$  counts per min per mg).

TABLE 1. *m*-RNA synthesis during thymine starvation<sup>a</sup>

Expt	Pulse-labeled RNA hybridizable with DNA (% of control)	Pulse-labeled RNA DNP decayable (% of zero-time)	Viable cells (% of zero-time)
a	69	67	61
b	23	16	10

<sup>a</sup> Cultures of control and thymine-starved cells were both labeled for 15 sec with uridine- $H^3$ ,  $2.5 \text{ mc}/\mu\text{mole}$ ,  $0.55 \mu\text{C}/\text{ml}$  (experiment a), or with uracil- $C^{14}$ ,  $25 \mu\text{C}/\mu\text{mole}$ ,  $5 \mu\text{C}/\text{ml}$  (experiment b). Their RNA was extracted, and the fraction of the incorporated label which hybridizes with DNA was determined, as described in Materials and Methods. Samples of the thymine-starved cultures were taken at the onset of starvation (zero-time) to measure the fraction of RNA labeled for 15 sec (uracil- $C^{14}$ ,  $25 \mu\text{C}/\mu\text{mole}$ ,  $0.1 \mu\text{C}/\text{ml}$ ) which is DNP decayable, as well as the number of viable cells per milliliter.

corporation, as determined after 15 min of incubation at 35 C, increased with the quantity of extract (normalized according to its DNA content) added to the reaction mixture for both

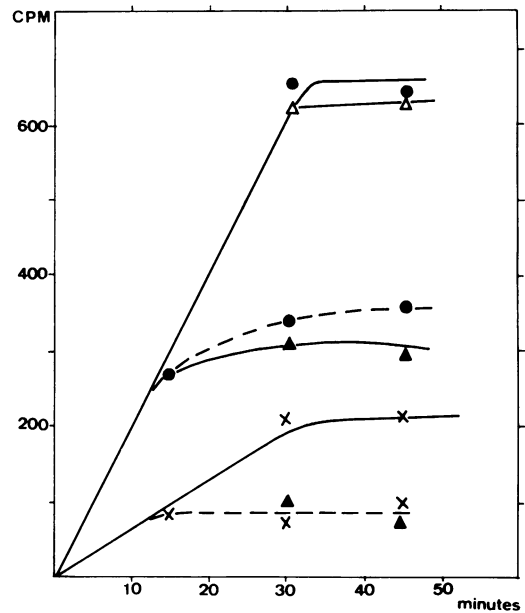


FIG. 3. Kinetics of incorporation of  $C^{14}$ -UTP by *Escherichia coli* 15  $T^-$  extracts. Symbols: ●, control extract ( $5 \mu\text{liters}$ ); ×, extract of cells thymine-starved for 120 min ( $10 \mu\text{liters}$ ). Effect of addition, after 15 min of incorporation, of: dashed line, unlabeled UTP; ▲, unlabeled UTP plus  $10 \mu\text{liters}$  of starved cell extract; △,  $10 \mu\text{liters}$  of starved cell extract [in this case, CPM = counts per minute of (control + starved extracts) minus (counts per minute of starved extract alone)];  $5 \mu\text{liters}$  of control extract = DNA,  $4.4 \mu\text{g}$ ; RNA,  $33 \mu\text{g}$ ; proteins,  $175 \mu\text{g}$ ;  $10 \mu\text{liters}$  of starved cell extract = DNA,  $3.3 \mu\text{g}$ ; RNA,  $85 \mu\text{g}$ ; proteins,  $270 \mu\text{g}$ .

types of extracts (Fig. 4-6), but the increase, though linear at a low extract concentration, leveled off later and reached a plateau value. This value was much lower for starved cell extracts than for control cell extracts (Fig. 4, 5, 6; Table 2). In both cases, the incorporation was completely inhibited by the addition of actinomycin D ( $10 \mu\text{g}$  per tube) to the reaction mixture (Fig. 4).

This decreased RNA polymerase activity of thymine-starved cell extracts was not unique to *E. coli* 15  $T^-$ , but was also observed with extracts of other thymineless strains, such as *E. coli* B<sub>3</sub> and *E. coli* CR 34 (Fig. 5).

Addition of chloramphenicol during thymine starvation at a concentration ( $100 \mu\text{g}/\text{ml}$ ) which completely inhibits thymineless death (30; Revel, Ph.D. Thesis, 1963), also prevented the decrease of RNA polymerase activity of the starved cell extracts (Fig. 6).

The observed decrease in the capacity of

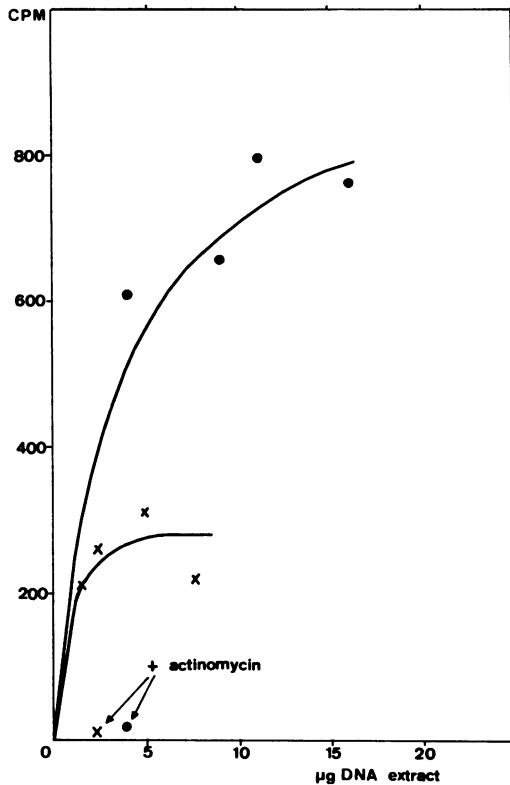


FIG. 4. Rate of incorporation of  $C^{14}$ -UTP by *Escherichia coli* 15  $T^-$  control and thymine-starved cell extracts as a function of the extract concentration. Symbols: ●, control extract; X, starved cell extract (same extracts as Fig. 3). Amount of  $C^{14}$ -UTP incorporated after 15 min of incubation at 35 C as a function of amount of extract, normalized on the basis of DNA content, added to the reaction mixture.

starved cell extracts to synthesize RNA may result from a number of different causes. Among the most probable causes are: (i) an increase in nuclease activity of the starved cell extracts, which could destroy the template or the product of the reaction, or an increase in enzymes capable of degrading the substrate; (ii) the synthesis during the course of thymine starvation of a soluble inhibitor of RNA polymerase; (iii) an inactivation or degradation of RNA polymerase itself; and (iv) a decrease of the capacity of the DNA from starved cells to serve as a template for RNA polymerase.

The first alternative was tested by diluting the labeled nucleoside triphosphate (Fig. 3) with unlabeled nucleoside triphosphate after 15 min of incubation. This dilution stopped further incorporation of the label, but the labeled product, which had been previously synthesized by either

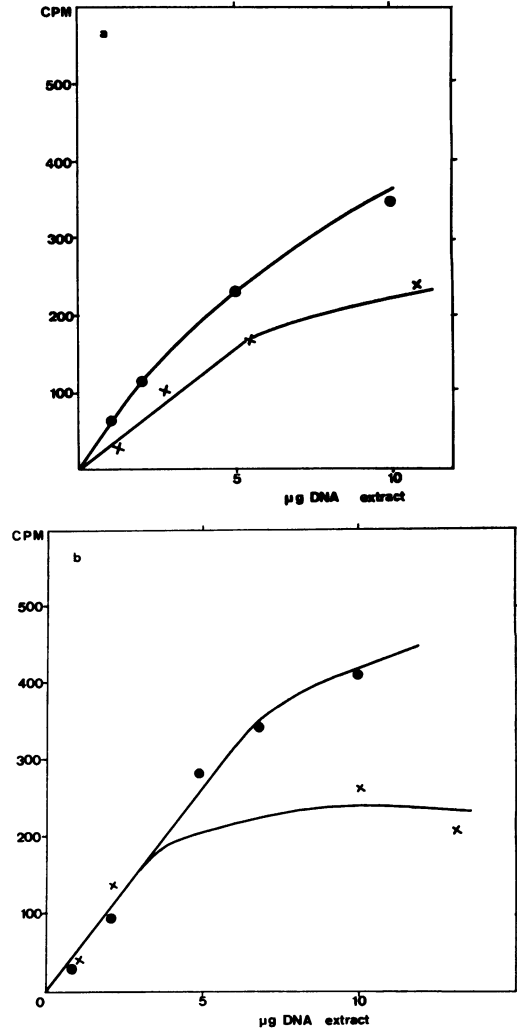


FIG. 5. Rate of incorporation of  $C^{14}$ -UTP by control and thymine-starved extracts of *Escherichia coli*  $B_8$  and *E. coli* CR 34 as a function of the extract concentration. (a) *E. coli*  $B_8$ . (b) *E. coli* CR 34. Symbols: ●, control extract; X, starved cell extract (*E. coli*  $B_8$ , starved for 150 min; *E. coli* CR 34, starved for 180 min).  $C^{14}$ -UTP incorporation after 15 min of incubation at 35 C as a function of extract concentration, normalized as in Fig. 4.

control or starved cell extracts, did not appear to be degraded upon further incubation, even when starved cell extract was added concomitantly (Fig. 3). Furthermore, addition of starved cell extracts to a reaction mixture containing control extract did not inhibit the incorporation of labeled nucleoside triphosphate catalyzed by the control extract. These results indicate that the observed difference between the RNA polymerase

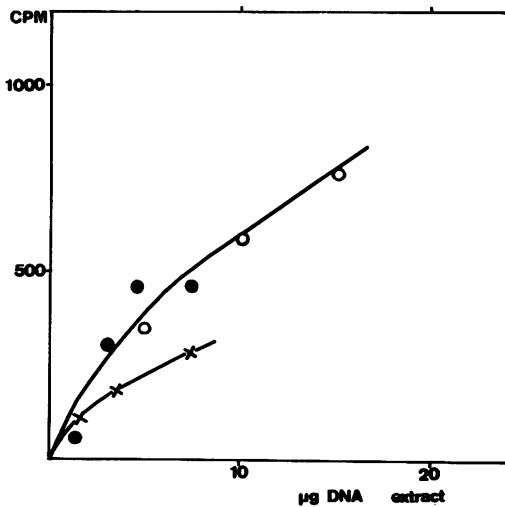


FIG. 6. Effect of chloramphenicol addition, during thymine starvation, on RNA polymerase activity of starved *Escherichia coli* 15 T<sup>-</sup> extracts. Symbols: ●, control extract; ×, extract of cells starved for thymine for 120 min (1.7% viable cells); ○, extract of cells starved for thymine for 120 min in the presence of 100 µg/ml of chloramphenicol (99% viable cells). C<sup>14</sup>-UTP incorporated after 15 min at 35 C as a function of extract concentration, normalized as in Fig. 4.

activity of control and starved cell extracts is due neither to an increase of nuclease activity for either RNA or DNA, nor to the production of a soluble inhibitor of the RNA polymerase. Regarding the possibility that the starved cell extracts could contain a greater amount than the control extracts of enzymes which would degrade the nucleoside triphosphates, it can be said that the leveling off of the curves corresponding to the rate of incorporation as a function of the amount of extract added to the reaction mixture (Fig. 4-6) could be explained on this basis. However, this leveling off is observed with both control and starved cell extracts, and the difference in the rate of incorporation between both extracts can be observed even at a very low concentration of extract (while the concentration of substrate is kept constant). Furthermore, addition of starved cell extract does not modify the rate or the amount of incorporation obtained with the control extract (Fig. 3), indicating that degradation of substrate does not play an important role in the peculiarity of the kinetics of the incorporation of nucleoside triphosphates catalyzed by starved cell extracts.

The second possibility, namely, that the presence of a soluble inhibitor of RNA polymerase is responsible for the decreased activity of starved cell extracts, can be further dismissed on the basis

TABLE 2. Comparison of RNA polymerase activity of extracts from control and thymine-starved cells<sup>a</sup>

Expt. no.	Strain	Concn of extract/tube (µg of DNA)	Count/min	Per cent incorporation
1	<i>Escherichia coli</i> 15 <sup>-</sup>	Control, 8.0	860	100
		Starved, 8.0	275	36
2		Control, 13.5	1,005	100
		Starved, 13.5	380	38
3		Control, 9.75	780	100
		Starved, 9.8	410	51
4	<i>E. coli</i> B <sub>2</sub>	Control, 9.8	409	100
		Starved, 10.0	264	65
5		Control, 9.75	454	100
		Starved, 9.8	300	66
6	<i>E. coli</i> CR 34	Control, 10.0	352	100
		Starved, 10.0	240	67

<sup>a</sup> Incorporation of cytidine triphosphate-C<sup>14</sup> or uridine triphosphate-C<sup>14</sup> by extracts from control and thymine-starved cells from *E. coli* 15 T<sup>-</sup> (120-min starvation), *E. coli* B<sub>2</sub> (150-min starvation), and *E. coli* CR 34 (180-min starvation), after 15 min of incubation at 35 C. The concentrations of DNA reported correspond to plateau incorporation (see Fig. 4). Each experiment corresponds to a different set of extracts.

of experiments in which purified thymus DNA and purified *E. coli* polymerase are added to the extracts. As shown in Fig. 7, the transcription of the exogenous DNA by an exogenous polymerase was not disturbed by the addition to the reaction mixture of control or of starved cell extracts.

To test the possibility of an inactivation of RNA polymerase during starvation, we added exogenous purified DNA (thymus or T4) to reaction mixtures containing equivalent amounts (on the basis of their DNA contents) of either control or starved cell extracts (Fig. 8). Although addition of exogenous DNA stimulated only to a small extent (1.2 to 1.7 times) the incorporation of nucleoside triphosphates into RNA catalyzed by the RNA polymerase present in control extracts, it increased two to six times the incorporation catalyzed by the RNA polymerase present in starved cell extracts. Thus, it appeared that, in control extracts, the endogenous polymerase was already saturated by the endogenous DNA, whereas the enzyme present in the starved cell extracts, even if transcribing poorly the endogenous DNA, was able to utilize an exogenous template. The enzyme present in starved cell extracts seems, thus, to be functional, and its amount, relative to bacterial DNA, has ap-

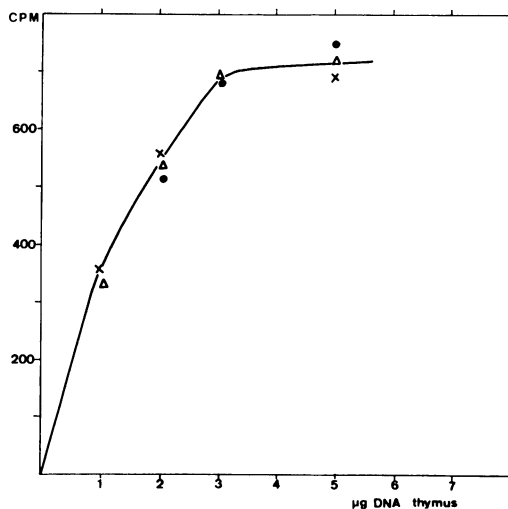


FIG. 7. Effect of the addition of control or starved cell extracts on the transcription of purified DNA by purified RNA polymerase. Symbols: ●, amount of  $C^{14}$ -CTP incorporated into RNA by purified RNA polymerase, after 15 min of incubation at 35 C, as a function of the amount of purified calf thymus DNA added as primer; ×, same, in the presence of 5  $\mu$ liters (1.2  $\mu$ g of endogenous DNA) of control extract. CPM = observed incorporation (counts per minute) of the labeled nucleoside triphosphate minus the incorporation observed by incubating under the same conditions the extract and varying amounts of thymus DNA (without purified polymerase);  $\Delta$ , same, in the presence of 20  $\mu$ liters (1.2  $\mu$ g of endogenous DNA) of starved cell extract; CPM as above (cells starved for thymine for 120 min).

parently increased two- to threefold during starvation, in parallel with overall protein synthesis.

According to the above experiments, the decrease of RNA polymerase activity of starved cell extracts cannot be ascribed to the presence of nucleases or of enzymes degrading the substrates, to a soluble inhibitor of RNA polymerase, nor to an inactivation of RNA polymerase. It could thus depend on the ability of DNA to serve as template for RNA polymerase.

When the template ability of starved cell extract DNA was tested by adding purified RNA polymerase to the extract, it was found to be just as poor a template for exogenous as for endogenous RNA polymerase (Fig. 9). The rate of incorporation of nucleoside triphosphate catalyzed by both enzymes (compare Fig. 3 and 9) was only 30 to 50% of that obtained by using as template the DNA present in control extracts.

The DNA from thymine-starved cells was purified in two different ways (as described in

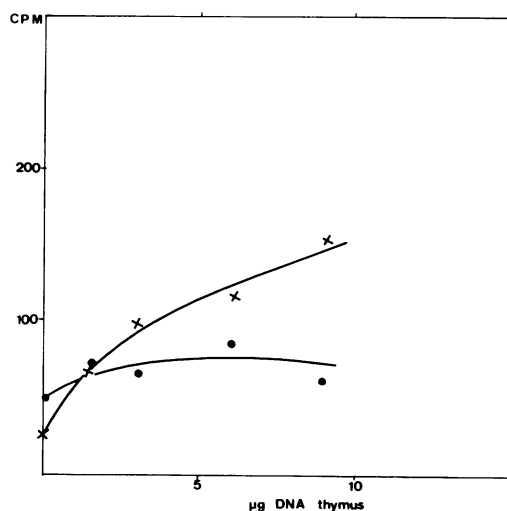


FIG. 8. Effect of addition of exogenous DNA on the incorporation of  $C^{14}$ -CTP by control and starved cell extracts. Symbols: ●, control extract (5  $\mu$ liters); ×, starved cell extract (20  $\mu$ liters). Same extracts as Fig. 7. Amount of  $C^{14}$ -CTP incorporated after 15 min of incubation at 35 C, as a function of the amount of purified calf thymus DNA added to the reaction mixture.

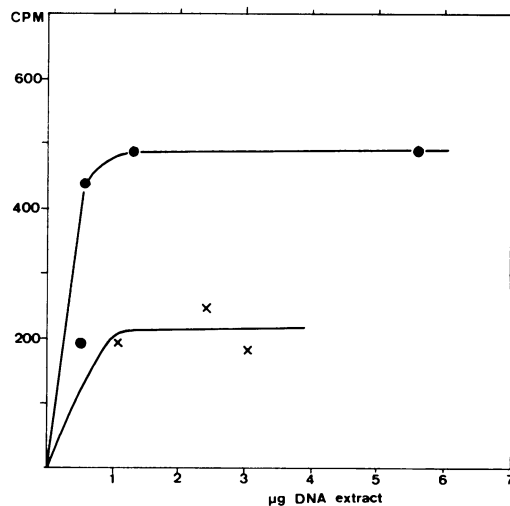


FIG. 9. Ability of DNA from control and starved cell extracts to serve as primer for purified RNA polymerase. Same extracts as in Fig. 7 and 8. Symbols: ●, control extract; ×, starved cell extract. Differential incorporation:  $\Delta$  CPM = ( $C^{14}$ -CTP incorporated after 15 min of incubation at 35 C by the extract plus RNA polymerase) minus ( $C^{14}$ -CTP incorporated under the same conditions by the extract alone) as a function of the amount of extract, normalized with respect to DNA content, added to the reaction mixture.



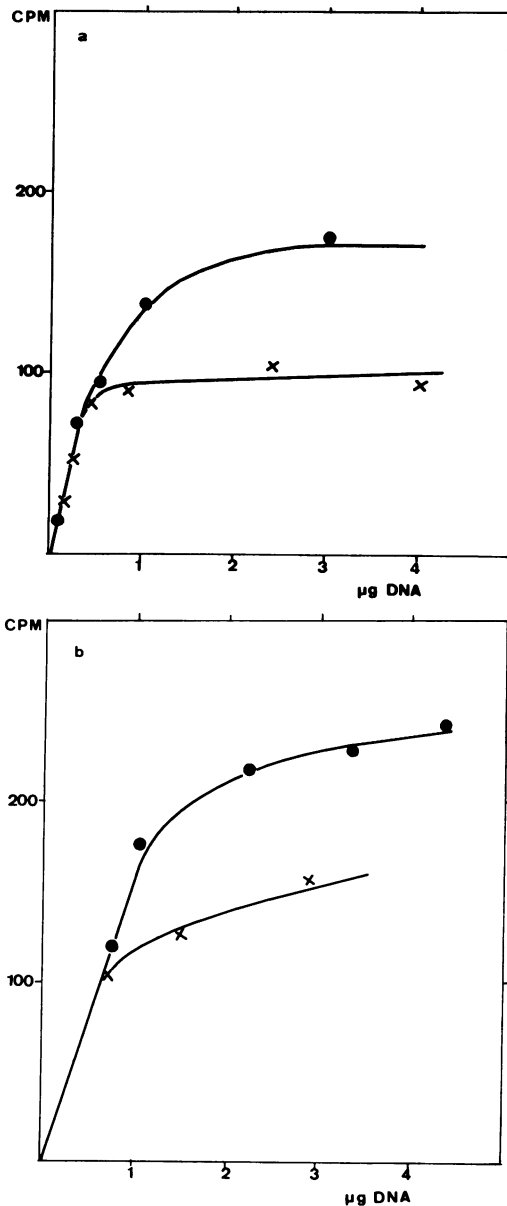


FIG. 10. Ability of purified DNA from control and thymine-starved cells to serve as primer for purified RNA polymerase. Symbols: ●, DNA from control cells; ×, DNA from starved cells.  $C^{14}$ -CTP incorporated, after 15 min of incubation at 35 C by purified *Escherichia coli* polymerase as a function of the amount of primer added to the reaction mixture. (a) DNA purified by method 1 (see Materials and Methods). (b) DNA from the same extracts as in Fig. 3 and 4, purified by method 2.

Materials and Methods): (i) by lysis of the cells with SDS, elimination of RNA by ribonuclease treatment and extensive deproteinization with chloroform-isoamyl alcohol (26), followed by

phenol extraction; or (ii) by centrifugation in a cesium chloride gradient of a portion of the extracts. Figure 10 shows that, in both cases, the ability of purified DNA from starved cells to serve as template for purified RNA polymerase was lower than that of control cell DNA. The rate of incorporation of nucleoside triphosphates into RNA catalyzed by the enzyme at a saturating level of starved cell DNA was only 63 to 66% of the observed rate of incorporation at a saturating control DNA concentration. Purification thus increased by 15 to 35% the ability of starved cell DNA to serve as template for purified RNA polymerase, without, however, allowing it to reach the efficiency of control cell DNA. This observation indicates that, in the extracts, the DNA of starved cells is probably associated with some other cell component which contributes to the impairment of its ability to be transcribed by RNA polymerase.

#### DISCUSSION

The experiments related above show that thymine starvation produces, after a certain lag, an exponential decrease in the rate of synthesis of mRNA which is parallel to the exponential decrease in the number of viable bacterial cells. This decrease in the capacity of starved bacteria to synthesize mRNA is reflected in vitro in all thymineless *E. coli* strains examined, by a decreased RNA polymerase activity of starved cell extracts which seems to reside in a decreased ability of their DNA to serve as a template for RNA polymerase.

These results confirm and extend previous observations (23; D. Luzzati, Abstr. Intern. Congr. Biochem., I-126, 1964) that suggested, on the basis of an observed decrease in the rate of labeling of RNA (especially the 4 to 10S fraction) or on the basis of the behavior of starved cell extracts with respect to amino acid incorporation into proteins, that a decreased ability to synthesize mRNA could account for the decreased ability of *E. coli* cells (or extracts) to synthesize protein after thymine starvation (22).

There seems to be some correlation between the lethal effect of thymine starvation and its effect on DNA template activity. Both effects are simultaneously abolished by addition of high concentrations of chloramphenicol during starvation, and the kinetics of both effects are parallel; the exponential decrease in rate of synthesis of mRNA as well as the exponential decrease in number of viable cells begin after a certain lag, and both then proceed at the same rate. However, the length of the lag is not the same for both effects, the exponential decrease in the rate of mRNA synthesis beginning only (for *E. coli* 15

T<sup>-</sup>) 13 to 20 min after the beginning of thymineless death. This shift could depend on a qualitative change concerning the proportion of different species of mRNA among the total mRNA population preceding the quantitative manifestations of the disturbances brought about by thymine starvation in the transcription of bacterial DNA. The qualitative variations alluded to may be related to the induction of some early functions of the defective phage (11, 12, 27) which *E. coli* 15 T<sup>-</sup> is known to harbor. No data are available concerning the possibility of B<sub>3</sub> or CR 34 harboring a defective prophage.

Concerning the nature of the alteration of the DNA from starved cells, which impairs its ability to serve as a template for RNA polymerase, three major types of alterations have to be considered: modification of its physical structure, modification of its chemical composition, or an association during thymine starvation between the DNA and some other constituent from which it cannot be dissociated upon purification.

This last possibility is suggested by the fact that purification increases by 15 to 35% the efficiency with which starved cell DNA acts as template for purified RNA polymerase. It could thus be argued that the decreased primer ability of the purified starved cell DNA could depend upon the persistence, throughout the purification procedures, of this type of complex. However, the same difference in priming activity (34 to 37%) between DNA from starved and control cells has been found after purification by two widely different methods, imparting to the DNA preparations purity of very different degree and nature. It thus seems likely that the impaired template activity of purified starved cell DNA is of an intrinsic nature.

The analysis of the results concerning the rate of transcription by purified RNA polymerase of normal and starved cell purified DNA throws some light on the possible type of alteration of the latter (Fig. 10). At low DNA concentrations, the rate of incorporation of nucleoside triphosphates into RNA is the same, whatever the origin of the DNA, but at higher DNA concentrations, when the concentration of enzyme is limiting, the rate of transcription of starved cell DNA is lower than that of control DNA. From the results of Bremer and Konrad (4, 5), as well as of J. P. Richardson (*J. Mol. Biol.*, *in press*), it is known that, when purified RNA polymerase is added to a reaction mixture containing the four ribonucleoside triphosphates and purified DNA, the enzyme molecules become attached to DNA at various "binding sites" and begin the transcription of DNA, which proceeds without enzyme or

product detaching from the template. With excess enzyme, several stretches of DNA can be transcribed by a succession of several RNA polymerase molecules (5), which might account for the absence of differences in the rate of nucleotide incorporation with low amounts of control or starved cell DNA as template. At limiting enzyme concentration, however, the rate of nucleotide incorporation is essentially dependent on the number of RNA chains, whose synthesis is proceeding, and on the growth rate of each chain (4). The decreased rate of nucleoside triphosphate incorporation primed under these conditions by starved cell DNA can thus be due to a decrease in the number of RNA polymerase molecules that it can bind, to a decrease in the growth rate of the RNA chains, or to an increase in the number of stopping signals on the template. Present results do not allow us to distinguish among these alternative modes of impairment of the priming activity of DNA from thymine-starved cells.

With regard to the exact nature of the alteration that the DNA undergoes in the course of thymine starvation, it is tempting to envisage it as a structural modification, such as the formation, during starvation, of single-stranded regions, on which transcription is known to proceed at a slower rate than on native DNA (5). However, analysis of the physicochemical properties of thymine-starved *E. coli* DNA has not shown the slightest evidence in favor of lesions of this type (18, 37; Revel, Ph.D. Thesis, 1963). The purified starved cell DNA preparation used in the experiments reported above (see Fig. 10a) had the same molecular weight, melting point, and density in a CsCl gradient as the control DNA samples, as well as a similar viscosity. The recent observations of Pauling and Hanawalt (33), indicating that after 40 min of thymine deprivation some amount of "repair synthesis" of 15 T<sup>-</sup> DNA is detectable, suggest that, during thymine starvation, a certain amount of chain breakage does occur, which, in the absence of immediate repair, evolves towards the formation of gaps on one of the DNA strands. It is difficult to decide whether such lesions, undetectable by physical methods, occur in sufficient number to account for the effect of thymine starvation on DNA template activity, and whether these are the only type of alteration that *E. coli* DNA can undergo during thymine starvation.

In fact, it is known that DNA modifications of other types, such as hypermethylation (13), do occur during thymine starvation, especially with *E. coli* 15 T<sup>-</sup>. Our observation concerning the effect of the addition to the starvation medium of chloramphenicol, which prevents the decrease

of RNA polymerase activity of starved cell extracts, could be interpreted as indicating that this decrease of transcribability is due to the action upon DNA of some enzyme whose synthesis is induced by thymine starvation and inhibited by chloramphenicol. However, no effect of hypermethylation on DNA template activity has yet been observed in vitro by action of different methylating enzymes upon a variety of DNA preparations (14, 15; A. Novogrodsky et al., *J. Biol. Chem.*, *in press*). If a chemical modification of DNA, mediated by an enzyme induced or activated by thymine starvation, is responsible for the decrease of DNA template activity after thymine starvation, either it concerns some modification other than methylation, or a methylation the specificity of which would be different from those examined up to now.

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