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Stimulation of Unbalanced Ribonucleic Acid Synthesis in *Escherichia coli* by Methanol

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Addition of methanol to a stringent strain of *Escherichia coli*, starving of methionine, stimulates unbalanced ribonucleic acid (RNA) synthesis. The newly formed RNA and ribonucleoprotein species sediment between 4*S* and 30*S*. As a result of methanol treatment, cells become permeable to actinomycin D. Damage to cellular membrane appears to influence the control for RNA synthesis.

Stringent strains of microorganisms stop net ribonucleic acid (RNA) synthesis when deprived of their essential amino acids (18). The means by which the synchrony between RNA and protein biosynthesis is achieved has been the subject of research since the discovery of the relaxed mutant (2). This strain is genotypically capable of net RNA synthesis in the absence of net protein production. It was demonstrated that a single point mutation of the RC gene caused this aberrant behavior (24).

The many details available on RNA and protein biosynthesis (25, 26) do not appreciably help us in our understanding of the nature of the correlation between the two.

A working hypothesis of the amino acid control of RNA synthesis stresses the availability of ribosomal proteins as the controlling factor of RNA synthesis (7, 17). Another theory is based on the amino acid control of the biosynthesis of RNA precursors (4, 11, 13). Some of the assumptions of this approach have been challenged (9).

A considerable amount of data has been introduced also in favor of guanosine tetraphosphate as an important factor in the "stringent response" (3, 5, 6). This notion, too, has not been left unchallenged (14, 30).

Yet another theory centers around the inhibition of active transport by amino acid starvation as the chief cause of the stringent response (22, 23). Their assertion that lipid biosynthesis is controlled by the RC gene has been contradicted (29). It is evident that the reasons behind the amino acid control of RNA synthesis are not yet fully understood.

Isogenic strains of relaxed and stringent cells of *Escherichia coli* appear to differ in more ways than just the capacity to synthesize RNA in the absence of protein production (15, 22, 23). Some of these differences exist even during logarithmic growth (12). The pleiotropic nature of the mutation of the RC locus suggests the possibility that the RC gene product affects RNA synthesis only indirectly.

A number of seemingly unrelated ways have been found which induce unbalanced RNA synthesis in stringent strains: by various inhibitors of protein synthesis (1, 10), by spermidine (20, 21), recovery from magnesium starvation (17), and by potassium deprivation of certain strains of *E. coli* (29).

This report introduces yet another class of agents capable of causing unbalanced RNA synthesis phenotypically in certain stringent strains of *E. coli*. These chemicals are fairly inert, and their suspected target area is the cellular membrane. We advance the suggestion that the structural integrity of the deoxyribonucleic acid (DNA)—membrane complex is an important controlling element of RNA synthesis.

MATERIALS AND METHODS

Chemicals. DL-Leucine-*1*-^{14}C and uracil-2-^{14}C were purchased from Cal-Atomic Corp., Los Angeles, Calif. Actinomycin D was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Density gradient grade sucrose was purchased from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were commercially available products.

Bacteria. The RC^{str} strains of *E. coli* used are shown in Table 1.

Media. Organisms were grown in a minimum salts medium (8) at 37 C., supplemented with glucose (0.3%, w/v); essential DL-amino acids (50 mg/liter); methionine (30 mg/liter); and, when appropriate, uracil, thymine, or DL-leucine (10 mg/liter each). A gyrotory water-bath shaker was employed, manufactured by the New Brunswick Scientific Co., New Brunswick, N.J. Growth was followed by nephelometry with a Coleman Nephco-colorimeter.

TABLE 1. *RC^{str} strains of Escherichia coli tested for stimulation of unbalanced RNA synthesis by methanol*

Strain	Source	Growth requirements
W122-33	E. Borek	Methionine
K_{12}W6-1R	Revertant of K_{12}W6	Methionine
NF126	N. Fiil	Threonine, leucine, histidine, arginine, lysine, thiamine
NF156	N. Fiil	Histidine, tryptophan, arginine
15THU	S. Cohen	Thymine, histidine, uracil

Colorimetric determinations. Three-milliliter samples of cultures were collected into 0.3 ml of ice-cold 50% trichloroacetic acid. After a minimum of 30 min, these were centrifuged and washed once with cold 5% trichloroacetic acid. The pellets were treated with 5% trichloroacetic acid for 15 min at 90 C. The cells were again centrifuged, and a sample of the supernatant fluid was assayed by the orcinol method (16).

Determination of radioactive label uptake. One-milliliter samples were withdrawn into ice-cold 0.1 ml of 50% trichloroacetic acid and stored for a minimum of 30 min. The cells were collected on a glass-fiber filter paper (2.4 cm diameter), washed with 30 ml of 5% trichloroacetic acid, and dried. The filter papers were placed in vials with toluene-based scintillation liquid, and the radioactivity was determined on a model 2111 Packard liquid scintillation spectrometer.

Preparation of cell extracts. Frozen pellets were ground in the cold with alumina (bacteriological grade A 305, manufactured by Alcoa Chemical Corp.). The homogenate was suspended in a small volume of ice-cold buffer [0.01 M tris(hydroxymethyl)-aminomethane (Tris)- 10^{-4} M $MgCl_2$, *p*H 7.2] containing 5 μg of deoxyribonuclease (Worthington Biochemical Corp.) per ml. The solution was centrifuged at 15,000 rev/min for 15 min. The supernatant fluid is the cell extract.

Sucrose density gradient centrifugation. Onto a 4.6-ml linear sucrose gradient (4 to 20%, w/v) prepared in buffer of the same composition as that of the cell extract, 0.2 ml of cell extract was layered. The gradients were centrifuged in a SW50 swinging bucket rotor at 38,000 rev/min for 2 hr. A Beckman model L40 ultracentrifuge was employed for these centrifugations. The gradients were fractionated with an Isco density gradient fractionator.

RESULTS

A number of organic solvents appear to stimulate the incorporation of ^{14}C-uracil into the cold trichloroacetic acid-insoluble fraction during methionine starvation in strains W122-33 and K_{12}W6-1R. Among these agents are acetone, ethanol, ethyl acetate, isopropyl alcohol, methanol, and *N,N*-dimethylformamide. We have worked with methanol most extensively. However, it is most likely that qualitatively similar results can be obtained by the other compounds listed also. This list of solvents is by no means exclusive; undoubtedly there are others that also have this property of stimulating RNA synthesis. Figure 1 shows the effect of varying concentrations of methanol. RNA synthesis is most rapid, after a lag period, between 20 and 40 min of methanol treatment. Very little additional synthesis of RNA occurs after 1 hr of methanol treatment under these conditions. Four per cent methanol concentration creates the largest burst of RNA synthesis. This amount of methanol kills approximately 80% of the bacterial population in 1 hr (Fig. 2). Comparing Fig. 1 and 2, it is noted that the most rapid rate of death precedes the fastest period of RNA synthesis. A trivial explanation of the stimulatory effect of methanol of RNA synthesis would be possible, could it be shown that methionine is liberated into the medium by the killed microorganisms. Under such a condition, however, stimulation of protein synthesis would also be expected. This is not the case, as shown in Fig. 3. There appears to be preferential RNA synthesis in the presence of 4% methanol. Even logarithmically growing cultures exhibit this phenomenon upon addition of methanol (Fig. 4).

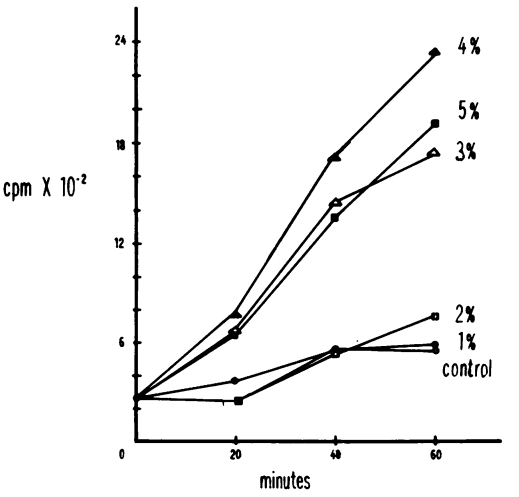

FIG. 1. *Stimulation of RNA synthesis during methionine starvation in E. coli W122-33. Cells deprived of methionine, in the presence of ^{14}C-uracil (0.1 μC, 10 μg/ml), were divided into six fractions. To five of these, enough methanol was added for the final concentrations indicated (v/v).*

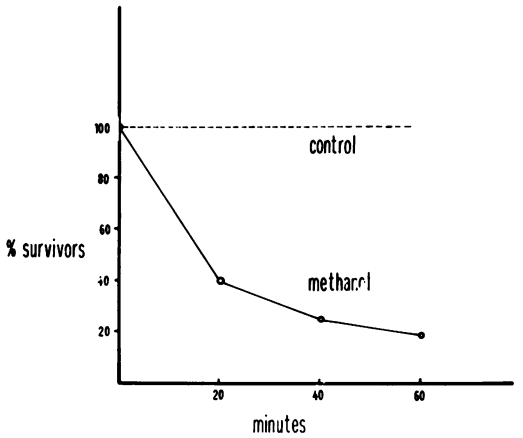

FIG. 2. *Survival rate of E. coli during exposure to 4% methanol. Methionine-starved culture was divided into halves, and one portion received enough methanol for a final concentration of 4% (v/v). Samples were serially diluted and plated on nutrient agar surfaces.*

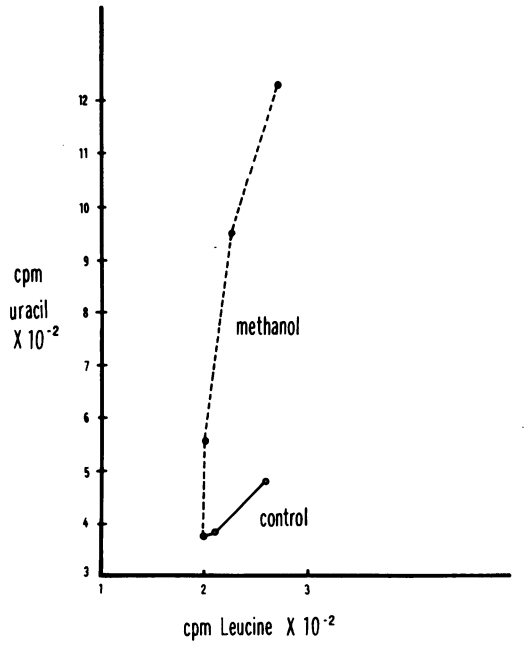

FIG. 3. *Differential rate of RNA and protein synthesis during methanol treatment of methionine-starved cells. Logarithmically growing cells were supplemented with 10 μg of* DL*-leucine per ml and 10 μg of uracil per ml. During methionine starvation, the cells were divided into halves, one receiving* ^{14}C*-uracil (0.1 μCi, 10 μg/ml) and* ^{14}C*-leucine (0.1 μCi, 10 μg/ml). Both cultures were halved again, one portion of each receiving enough methanol to a 4% final concentration.*

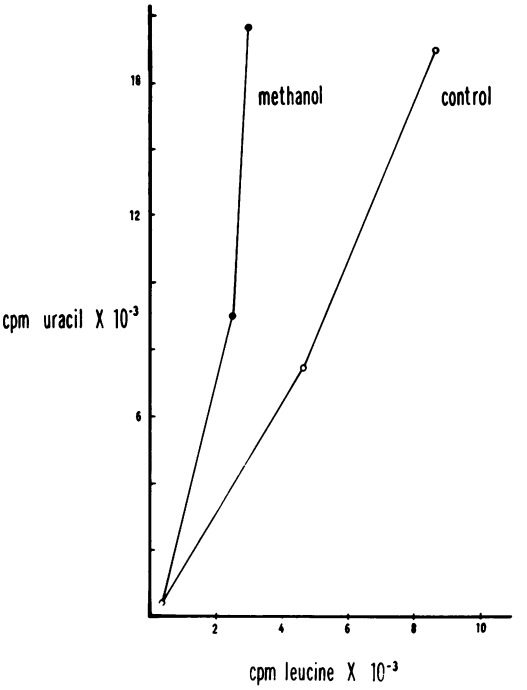

FIG. 4. *Differential rate of RNA and protein synthesis during methanol treatment of logarithmically growing cells. Experimental details were identical to those in Fig. 3, except the methionine starvation is omitted.*

Some of the radioactive uracil incorporated during methionine starvation enters into DNA, but there is no preferential labeling of DNA during methanol treatment (*unpublished observations*).

Is the increased RNA synthesis a reflection of increased breakdown and resynthesis of preexisting RNA? Organisms prelabeled with leucine or uracil were treated with 4% methanol, and the amounts of the label released into the culture medium and into the cold trichloroacetic acid-soluble pool were assessed. In 1 hr, about 10% of the label from both protein and RNA was released into the medium and another 10% was released into the soluble pool. There was no preferential release of label during methanol treatment. Thus, whereas turnover of RNA and protein is occurring during amino acid starvation, it is not the explanation of the stimulatory effect.

Examining the amount of net RNA synthesis by the orcinol method, we found only about 10 to 15% increase in 1 hr of methanol treatment. Methanol treatment adversely affected the ability to reproduce of a large fraction of the population. It is likely that some

cells lost all biosynthetic capacity as well, whereas others accumulated considerably more RNA.

Unbalanced RNA synthesis may be stimulated by the addition of methanol after the elapse of various periods of methionine starvation. However, the degree of stimulation diminishes to a very low level beyond 2 hr of amino acid deprivation (Fig. 5).

Methanol need not be present in the solution during unbalanced RNA synthesis. Pre-

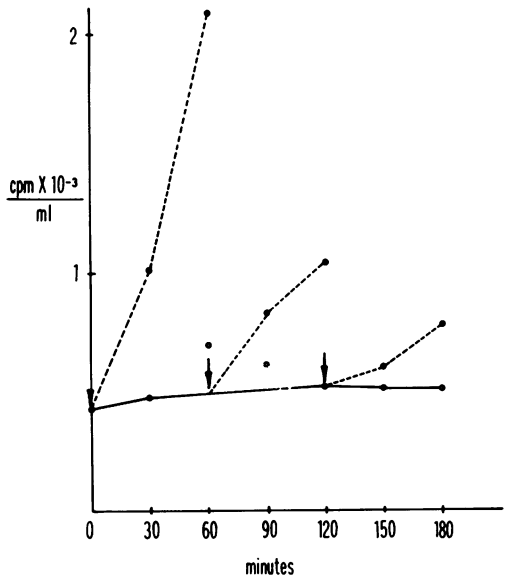

FIG. 5. *Stimulation of unbalanced RNA synthesis by methanol after various periods of methionine starvation. Portions of a methionine-starving culture, supplemented with ^{14}C-uracil (0.1 μCi, 10 μg/ml), were removed at times indicated by the arrow and treated with methanol, 4% (v/v) final concentration.*

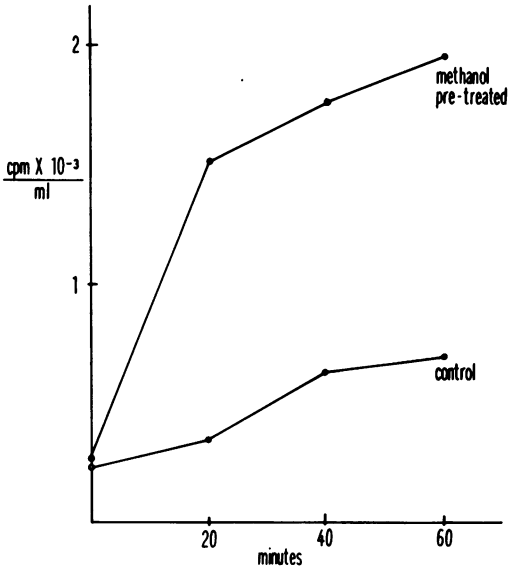

FIG. 6. *Stimulation of unbalanced RNA synthesis by pretreatment with methanol. Logarithmically growing cells, supplemented with uracil (10 μg/ml) were harvested, washed, and starved of methionine and glucose. One-half of this culture was treated with methanol, final concentration 4% (v/v). After 60 min, both halves were centrifuged, washed, and suspended in methionineless medium, supplemented with glucose, ^{14}C-uracil (0.1 μCi, 10 μg/ml).*

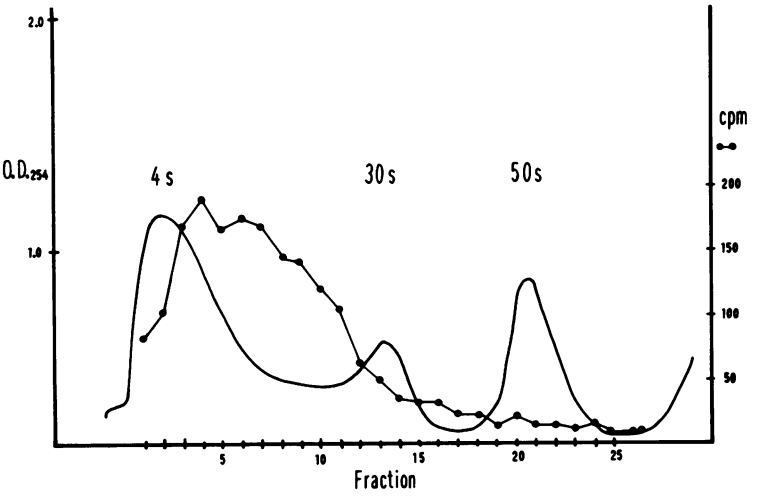

FIG. 7. *Sucrose density gradient of a cell extract after methanol treatment during methionine starvation. A methionine-starved culture, supplemented with ^{14}C-uracil (0.1 μCi, 10 μg/ml) was treated with methanol for 1 hr.*

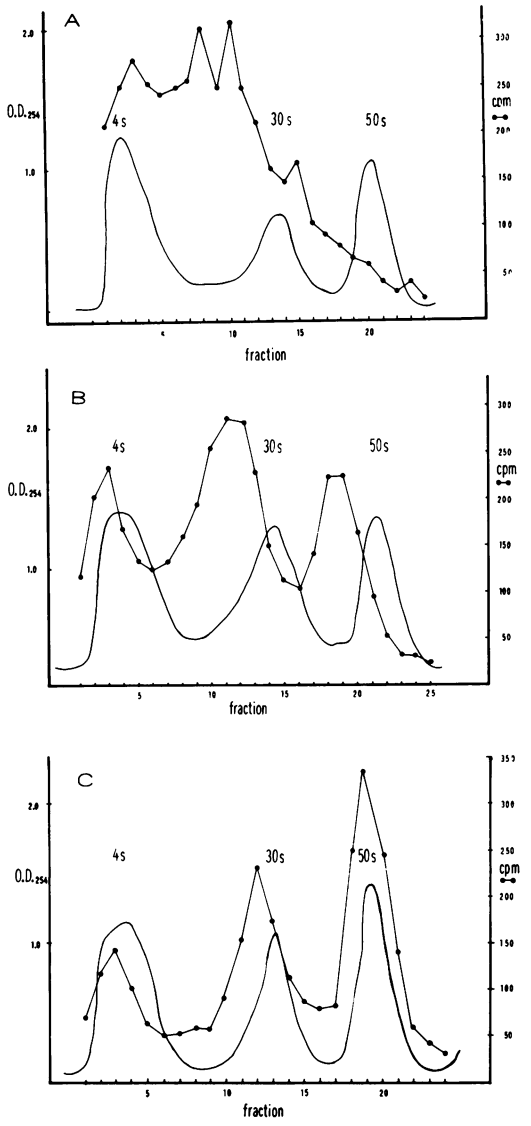

FIG. 8. *Recovery from methionine starvation and methanol treatment. A methionine-starved culture, supplemented with ^{14}C-uracil and treated with methanol for 60 min was centrifuged, washed, and suspended in growth medium containing 100 μg of uracil per ml. Fractions A, B, and C represent cell extracts prepared after 0, 45, and 90 min of recovery, respectively.*

treatment of cells with methanol in the absence of glucose will yield results shown in Fig. 6. It may be noted that in this experiment there is no lag in RNA synthesis.

Sucrose density gradient centrifugation of cell extracts indicate that the predominant RNA and ribonucleoprotein species synthesized during methanol treatment sediment between 4*S* and 30*S* (Fig. 7). If methionine is restored to the culture and methanol is removed, the accumulated ribonucleoprotein particles are incorporated into 30*S* and 50*S* particles in 90 min without appreciable loss of their specific activity even in the presence of large excess of "cold" uracil (Fig. 8). It should be noted that the radioactivity of the smaller ribosomal subunit occurs in the 28 or 29*S* region instead of 30*S*. This observation can be explained by assuming that some of the cells were killed by methanol following RNA accumulation. The emphasis, however, is on the very large extent to which recovery is accomplished.

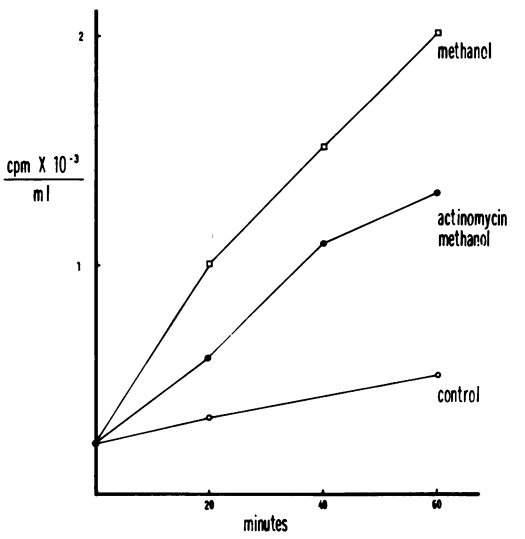

FIG. 9. *Inhibition of unbalanced RNA synthesis by actinomycin D (100 μg/ml).*

Methanol treatment alters the permeability of *E. coli* to such an extent that actinomycin D can exert an inhibitory effect on unbalanced RNA synthesis. Although the inhibition is not complete, it is significant (Fig. 9).

DISCUSSION

Methanol and the other substances listed above are chemically rather inert compounds. One common property of these reagents is their solubility in both aqueous and nonpolar substances. Tests conducted with nonpolar solvents such as chloroform and toluene gave negative results in our hands, with indications that the organisms were killed by these reagents rather rapidly. Toluene-treated nonviable cells were recently shown to be capable of RNA synthesis (19). However, in toluene-

treated cells, RNA production appears to depend on the presence of ribonucleoside triphosphates for optimal results. Moreover, these cells are incapable of protein synthesis, whereas methanol-treated cells are capable of protein synthesis during recovery from methionine starvation (Fig. 8). These differences indicate that our finding, reported above, is not identical to the one reported by Peterson et al (19). Methanol-treated cells, in all probability, sustain a milder damage than the toluene-treated ones. What is significant in both instances is that RNA synthesis can continue in the absence of protein synthesis.

We suspect that our solvents, as well as toluene, damage the cellular membrane system. Another way to injure the cell membrane is by plasmolysis. We have plasmolyzed *E. coli* W122-23 cells for 20 to 30 min by 20% sucrose and then resuspended them in methionineless growth medium. Under these conditions, stimulation of unbalanced RNA synthesis was observed also, as monitored by simultaneous incorporation of ^{3}H-uracil and ^{14}C-leucine (*unpublished observations*).

Tremblay et al. have demonstrated that the cellular membrane, DNA, and the RNA-synthesizing machinery form an integral complex (27). On the basis of our observations, we view the unbalanced synthesis of RNA in the presence of methanol as a manifestation of structural aberration of the cytoplasmic membrane. It is possible that some of the treatments (referred to above) that induce unbalanced RNA synthesis phenotypically also damage the cellular membrane.

However, our findings may not have a general application. We have tested four other stringent strains of *E. coli*, and only one, K_{12}-W6-1R, responded to methanol treatment. Unbalanced RNA synthesis was not stimulated by methanol in strains 15THU, NF126, and NF156. At the present time, we cannot account for this difference in behavior among the strains mentioned.

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