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Effect of Elevated Temperatures on Protein Synthesis in *Escherichia coli*

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Upon a temperature shift from 30 C to 42 to 44 C, *Escherichia coli* experiences a transient slowdown in rate of optical density increase, uridine incorporation, and amino acid incorporation. The data show that the primary target for thermal inactivation is a molecule(s) required for the initiation of protein synthesis. The effect on ribonucleic acid synthesis is a secondary effect.

It was recently reported that upon elevating the temperature of an exponentially growing culture of HeLa cells from 37 and 42 C, the ability of the cells to carry out protein synthesis is transiently altered, as measured either by radioisotope incorporation or by the amount of ribosomal material present in polyribosomes (12). This alteration was hypothesized to involve an unstable ribonucleic acid (RNA) species which is normally required for protein synthesis, and which becomes temporarily depleted when the cells are shifted to 42 C.

The present study was designed to examine a similar transient depression of protein and RNA synthesis observed in *Escherichia coli*. Understanding this effect has made it possible to interpret data obtained in experiments on temperature-sensitive mutants (2, 6, 7, 15, 16). In addition, the effects observed in vivo have been reproduced in an in vitro protein-synthesizing system. This has allowed a tentative assignment of the site of inactivation to some step in protein synthesis prior to chain elongation.

MATERIALS AND METHODS

Bacteria and media. *E. coli* A2325 is a derivative of *E. coli* K-12 and was previously described (2). Minimal medium 3xPATHB$_1$ was also described (16) Cells used for the preparation of extracts for in vitro protein synthesis were grown in the medium of Müller-Hill et al. (13).

Pulse labeling. The pulse-labeling methods were described in detail (16). Briefly, 1.0 ml of cells grown in 3xPATHB$_1$ minimal medium was labeled for 1 min with 0.5 μCi of ^{14}C-leucine (255 mCi/mmole) and 5.0 μCi of ^{3}H-uridine (26.2 Ci/mmole). Acid-precipitable radioactivity was determined. Temperature shifts were complete in less than 30 sec, and were accomplished by diluting cell cultures into two volumes of medium prewarmed to the desired temperature (16).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of ^{3}H-labeled RNA. One milliliter of pulse-labeled cells, in this case labeled with 25 μCi of ^{3}H-uridine, was placed in two volumes of 95% ethanol, precooled to −20 C. RNA was isolated by the modification of the method of Summers (23), described by Gillespie and Gillespie (4). SDS-polyacrylamide gel electrophoresis was carried out essentially by the method of Bishop et al. (1). After electrophoresis, gels were sliced into 2-mm sections, and the RNA from each slice was eluted with 10 ml of a toluene-based liquid scintillant containing 3.5% NCS (Nuclear-Chicago Corp.) and counted in a liquid scintillation counter.

Preparation and analysis of polyribosomes. Polyribosomes were prepared by a slight modification of the procedure of Godson and Sinsheimer (5). The polyribosome extracts were analyzed on 16-ml, 15 to 50% linear sucrose gradients, in buffer containing 0.005 N *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES) (*p*H 7.0), 0.06 M NH_4Cl, and 0.01 M $MgAc_2$, by centrifugation at 2 C for 5 hr in a Spinco SW27.1 rotor. Gradients were scanned at 260 nm using a Beckman Kintrac VII recording spectrophotometer with a flow-through cell.

Preparation and fractionation of cell-free extracts. S30 extracts for in vitro protein synthesis were prepared by the method of Lodish (10), except that cells were broken by sonic disruption in 0.03 M HEPES (*p*H 7.0), 0.01 M magnesium acetate, 0.06 M NH_4Cl, and 0.003 M β-mercaptoethanol (HMN + MSH). The extracts were dialyzed against HMN containing 1 mg of glutathione per ml (HMN +

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GSH). These extracts were fractionated into S100, washed ribosomes, and high-salt ribosome wash by the method of Revel et al. (18), with minor modifications. HMN + GSH was used as a buffer throughout the preparation. The salt-washed ribosomes (2 M NH_4Cl) were washed three times with high-salt buffer and once with HMN + GSH. All fractions were stored at −70 C and were used immediately upon thawing.

Binding of ^{35}S-N-formyl-methionyl-tRNA$_F^{met}$ to ribosomes. The binding assay used was described (10). ^{35}S-methionine prepared from yeast and uncharged tRNA$_F^{met}$ were generous gifts of H. F. Lodish. Charging and formylation of this transfer RNA (tRNA) was carried out essentially as described by Lodish (9), except that purified tRNA$_F^{met}$ was used in place of unfractionated *E. coli* tRNA.

Incorporation of radioactive amino acid by cell-free extracts. Reaction mixtures (50 μliters) contained, in addition to the biological materials specified in each figure legend: 0.056 M HEPES (*p*H 7.0); 0.11 M NH_4Cl; 8 mM magnesium acetate: 0.01 M β-mercaptoethanol; 2 mM adenosine triphosphate; 4.2 mM phosphoenol pyruvate; 0.3 mM guanosine triphosphate; 0.1 mM of each of 19 nonradioactive amino acids; 0.013 mM ^{14}C-phenylalanine (100 μCi/ml, 384 mCi/mmole, from New England Nuclear Corp.); and 12.5 μg of f2 RNA. At the appropriate times, samples were removed into 0.5 ml of 1 N NaOH and incubated at 37 C for 10 min. A 0.5-ml amount of 10% trichloroacetic acid was then added, and the samples were chilled, collected on glass fiber filters (H. Reeve Angel Co., 934AH), dried, and counted in a toluene-based scintillation fluid in a Nuclear-Chicago Mark I liquid scintillation counter.

RESULTS

In vivo studies. The effect of shifting a culture of *E. coli* A2325 from 30 C to various higher temperatures is shown in Fig. 1 (30 and 43 C-grown cells refer to cells cultured for many generations at 30 C and to cells subsequently shifted for 1 hr to 43 C, respectively). At each elevated temperature there is an initial burst of growth, as measured by optical density (OD) at 660 nm. This is followed by the establishment of a new exponential growth rate characteristic of the new temperature. At 44 C there is a lag of about 45 min before the establishment of the new growth rate. This lag is noticeable, but much shorter, at 42 C and undetectable at 40 or 37 C.

Pulse-labeling data, measuring the rate of incorporation of uridine into RNA and leucine into protein, are shown in Fig. 1B and C. There is a drop in the rate of uridine incorporation immediately after a temperature elevation, which becomes more pronounced as the final temperature is raised. At 44 C, the rate of uridine incorporation 10 min after the shift is only 20% that of the initial rate. After the drop, the rate of uridine incorporation gradually increases until it reaches the initial level.

The initial effects of temperature elevation on the rate of amino acid incorporation are less drastic. Up to temperatures of 40 C, the initial rate of amino acid incorporation is high and remains at the new, higher level. At 44 C, however, the rate of leucine incorporation falls rapidly to one-half of the initial high level, and within the times assayed, the cells do not completely recover.

In vivo incorporation experiments of this type do not rule out the possibility that the observed effects are secondary and do not affect RNA synthesis and protein synthesis directly. It is clear, however, that with respect to RNA synthesis, the incorporation observed in the direct shift experiments does represent incorporation into RNA molecules, and the radioactive RNA synthesized at various times after a shift to 44 C does not have an unusual size distribution (Fig. 2). The crude incorporation data of Fig. 1B reflect the actual specific activity of isolated RNA (data not shown).

To investigate further the ability of cells to synthesize proteins when shifted to elevated temperatures, the state of the polyribosomes in these cells was examined at several times after a temperature shift. When cells are shifted to 44 C, there is a displacement of ribosomes out of the polyribosomal region of a sucrose gradient (Fig. 3). As the time after the temperature shift increases, however, the amount of ribosomal material associated with polyribosomes increases, although it does not reach the 30 C level.

In vitro activity of extracts. The effects described thus far could be a consequence of an initial effect on either RNA or protein synthesis. In the simplest case, one would expect that the component which is directly inactivated at elevated temperatures in vivo would be defective in vitro when assayed in an uncoupled system.

Table 1 presents the RNA polymerase activity of crude extracts from cells grown at 30 C or exposed to 44 C. Brief treatment of the cells at 44 C results in a small loss of activity, using calf thymus deoxyribonucleic acid (DNA) as a template, but the effect is minimal compared to the in vivo loss of activity.

Protein synthesis activity, on the other hand, is dramatically reduced when the cells are exposed to 44 C before preparing the extracts. S30 extracts prepared from 30 C-grown cells can synthesize the same amount of protein at 30 and 43 C (Fig. 4). The rate of synthesis, however, is greater at 43 C. Extracts

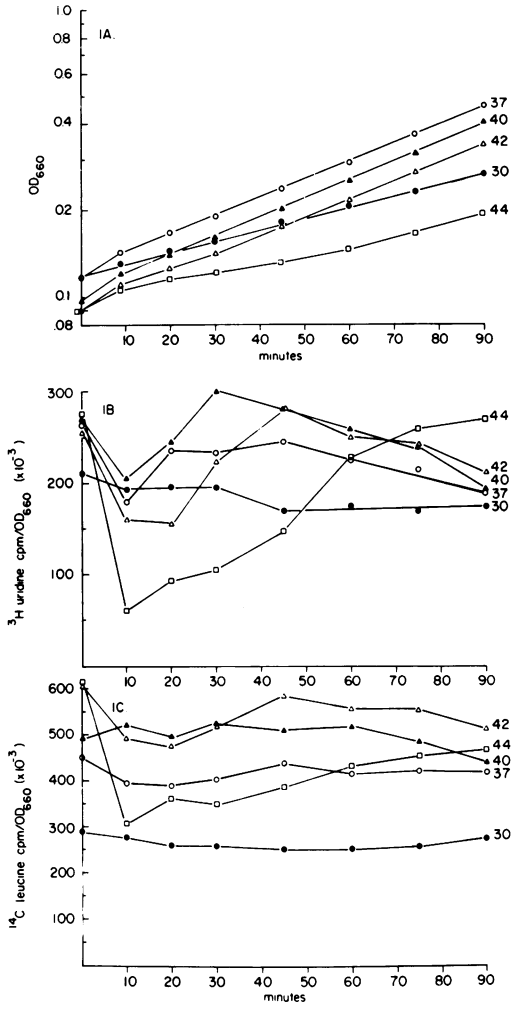

FIG. 1. *Effect of elevation of temperature on rate of growth, ^3H-uridine incorporation, and ^{14}C-leucine incorporation. (For exact experimental details, see Materials and Methods.)*

prepared from 43 C-grown cells are only 20 to 30% as active as the standard extracts, regardless of the temperature of the in vitro reaction.

Table 2 demonstrates the activity of extracts which have been isolated from 30 or 42 C-grown cells and fractionated into S100, washed ribosomes, and ribosomal wash proteins. This table shows that the activity of washed ribosomes (samples 1 and 2) is slightly higher from 43 C-grown cells, and that the activity of supernatant enzymes (samples 1 and 3) is slightly lower. The activity of ribosomal wash proteins (samples 1 and 4) is markedly lower from 43 C-grown cells. The amount of ribosomal wash proteins from 43 C-grown cells is saturating (sample 8). The low activity in reactions containing ribosomal wash proteins from 43 C-grown cells is not a consequence of an inhibitor present in the preparation, since full activity can be restored by adding ribosomal wash proteins from 30 C-grown cells (sample 9).

It is possible to duplicate the in vivo thermal inactivation in an in vitro system. Although S30 and ribosomal wash protein fractions obtained from 30 C-grown cells can be inactivated in vitro by incubation at 43 C, the ribosomal wash proteins obtained from 43 C-grown cells are largely resistant to inactivation (Fig. 5).

RNA synthesis activity in extracts prepared from 30 C-grown cells is not inactivated by a 30-min preincubation at 44 C, even when as-

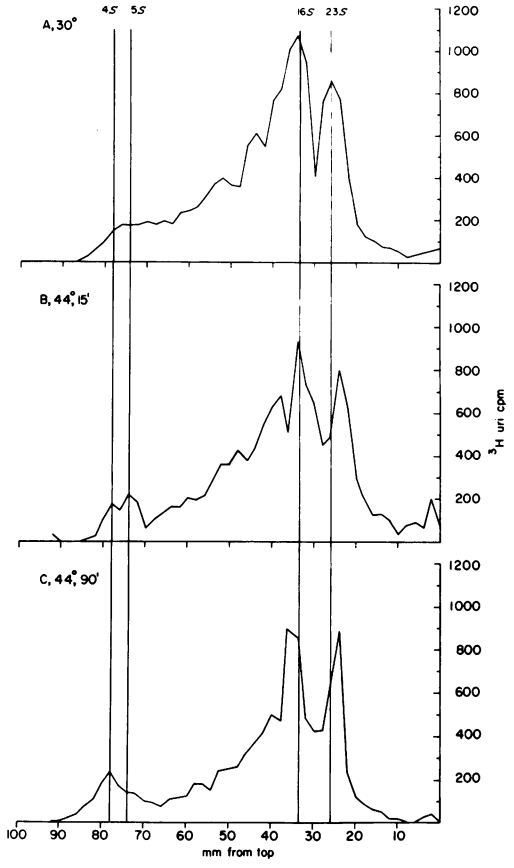

FIG. 2. *SDS-polyacrylamide gel electrophoresis of pulse-labeled RNA from cells growing at 30 and at 44 C. RNA was prepared and subjected to electrophoresis, as described in Materials and Methods. Recovery of ^3H-RNA counts/min was greater than 90% for all gels. Between 6,500 and 14,000 ^3H counts/min was applied to each gel.*

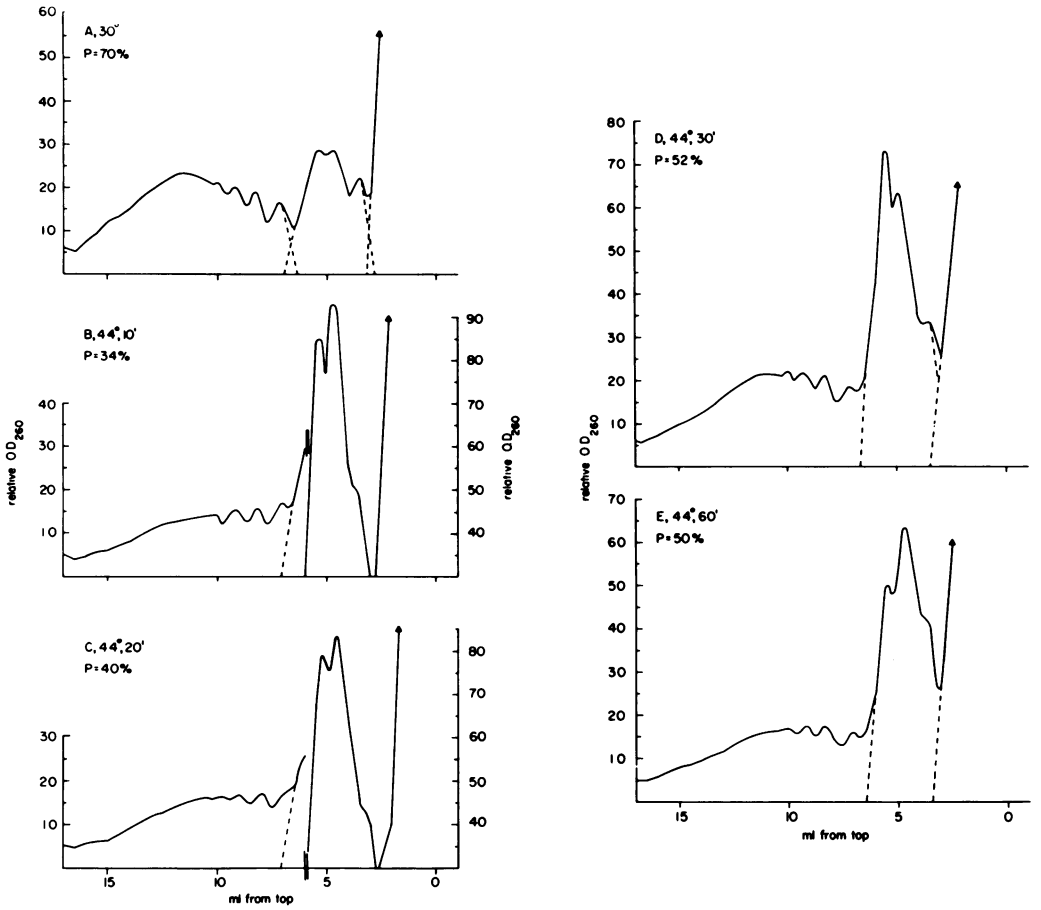

FIG. 3. *Polyribosome content of E. coli A2325 at various times after a temperature shift from 30 to 44 C. Polyribosome profiles were analyzed as described in Materials and Methods. Percentages of polyribosomal material, P_n, were determined by weighing the appropriate areas under the curves, indicated by dotted lines.*

sayed in reaction mixtures containing T7 DNA as template. It is likely, from these data, that the inactivation of protein synthesis in vivo is a direct thermal inactivation, but the inactivation of RNA synthesis is a secondary effect.

The defect in 43 C-grown cells for protein synthesis resides in the ribosomal wash fraction, a fraction which is known to contain the initiation factor activity of *E. coli* extracts (18). Accordingly, the ability of these extracts to support one of the steps of initiation of protein synthesis was assayed, namely f2 RNA-dependent binding of ^{35}S-*N*-formyl-methionyl-$tRNA_F^{met}$ to ribosomes.

Ribosomal wash proteins from 43 C-grown cells are deficient when compared to those from 30 C-grown cells with respect to ability to stimulate f2 RNA-directed binding of ^{35}S-*N*-formyl-methionyl-$tRNA_F^{met}$ at all concentrations of ribosomal wash protein tested. The deficiency is observable when the results are expressed as volume of fraction added or as micrograms of protein added.

DISCUSSION

The data presented demonstrated that, upon elevation of the growth temperature of *E. coli* A2325 from 30 to 44 C, this strain becomes transiently defective in its ability to synthesize both protein and RNA molecules. The same phenomenon has been reported for HeLa cells (12). In the *E. coli* case, the defect resides in one or more proteins released from ribosomes by a 2 M NH_4Cl wash procedure. This is the fraction in which initiation factor activity is found (18).

The inactivation of a protein(s) in this fraction appears to be the primary target of a shift to high temperature, since (i) this fraction, when obtained from cells grown at 30 C, is

TABLE 1. *RNA polymerase activity from cells grown at 30 or 43 C*[a]

Growth conditions	Activity (counts/min)
30 C	669
44 C, 10 min	529
44 C, 30 min	778

[a] Cells were broken, and crude extracts were prepared and assayed as described earlier (16). Endogenous DNA was removed from these extracts by treatment with 10 μg of micrococcal nuclease per ml (Worthington Biochemical Corp., Freehold, N.J.) for 30 min at 30 C, in the presence of 0.001 M $CaCl_2$. Calcium was then removed by dialysis. All of the activity is abolished by antiserum directed against pure *E. coli* RNA polymerase. The reaction uses 25 μg of calf thymus DNA as template.

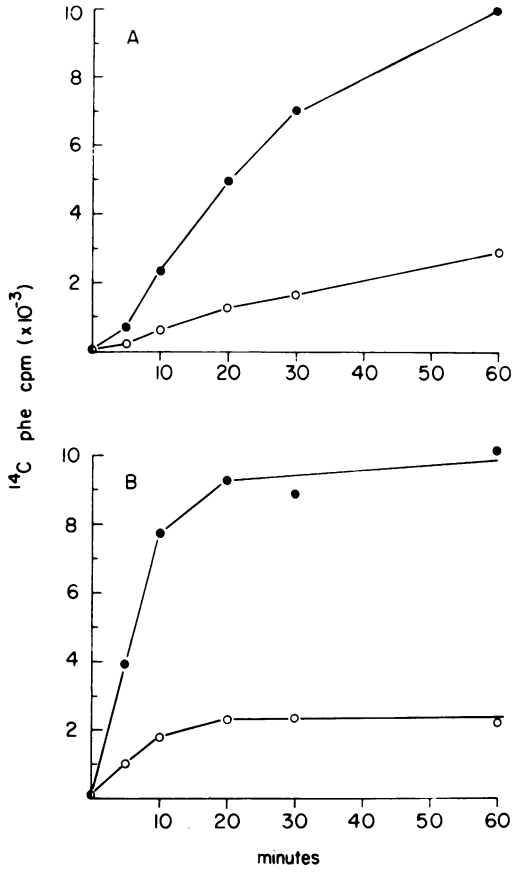

FIG. 4. *Kinetics and extent of ^{14}C-phenylalanine incorporation by S30 extracts from 30 and 43 C-grown cells. Reaction mixtures (100 μliters) contained either 210 μg of S30 prepared from 30 C-grown cells or 164 μg of protein of S30 from 43 C-grown cells plus 25 μg of f2 RNA. At the times indicated, 10-μliter samples were collected as described in Materials and Methods. A, Reactions incubated at 25 to 30 C. B, Reactions incubated at 43 C. Symbols: ●, S30 extracts from 30 C-grown cells; ○, S30 extracts from 43 C-grown cells. Incorporation in the absence of f2 RNA was 200 counts/min or less, in all conditions.*

inactivated by about 70% during incubation at 44 C in vitro, and (ii) the equivalent fraction obtained from cells shifted to 44 C has the same activity as its heat-inactivated counterpart from 30 C-grown cells and is resistant to thermal inactivation in vitro. Our data suggest that the ribosomal wash proteins from 43 C-grown cells are defective in the ability to promote binding of initiator formyl-methionyl-$tRNA_F^{met}$ to ribosomes in response to f2 bacteriophage RNA.

On the basis of experiments with inhibitors of RNA and protein synthesis, McCormick and Penman (12) hypothesized that, at the elevated temperature, HeLa cells had to increase the rate of synthesis of an unstable nonmessenger RNA molecule which regulated the rate of initiation of protein synthesis. We feel that this explanation does not explain satisfactorily the data with *E. coli*, since the thermal inactivation we observe in vitro is characteristic for proteins and not for RNA. Purification of the stimulating factor in extracts from 30 C-grown cells should clarify this point.

TABLE 2. *Protein synthetic capacity in vitro by subcellular fractions from 30 and 43 C-grown cells*[a]

Expt	SUP[a]	RW	Ribosomes	^{14}C-Phe (counts/min)
1	30[b]	30	30	8,486
2	30	30	43	11,514
3	43	30	30	5,405
4	30	43	30	2,648
5	43	43	43	2,915
6	43	43	30	2,664
7	43	30	43	6,145
8	30	2 × 43	30	3,031
9	30	30 + 43	30	12,123
10		30	30	657
11	30		30	601
12	30	30		540
13		43	43	314
14	43		43	346
15	43	43		542

[a] Extracts were fractionated and reactions were run as described in Materials and Methods, by using 38 μg of supernatant enzymes (SUP), 16 μg of ribosomal wash proteins (RW), and 1.7 absorbancy units at 260 nm of ribosomes.

[b] 30 and 43 indicate extracts prepared from 30 and 43 C-grown cells, respectively.

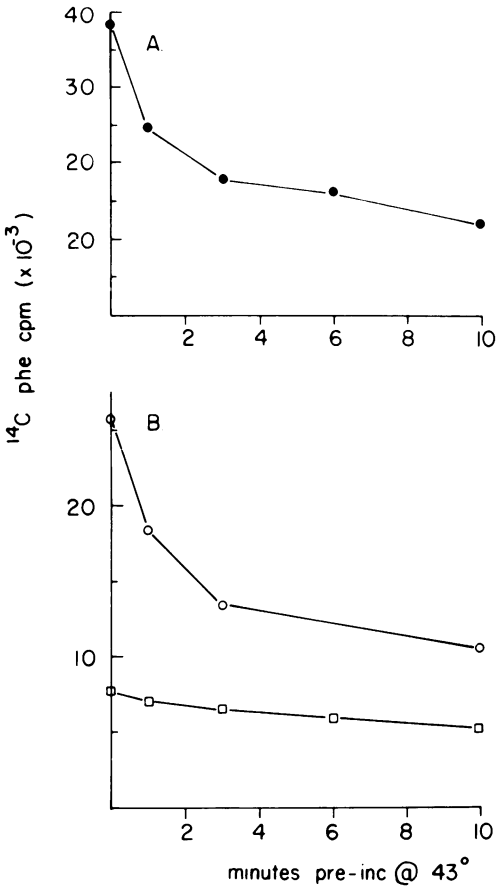

FIG. 5. *In vitro inactivation of S30 and ribosomal wash protein fractions. A, Complete reaction mixtures minus S30 were prepared and kept at 0 C. A standard S30 was then incubated at 43 C. At the times indicated, 10 μliters was added to 40-μliter samples of reaction mixture at 0 C and then immediately shifted to 30 C and processed as described in Materials and Methods. B, Complete reaction mixtures containing 17 μg of 30 C supernatant protein and 1.7 OD_{260} units of washed 30 C ribosomes in 40 μliters were prepared and kept at 0 C. Ribosomal wash fractions were then incubated at 43 C. At the times indicated, 10 μliters (29 μg of 30 C-grown ribosomal wash proteins and 19 μg of 43 C-grown ribosomal wash proteins) was removed and added to 40-μliter samples of reaction mixture at 0 C and then immediately shifted to 30 C, incubated for 30 min, and processed as described in Materials and Methods. Symbols: ○, 30 C ribosomal wash; □, 43 C ribosomal wash.*

Several conditions lead to a loss of activity of the protein synthetic complex (in vivo). These include infection with T4 or T7 viruses (3, 8, 17), treatment with chloramphenicol (24), or growth into stationary phase (21). In all of these cases, a defect in the initiation of protein synthesis was documented. Some of these defects are reversed by purified initiation factor proteins (8, 24). We were unable to reverse the defect caused by growth at elevated temperature with purified preparations of factors F1 or F2 (generously donated to us by P. Leder). Nevertheless, our data suggest an inactivation of a protein required for the initiation of protein synthesis.

TABLE 3. *Binding of ^{35}S-formyl-methionyl-tRNA$_F^{met}$ to ribosomes*[a]

Sample no.	Protein (μg)	^{35}S bound (counts/min)	
		30 C-grown	43 C-grown
1	3	37	41
2	9	1,317	413
3	20	3,181	1,386
4	28	3,481	2,325

[a] Binding reaction was carried out as described by Lodish (9), by using the indicated amounts of ribosomal wash protein (see Materials and Methods) obtained from 30 or 43 C-grown cells. The yield of the ribosomal wash fraction per cell was 1.6 times greater in the case of 30 C-grown cells. Protein was determined according to Lowry et al. (11).

RNA synthesis also becomes restricted at elevated temperatures, but this appears to be an indirect consequence of the temperature shift. RNA polymerase activity assayed in vitro by using calf thymus DNA as template is not diminished by growth of the cells at high temperature. The restriction of RNA synthesis is seen in cells which control RNA synthesis stringently but not in cells where RNA control is relaxed (14). The data taken together suggest that the close coordination of protein synthesis and RNA synthesis in *E. coli* strains possessing stringent control of RNA synthesis is mediated by the activity of one or more steps in the initiation of protein synthesis. Our data suggest that the step is at or prior to the binding of *N*-formyl-methionyl-tRNA$_F^{met}$ to ribosomes. A similar conclusion was reached by Shih et al. (22).

In *E. coli*, the restriction of RNA and protein synthesis at elevated temperatures is temporary in vivo. The in vitro capacity of extracts decreases, however, when taken from cells incubated at 43 C for extended periods (e.g., 60 min). Polyacrylamide gel electrophoresis of S30 extracts revealed the accumulation of one protein band in cells grown at 43 C (data not presented). This band is characteristically recovered in the ribosomal wash and supernatant fractions, but is not present in

washed ribosomes. A temperature-sensitive protein synthesis mutant of *E. coli* (15) does not accumulate this protein at 43 C. These data suggest that the mechanism of recovery at high temperature involves the overproduction of some protein, possibly the protein which is inactivated at the high temperature.

Ron and co-workers (19, 20) suggested that the primary effect of a temperature shift-up on metabolism in *E. coli* is to inactivate methionine biosynthesis. In their case and in ours, the rate of inactivation of the thermolabile activities in vitro is extremely rapid. Moreover, extracts prepared from cells incubated at elevated temperatures for brief periods lack both activities. The effect observed by Ron and associates is reversed by the addition of methionine to the medium and persists for at least 1 hr, whereas the effect we studied is apparently not dependent on the composition of the medium and is temporary. The two effects may be separate.

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