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# Mechanism of growth delay induced in *Escherichia coli* by near ultraviolet radiation

(guanosine tetraphosphate/transfer RNA/*E. coli rel*/RNA accumulation)

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**ABSTRACT** **Continuously growing cultures of *E. coli* B/r were irradiated with a fluence of broad-band near-ultraviolet radiation (315–405 nm) sufficient to cause extensive growth delay and complete cessation of net RNA synthesis. Chloramphenicol treatment was found to stimulate resumption of RNA synthesis, similar to that observed with chloramphenicol treatment after amino-acid starvation. *E. coli* strains in which amino-acid starvation does not result in cessation of RNA synthesis ("relaxed" or *rel*$^-$ strains) show no cessation of growth and only a slight effect on the rate of growth or of RNA synthesis. These findings show that such near-UV fluences do not inactivate the RNA synthetic machinery but affect the regulation of RNA synthesis, in a manner similar to that produced by amino-acid starvation. Such regulation is believed to be mediated through alterations in concentration of guanosine tetraphosphate (ppGpp), and our estimations of ppGpp after near-UV irradiation are consistent with such an interpretation. These data, combined with earlier published data, strongly suggest that the mechanism of near-UV-induced growth delay in *E. coli* involves partial inactivation of certain tRNA species, which is interpreted by the cell in a manner similar to that of amino-acid starvation, causing a rise in ppGpp levels, a shut-off of net RNA synthesis, and the induction of a growth delay.**

Near-ultraviolet radiation (near-UV; 300–380 nm) is present in sunlight, but is absorbed only marginally, if at all, by proteins and nucleic acids. Yet the well-known deleterious effects of far-ultraviolet radiation (far-UV; below 300 nm), such as killing, induction of mutation, and inhibition of growth, are found to occur in bacteria throughout the near-UV region (1, 2).

Of these effects, the one induced by the lowest fluences of near UV is the inhibition of growth*. Bacterial studies have shown that growth delay has a narrow action spectrum, peaking at 340 nm. Earlier suggestions that quinones might be the chromophores and oxidative phosphorylation the cellular target (1) have not been supported by recent work showing that coenzyme Q is resistant to near-UV radiation *in vivo* (3) and that ATP synthesis shows a threshold response (B. Lakchaura, T. Fossum, and J. Jagger, *J. Bacteriol.*, **125**, in press). In addition, it is unlikely that DNA is the target for near-UV-induced growth inhibition (4).

Inhibition of growth clearly must involve inhibition of macromolecular synthesis. Swenson *et al.* (5) present data showing that RNA and protein synthesis in *E. coli* B/r grown in glycerol are somewhat more sensitive to near UV than DNA synthesis, although they do not discuss this.

Ramabhadran (6) has recently shown a more clear-cut effect, in which fluences of near UV capable of inducing a large growth delay in *E. coli* B/r grown in glucose result in a complete (but temporary) cessation of net RNA synthesis, the effects on protein and DNA synthesis being less drastic. He has also shown that the action spectrum for induction of this inhibition of RNA synthesis is similar to the action spectrum for induction of growth delay in *E. coli*, and that the growth delay action spectrum fits very closely to the absorption spectrum of tRNAs that contain the unusual nucleoside 4-thiouridine ($^4$Srd). It is known that near-UV irradiation *in vitro* of such tRNAs can induce an adduct between the $^4$Srd in the 8-position and a cytidine in the 13-position of the tRNA, and that this lowers the rate at which the tRNA accepts amino acids (7). The fluences required to inactivate the tRNA are similar to those required for inhibition of bacterial growth (6). [Similar results have been obtained by irradiation *in vivo* of *E. coli* B/r (8,†).] These findings led Ramabhadran to suggest that the chromophore for near-UV-induced growth delay in *E. coli* is $^4$Srd, that the target is tRNA, and that the mechanism is the near-UV-induced lowering of the activity of tRNA which leads to a lowered amino-acid availability, and thence to a temporary but complete shut-off of net RNA synthesis.

The present paper provides data that support this hypothesis. This work was first reported at the meetings of the American Society for Photobiology in June, 1975 (9).

## MATERIALS AND METHODS

**Chemicals.** [5-$^3$H]Uridine (8 Ci/mmol, 0.5 mCi/ml); [4,5-$^3$H]leucine (6 Ci/mmol, 1.0 mCi/ml); [2-$^{14}$C]uridine (55 mCi/mmol, 10 $\mu$Ci/ml); and [8-$^{14}$C]guanine (50 mCi/mmol, 100 $\mu$Ci/ml) were obtained from Schwarz-Mann, New York. Nonradioactive uridine, leucine, L-fucose, and purine ribonucleoside mono-, di-, and triphosphates were obtained from Sigma Chemical Co., St. Louis, Mo. Polyethylene imine-cellulose thin-layer chromatography plates were obtained from Brinkmann Instruments, Inc., New York.

**Bacteria.** *Escherichia coli* B/r ATCC 12407 was obtained from Dr. H. Bremer. *E. coli* B/r NC32, a *lac*$^-$ *tRNA*$^{Val}$ *S*$^{ts}$ derivative of the above strain carrying a temperature-sensitive valine synthetase, was kindly supplied by Dr. F. C. Neidhardt, University of Michigan. This strain lacks the ability to restrict phage $P_1$ grown on *E. coli* K12 strains. *E. coli* K12 W6, *met*$^-$*rel*$^-$ and phage $P_1$*kc* were obtained from Dr. R. C. Clowes.

**Growth.** Cells were grown with vigorous shaking in M9 medium (described in ref. 10). The temperature-sensitive B/r strains NC51 and NC52 were grown at 30°, whereas the wild-type B/r ATCC 12407 was grown at 37°. Growth was monitored by optical density in a 1 cm light-path cuvette at

Abbreviations: TCA, trichloroacetic acid; $^4$Srd, 4-thiouridine; ppGpp, guanosine-3′-diphosphate-5′-diphosphate.

* We use the phrase "inhibition of growth" to include either a decrease of the initial rate of growth or a complete, but temporary, cessation of growth ("growth delay").

† T. V. Ramabhadran, T. Fossum, and J. Jagger (1976) *Photochem. Photobiol.* **23**, in press.

460 nm ($OD_{1.0}^{460}$) with a Zeiss PMQII spectrophotometer. The doubling times were close to 60 min at 30° and 45 min at 37°.

**Transduction.** Deprival of a required amino acid causes net RNA synthesis to stop in normal ("stringent"; *rel*$^+$) strains of *E. coli*. In a "relaxed" (*rel*$^-$) mutant, however, RNA synthesis continues under these conditions (11). An isogenic pair of *E. coli* B/r strains differing only at the *rel* locus was constructed by transducing the *rel*$^-$ gene from the W6 strain via phage $P_1$ into B/r NC32, using the transduction procedure of Miller (12). The *rel* marker, which maps around 54 min on the *E. coli* chromosome, is close to the marker for the ability to utilize L-fucose (13). Strains of *E. coli* B/r are unable to grow on L-fucose, whereas K12 strains can use this sugar as a carbon source (14). Hence the *rel* gene was cotransduced with the fucose gene via phage $P_1kc$ grown on the W6 strain. The transduction was done at a multiplicity-of-infection of 1/100 and the *fuc*$^+$ transductants were selected on fucose minimal plates. The response of such a pair of B/r strains, NC51 *rel*$^+$ and NC52 *rel*$^-$, to an effective amino-acid starvation caused by a temperature shift is shown in Fig. 1.

**Irradiation.** Two General Electric T-15 BLB "blacklight" lamps (emission chiefly at 320–405 nm) were used as a source of polychromatic near-UV radiation. Presence of a DuPont Mylar film (cut-off at 310 nm) did not change the results appreciably. Growing bacterial suspension (20–30 ml) of $OD_{1.0}^{460}$ = 0.3 was transferred to flat-bottomed petri dishes of 5 cm diameter in a water bath at 37° or 30°. An air jet mixed the suspension to keep the growth rate the same as before transfer. The fluence rate at the suspension surface (6 cm from the BLB lamps) was determined by ferrioxalate actinometry (15) to be 35 ± 3 W/m$^2$. After irradiation the cultures were returned to the shaker or were aerated in test tubes.

**Measurement of DNA, RNA, and Protein Synthesis.** The measured net synthesis of these macromolecules is the same, whether followed by colorimetric methods or radioactive labeling (6). In the present experiments, macromolecular synthesis was measured by incorporation of radioactive precursors.

[2-$^{14}$C]Uridine was used to label both DNA and RNA. (Uridine is converted to dCTP and dTTP in wild-type cells.) Two samples were taken for each point; one was precipitated with cold trichloroacetic acid (TCA) and $^{14}$C incorporation into total nucleic acid was measured. The other was hydrolyzed at 37° with 0.5 ml of 1.0 M NaOH for 2 hr. The hydrolyzed sample was precipitated with TCA and the amount of $^{14}$C incorporated into DNA (alkali-resistant radioactivity) was determined. The difference between these counts is a measure of incorporation into RNA. [Under these growth conditions, $^{14}$C radioactivity in DNA is about 16% of that in total nucleic acids (16) and hence this method does not introduce large subtraction errors]. [$^3$H]Leucine was used simultaneously to label the proteins.

In all incorporation studies, the TCA precipitates were collected on glass fiber filters (Reeve Angel, 984H) and the radioactivity was determined by liquid scintillation counting in a toluene-based cocktail.

**Estimation of Guanosine-3′-diphosphate-5′-diphosphate (ppGpp).** Cells were labeled for 5 min with [$^{14}$C]guanine and were then irradiated for 10 min. Samples of 100 μl were withdrawn and added to 100 μl of 2 M formic acid. The samples were centrifuged to remove cell debris, and 100 μl of the supernatants were applied to polyethylene imine-cellulose thin-layer chromatography plates. The plates were developed to the top in an ascending manner thrice with distilled water, in order to move guanine, and nucleosides derived from guanine, away from the origin (17). This was followed by one ascending development in 1.5 M $KH_2PO_4$, pH 3.4, as described by Cashel (18). Autoradiograms were obtained by exposure to Kodak RP/R54 medical x-ray film for 1–2 weeks.

The spots corresponding to ppGpp were identified on the

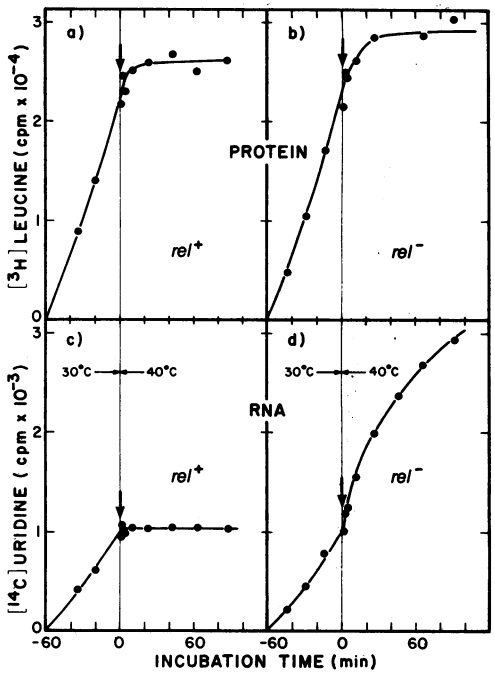


FIG. 1. Protein and RNA synthesis in *E. coli* B/r strains NC51 (*rel*$^+$) and NC52 (*rel*$^-$), both carrying a temperature-sensitive valine synthetase, following a temperature shift that induces an effective amino-acid starvation. Cells growing exponentially at 30° were labeled at $OD^{460} \simeq 0.15$ with uridine and leucine to give 0.04 μCi/ml of [$^{14}$C]uridine in 0.14 mM nonradioactive uridine and 1.4 μCi/ml of [$^3$H]leucine in 0.16 mM nonradioactive leucine. Sixty minutes later (arrows) (time = 0) the cultures were shifted to 40°. (a) and (c): NC51 (*rel*$^+$); (b) and (d): NC52 (*rel*$^-$).

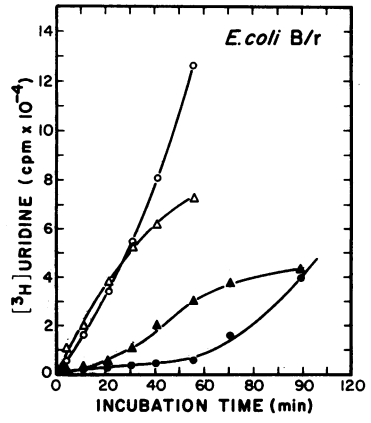


FIG. 2. Chloramphenicol-induced resumption of net RNA synthesis in *E. coli* B/r ATCC 12407 irradiated with near UV. Cells were grown to $OD^{460}$ = 0.3 at 37°, exposed to 20 kJ/m$^2$ of near UV and labeled with 0.6 μCi/ml of [$^3$H]uridine in 0.03 M nonradioactive uridine. (○) Unirradiated; (△) unirradiated + 100 μg/ml of chloramphenicol added at time zero; (●) irradiated; (▲) irradiated + 100 μg/ml of chloramphenicol added immediately after irradiation. Counts were not corrected for the small incorporation into DNA (see *Materials and Methods*).

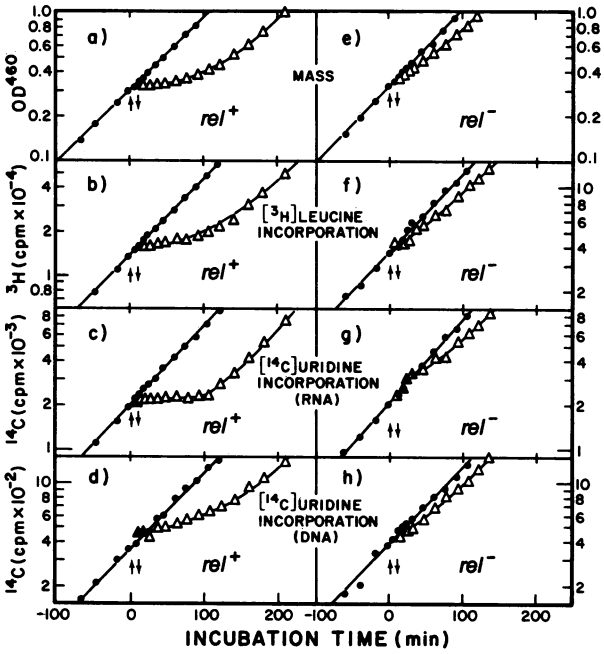


FIG. 3. Mass increase and protein, RNA, and DNA synthesis in *E. coli* B/r strains NC51 (*rel*$^+$) and NC52 (*rel*$^-$) irradiated with near UV. Cells were grown for 5 generations in M9 medium supplemented with uridine and leucine to give 0.03 $\mu$Ci/ml of [$^{14}$C]uridine in 0.11 mM nonradioactive uridine and 0.9 $\mu$Ci/ml of [$^3$H]leucine in 0.12 mM nonradioactive leucine. At OD$^{460}$ $\sim$ 0.3, cells were irradiated for 10 min starting at time = 0 (total fluence of 20 kJ/m$^2$). Arrows indicate the beginning (↑) and the end (↓) of irradiation. (●) Unirradiated; (△) irradiated. (a–d) *rel*$^+$; (e–h) *rel*$^-$.

following criteria: (*a*) an $R_F$ value lower than GTP and close to the value obtained by Cashel (18), (*b*) occurrence in the NC32 *valS*$^{ts}$ mutant (in contrast to wild-type cells) when subjected to amino-acid deprivation by elevating the temperature, and (*c*) high levels in the "stringent" strain NC51 and low levels in the "relaxed" strain NC52 when subjected to elevated temperatures.

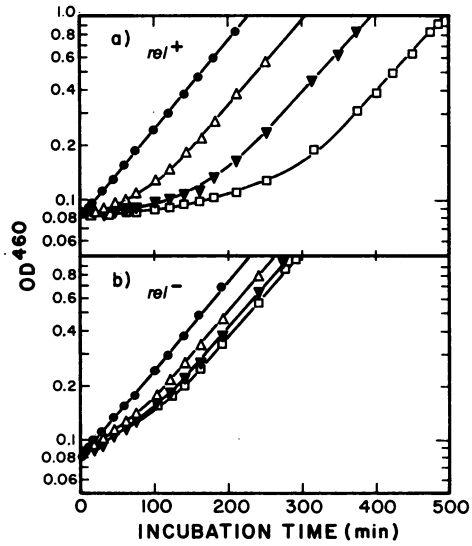


FIG. 4. Near-UV-induced growth inhibition in *E. coli* B/r strains NC51 (*rel*$^+$) and NC52 (*rel*$^-$). Cells growing exponentially at 30° were exposed at OD$^{460}$ $\simeq$ 0.3 to varying fluences of near UV. Following irradiation, the cultures were diluted ¼ in M9 and growth was followed by measuring OD$^{460}$. (●) 0 kJ/m$^2$; (△) 10 kJ/m$^2$; (▼) 20 kJ/m$^2$; (□) 30 kJ/m$^2$.

## RESULTS

Fig. 2 shows the inhibition of net RNA synthesis in *E. coli* B/r induced by a near-UV fluence of 20 kJ/m$^2$. Addition of chloramphenicol (100 $\mu$g/ml) stimulates an earlier resumption of net RNA synthesis. This is similar to the response of *E. coli* cells to amino-acid starvation (19, 20). However, addition of amino acids to the medium does not cause an early resumption of RNA accumulation or growth (data not shown).

If the mechanism of near-UV-induced inhibition of net RNA synthesis is similar to that operating in amino acid starvation, one would expect less inhibition of net RNA synthesis by near UV in relaxed (*rel*$^-$) strains. Fig. 3 shows that the synthesis patterns observed for strain NC51 (*rel*$^+$) (Fig. 3a–d) are similar to those reported earlier (6) for B/r ATCC 12407. Growth (Fig. 3a) and incorporation of leucine (Fig. 3b) show a strong inhibition after near UV, followed by a steadily increasing rate. Accumulation of RNA stops for about 100 min (Fig. 3c), whereas DNA synthesis proceeds at a gradually decreasing rate and recovers when RNA synthesis resumes (Fig. 3d).

In the *rel*$^-$ mutant NC52, RNA accumulation continues unchanged immediately after irradiation but shifts to a slightly lower rate after about 50 min (Fig. 3g). Growth (OD$^{460}$; Fig. 3e), protein synthesis (Fig. 3f), and DNA synthesis (Fig. 3h) also show lowered sensitivity to near UV.

Fig. 4 shows that increasing fluences of near UV produce increasing delays in the resumption of normal growth in the *rel*$^+$ strain. The *rel*$^-$ strain shows no cessation of growth, but continues to grow at a slightly reduced rate until attaining the growth rate of the unirradiated culture. A fluence as high as 60 kJ/m$^2$ does not change the growth pattern appreciably from what is observed for 30 kJ/m$^2$, whereas increasing delays are found in the *rel*$^+$ strain (data not shown). Another pair of isogenic *rel*$^+$ and *rel*$^-$ strains, CP78 (*rel*$^+$) and CP79 (*rel*$^-$), showed the same responses as the corresponding strains in Fig. 4 (data not shown). The donor strain, *E. coli* K12 W6 *rel*$^-$, behaves like the NC52 *rel*$^-$ transductant (data not shown).

Another event accompanying the stringent response of RNA synthesis during amino-acid starvation is the increase in levels of ppGpp. This increase is not seen in relaxed strains of *E. coli* (18). This and other observations have led to the hypothesis that ppGpp may be a regulatory factor in control of RNA accumulation during amino-acid starvation (21). Therefore, if near UV inhibits RNA synthesis through the equivalent of an amino-acid starvation, then levels of ppGpp should increase following near-UV irradiation.

Figs. 5a and b show the incorporation of guanine into TCA-precipitable material in strains NC51 *rel*$^+$ and NC52 *rel*$^-$ after near-UV irradiation. As shown in Fig. 3c and g, RNA accumulation ceases in *rel*$^+$ but not in *rel*$^-$ cells.

Fig. 5c (I2) shows that, by 7 min after the start of irradiation, ppGpp has increased substantially over the levels found at earlier times (I1) and exceeds the levels found in unirradiated controls (C1 through C3) at any time in the experiment. Following this increase, the level falls off slowly, but, even in the last sample (I7), is above that observed in controls. In contrast, Fig. 5d shows that *rel*$^-$ strains show little if any increase in the levels of ppGpp when irradiated with near UV.

## DISCUSSION

**Near UV Irradiation Mimics Amino-Acid Starvation.** In stringent strains of *E. coli* whose RNA accumulation has

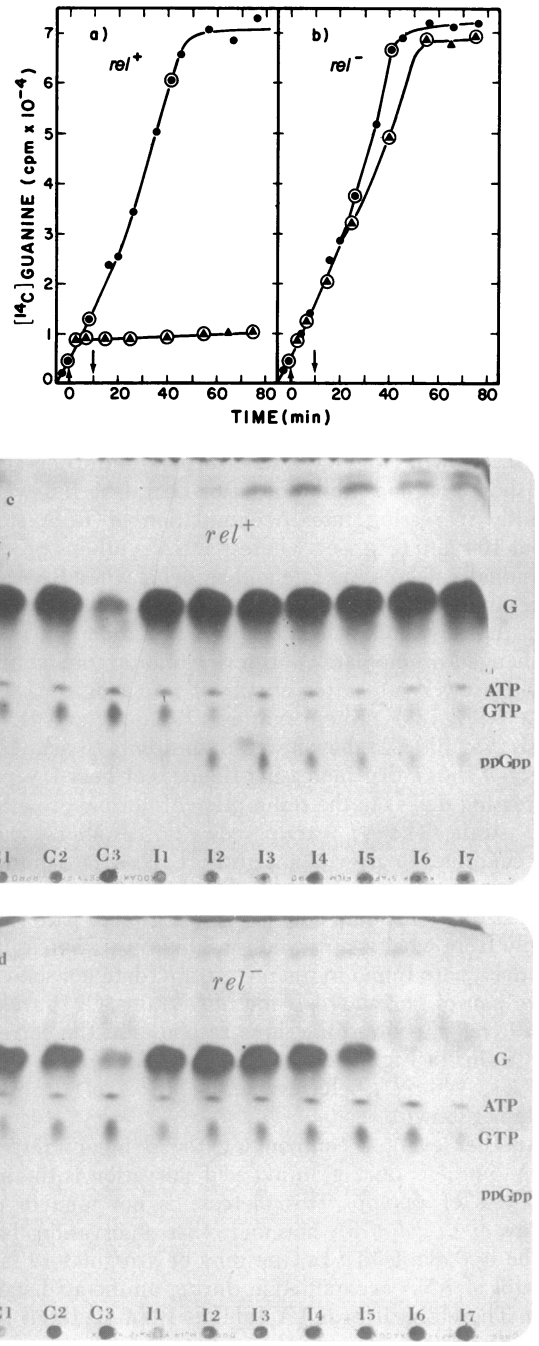


FIG. 5. Kinetics of guanine incorporation and levels of ppGpp in *E. coli* B/r strains NC51 (*rel*$^+$) and NC52 (*rel*$^-$) following near-UV irradiation. Cells growing exponentially at 30° were labeled with 0.8 $\mu$Ci/ml (16 $\mu$M) of [$^{14}$C]guanine at OD$^{460}$ $\simeq$ 0.3. Five minutes later (time = 0), part of the culture was irradiated for 10 min (↑, beginning; ↓, end of irradiation; total fluence 20 kJ/m$^2$). (a and b) Incorporation of guanine into TCA-precipitable material: (a) NC51 (*rel*$^+$); (b) NC52 (*rel*$^-$). (●) Unirradiated; (▲) irradiated; circles enclosing points represent where samples were taken for measurement of ppGpp. (c and d) Autoradiograms of thin-layer chromatograms of samples taken in a and b, respectively. C1 through C3 represent samples from unirradiated and I1 through I7 from irradiated cultures, placed in the chronological order of samplings in a and b. The lightening of guanine spots with time in c (C3) and d (C3, I6, I7) is due to exhaustion of guanine in the medium.

been curtailed by withdrawal of an essential amino acid, chloramphenicol causes a resumption of net RNA synthesis (19, 20, 22, 23). Our findings show that near-ultraviolet radiation inhibits the net synthesis of RNA in stringent cells of *E. coli*, and that such inhibition can be relieved by addition of chloramphenicol, which suggests that near-UV-induced inhibition of RNA synthesis is due to a stringent response.

Amino-acid starvation induces the synthesis of ppGpp and a shut-off of RNA accumulation in *rel*$^+$ strains. Neither of these responses is seen in *rel*$^-$ strains under similar conditions (18). The level of ppGpp in *E. coli* is found to be inversely correlated with the rate of net RNA synthesis, upon amino-acid starvation in a stringent strain (17). Many other lines of evidence also suggest that ppGpp is involved in the regulation of RNA synthesis in *E. coli* (21). We find that near-UV irradiation inhibits RNA accumulation and results in an increase in the level of ppGpp in the *rel*$^+$ strain, whereas neither of these effects is seen in *rel*$^-$ strains.

These observations indicate that near-UV irradiation creates conditions similar to those in amino-acid starvation. Addition of all the usual amino acids to the minimal medium following irradiation causes neither an early resumption of RNA accumulation nor a reversal of growth inhibition in the stringent strain, indicating that the amino-acid starvation is not caused by inability to synthesize an amino acid but by inability to make the amino acid available to the ribosome. Thus, by several criteria, it appears that near-UV irradiation mimics amino-acid starvation.

**tRNA as the Target Molecule for Growth Delay and Inhibition of RNA Synthesis.** The modified nucleoside, 4-thiouridine ($^4$Srd), occurs at the 8-position (24) in about 65% of all *E. coli* tRNAs (7). The absorption spectrum of such tRNAs shows a peak around 340 nm, which is due solely to the presence of the $^4$Srd moiety. Our earlier work (see Introduction) indicates that $^4$Srd is probably the chromophore for both growth delay and inhibition of net RNA synthesis by near UV.

Irradiation at 334 nm of a number of purified $^4$Srd-containing tRNAs produces an adduct between $^4$Srd and a cytidine in the 13-position (25). About three-fourths of the *E. coli* tRNA species that contain $^4$Srd (all those that have a cytidine at the 13-position) can form this adduct (7). Such adducts can be formed in acylated, nonacylated, or synthetase-bound tRNA (25). We have recently demonstrated that such $^4$Srd-Cyd adducts are also produced in irradiated tRNA extracted from *E. coli* B/r by fluences similar to those required *in vitro*, and also to those required for near-UV-induced growth delay and inhibition of net RNA synthesis (8). Thus it appears that $^4$Srd in tRNA is not only the *in vivo* chromophore but also the *in vivo* target for near-UV-induced growth delay and inhibition of RNA synthesis, and that the photoproduct involved is the $^4$Srd-Cyd adduct.

The question may arise as to whether or not the observed inhibition of $^4$Srd-containing tRNA is sufficient to account for the cessation of net RNA synthesis that is observed with these near-UV fluences. Irradiated *E. coli* tRNA$^{Phe}$ shows a drastic reduction of the initial rate of acylation, and a final level only 20% of that of the control, although other tRNAs show lesser effects (7). It has also been found that irradiated tRNAs, in an *in vitro* protein synthesis system, incorporate the corresponding amino acid into polypeptides at only half the normal rate (26, 27). Now, it is known that (*a*) the extent of the stringent response is dependent on the degree of starvation for a single amino acid (17), and (*b*) total withdrawal of a single amino acid will greatly decrease the rate of RNA accumulation (28). Since near UV can produce the adduct in a large number of tRNA species and some of the species will experience a drastic reduction in acylation rate, it is therefore evident that the fluences of near-UV radiation that lead

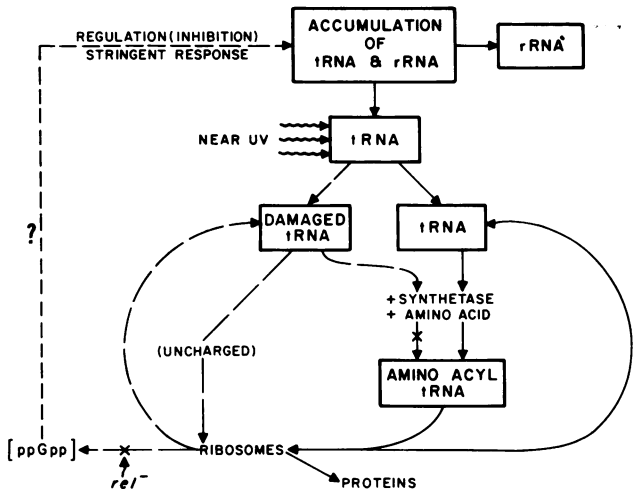


FIG. 6. Proposed scheme of events following near-UV irradiation of *E. coli* B/r, leading to inhibition of net RNA synthesis and delay in growth. Broken lines indicate events precipitated by near-UV irradiation, which then occur in addition to the normal events indicated by solid lines, and which may, at high fluences, stop the normal events for a short time.

to growth delay are quite sufficient to temporarily but completely inhibit net RNA synthesis through the induction of $^{4}$Srd-Cyd adducts in tRNA.

**Summary of Proposed Mechanism.** The events in near-UV-induced inhibition of growth of the stringent strains can be explained as in Fig. 6. Near-UV irradiation causes a fluence-dependent decrease in the level of amino-acid-charged tRNAs, which in turn decreases the rate of protein synthesis. The presence of uncharged tRNAs in the cell then leads to an increase in the level of ppGpp and a drastic decrease in the rate of stable RNA accumulation, with consequent growth delay. Similar events are induced by amino-acid starvation.

To resume normal growth, the cell might either repair its damaged tRNAs or dilute the damage through synthesis of new tRNA. The rate of new tRNA accumulation is expected to be a function of the rate of stable RNA accumulation (21), which in turn is controlled by the availability of acylated tRNA to the ribosome. This creates the positive feedback situation illustrated in Fig. 6. Thus, at all times during the recovery period, the rate of new tRNA synthesis would be controlled by the level of charged tRNAs, and the growth rate would approach that of unirradiated cells through a regenerative process.

The relaxed strain NC52 *rel*$^{-}$ used in these experiments has approximately the same amount of $^{4}$Srd in its tRNA as the stringent strain (T. V. Ramabhadran and T. Fossum, unpublished observations) and hence must have the same degree of near-UV damage in the tRNA. However, due to the mutation in the *rel* gene, ppGpp levels do not change after near-UV irradiation, and the rate of production of new tRNA is independent of the state of protein synthesis (29). This leads to a faster dilution of damaged tRNA and hence a faster recovery.

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