The effects of a series of alcohols on the stringent response system of *Escherichia coli* were studied. The alcohols used could be divided into two groups on the basis of the response of ppGpp and ppGpp to the growth downshift induced by the alcohols. The cells responded to the alcohols, methanol, ethanol, and propanol, as if they were being starved of amino acids. In the stringent strain CP78 these alcohols induced ppGpp and ppGpp accumulation and curtailed RNA synthesis, whereas in the relaxed strain CP79, both of these responses were absent. It was determined that this response was most likely due to an interference by these alcohols with the uptake of amino acids required by these strains.

By contrast both stringent and relaxed cells elevated their level of ppGpp and decreased RNA accumulation when treated with butanol or pentanol. This response is similar to the effect of carbon source limitation. It was determined that the elevation of ppGpp in the stringent strain was primarily the result of increased ppGpp synthesis in response to these alcohols. In the relaxed strain the rise in ppGpp was dependent on a decrease in ppGpp degradation coupled with a moderate increase in ppGpp synthesis. This stimulation of ppGpp synthesis in relaxed cells, although small, suggests the existence of an enzyme distinct from stringent factor which is capable of synthesizing ppGpp. Data are presented which suggest that the activity of this enzyme is coupled to the potential for protein synthesis and energy availability of the cell, perhaps being regulated by the overall ratio of uncharged to aminoacylated tRNA.

The accumulation of RNA in *Escherichia coli* is closely coupled to the growth rate of the culture. Under conditions of stress, such as amino acid limitation or carbon source downshift, the ability of *E. coli* to synthesize RNA, particularly rRNA, is rapidly curtailed. For a recent review see Nierlich (1978). The decrease in RNA accumulation under these conditions generally correlates with the appearance of guanosine 5'-diphosphate-3'-diphosphate in the acid-soluble nucleotide pool (Cassel and Gallant, 1969; Harshman and Yamazaki, 1971; St. John and Goldberg, 1978). The kinetics of the accumulation of ppGpp\(^1\) and its ability in vitro to modulate many cellular processes, including rRNA synthesis (van Ooyen et al., 1976), suggest that ppGpp regulates the "stringent response" to amino acid starvation.

Amino acid starvation of stringent cells leads to the synthesis of ppGpp by a ribosome-bound "stringent factor" on response to a codon-specific uncharged tRNA in the ribosomal A site (Haseltine et al., 1972). Cells harboring the "relaxed" mutation are deficient in this synthetic ability. These relaxed strains, however, do maintain a somewhat reduced basal level of ppGpp (Pao and Gallant, 1978) and like their stringent counterparts accumulate substantial quantities of the compound during carbon source downshifts (Harshman and Yamazaki, 1971). It has been suggested that this accumulation is due to a slowed ppGpp degradation rate (Friesen et al., 1975). The mechanism of its production under these conditions, however, remains obscure.

Relaxed cells also elevate their ppGpp level when treated with levallophan (Boquet et al., 1973) or subjected to hypertonic shock (Harshman and Yamazaki, 1972). This response may reflect some alteration of the cellular membrane. Ingram (1976) has shown that the addition of small amounts of alcohols to growing cultures of *E. coli* results in slower growth which is accompanied by an alteration in the fatty acid composition of the membrane. Further, ppGpp has been shown to be involved in the regulation of the synthesis of saturated fatty acids (Polakos et al., 1973) and thus may be involved in the adaptation of the membrane to the fluidity changes induced by the alcohols. This suggests that treatment with alcohols, like exposure to hypertonic shock, might alter the cellular level of ppGpp and thus be useful probes in the study of its metabolism.

In this communication we examine the response of growing cultures to downshifts caused by the addition of low levels of alcohols. The results indicate that an elevation in the ppGpp pool that occurs in both stringent and relaxed cells in response to a particular stimulus may depend upon different mechanisms. Further, they suggest the existence of a second enzyme for ppGpp synthesis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—The strains employed were CP78, an *E. coli* K12 strain, genotype *leu*, *arg*, *thr*, *his*, *thi*\(^{−}\), *relA*\(^{−}\), *spoT*\(^{−}\), *relx*\(^{−}\) and its isogenic relaxed (relA\(^{+}\)) partner, CP79 (Fiiil and Friesen, 1968). The growth medium was based on that used by Harshman and Yamazaki (1971) and was composed of a basal salts solution containing, per liter, 6.05 g of Tris, 5.80 g of maleic acid, 2.5 g of NaCl, 2.0 g of KCl, 1.0 g of NH4Cl, 1.015 g of MgCl\(_2\)-6H\(_2\)O, 0.142 g of Na\(_2\)SO\(_4\), and NaH\(_2\)PO\(_4\) to yield a final concentration of 0.3 m. The abbreviations used are: ppGpp, guanosine 5'-diphosphate-3'-diphosphate; ppGpp, guanosine 5'-triphosphate-3'-diphosphate; MS I, Magic Spot compound I, ppGpp; MS II, Magic Spot compound II, ppGpp; MS, Magic Spot compounds I and II.

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mm. The required amino acids were provided at 50 µg/ml each, thiamine at 10 µg/ml, and glucose at 2 mg/ml. Cultures were incubated in tubes (16 × 125 mm) at 37°C using a New Brunswick Rollodrum to enhance aeration. Growth was monitored by measuring the absorbance at 500 nm (A500) using a Beckman DU Gilford modified spectrophotometer.

Growth was carried out by the addition of a perturbing agent. Amino acid starvation (isoleucine) was induced by addition of valine to a final concentration of 500 µg/ml. Carbon source limitation was imposed by addition of α-methyl glucoside at 20 times the glucose concentration to inhibit glucose uptake. Alcohols were added at concentrations which decreased the growth rate by 30 to 50%; methanol 6%, ethanol 4%, propanol 1.6%, butanol 1%, and pentanol (any) 0.2%. These are the same concentrations employed by Ingram (1976).

Radioactive Labeling—Determination of nucleotide levels and RNA was carried out by monitoring the incorporation of H3-P04 into these substances. Routinely, carrier-free H3-P04 was added to a 2- to 4-ml portion of the culture, shortly after inoculation with bacteria, to yield a final specific radioactivity of 100 Ci/mM, 333 µCi/mM. When low levels of ppGpp were to be analyzed, in the degradation rate experiments, the amount of label was raised to 400 µCi/ml.

Nucleotide Pool Measurements—The procedure used was based on the technique of Caspel et al. (1969). Fifty-microliter aliquots of the 32P04-labeled culture were pipetted into 50 µl of ice-cold 2 M formic acid, and immediately frozen in a bath of dry ice in ethanol. Samples were stored frozen at −20°C for no longer than 24 h. Prior to spotting, the polyethylene-imregnated cellulose thin layer sheets (Brinkmann) were soaked for 20 min in 10% trichloroacetic acid, and then washed three times in distilled water and dried. Two microliters of nucleotide were applied to the plates, which were developed in the direction of migration using n-propyl alcohol 6%, ethanol 4%, propanol 5%, butanol 2%, and pentanol 1%.

Radioactivity in each of the spots was measured using a Nuclear Chicago model 45 counter and expressed as disintegrations per minute.

RESULTS

Alcohol-induced Downshifts—When low concentrations of alcohols were added to rapidly growing cultures of E. coli, a decrease in the growth rate was observed in both stringent and relaxed cells. The cellular response in growth, RNA synthesis, and ppGpp accumulation to a series of straight chain alcohols, methanol through amyl alcohol, is presented in Table 1. Amino acid starvation (isoleucine) was induced by addition of valine (500 µg/ml) or CP 79 (relA-). B) was subjected to carbon source limitation by the addition of a-methyl glucoside (αMG) (20 times glucose concentration). At an appropriate time thereafter (second arrow) the stressed culture was split and one portion subjected to alcohol treatment as indicated. Nucleotides were assayed as described under "Experimental Procedures." A) ATP; B) GTP; C) ppGpp; D) ppGpp.

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ular weight alcohols produce a condition similar to an amino acid limitation.

Butanol, a group II alcohol, appeared to add its effect to that of a carbon source limitation (Fig. 6D) in the relaxed cells but caused a complex response in the amino acid-limited stringent cells (Fig. 6C): ppGpp rose while pppGpp fell. This latter effect may have resulted from an alteration in the rate of interconversion of pppGpp and ppGpp or from a feedback inhibition of further ppGpp synthesis by the high level of ppGpp obtained under these conditions.

Although the response of the basal level of ppGpp to the addition of a group I alcohol (Figs. 1 to 3) was somewhat difficult to assay, the experiments represented in Fig. 6 demonstrated that these alcohols moderately depressed ppGpp accumulation in relaxed cells. The α-methyl glucoside prestressed condition provided an opportunity to examine the coupling of the rate of RNA accumulation to the ppGpp level when that level was abruptly altered by the addition of alcohol. Fig. 7 demonstrates that RNA accumulation ceased when ppGpp was substantially increased by butanol. The
addition of ethanol to the α-methyl glucoside-limited culture returned ppGpp to its original level and, although the rate of RNA accumulation recovered somewhat, it did not increase by as much as might be predicted by the fall in ppGpp.

Reversal of the Effect of Alcohols on ppGpp—Since the strains employed in this study require exogenous amino acids for growth, the low molecular weight alcohols might mimic amino acid starvation by interfering with the normal uptake of amino acids from the medium. An experiment designed to test this hypothesis is represented in Fig. 8. A culture was provided with three times the normal concentration of the required amino acids and then stressed by the addition of ethanol or butanol. The amino acids substantially lowered the response of ppGpp to ethanol (Fig. 8A). However, the ppGpp response to butanol was unaffected by the increase in amino acids (Fig. 8B). This experiment suggested that the response of the cells to group I alcohols was, in part, dependent upon a decline in amino acid availability. However, it provided little information on the mechanism by which group II alcohols influence ppGpp.

Since methanol has been shown to promote ppGpp synthesis by stringent factor (Sy et al., 1973), it was conceivable that the more hydrophobic solvent, butanol, might also activate the enzyme, perhaps sufficiently to allow some ppGpp synthesis from the mutant stringent factor in relaxed cells. However, butanol at 1% v/v proved to be inhibitory to the synthesis of ppGpp by stringent ribosomes assayed by the method of Haseltine et al. (1972) (data not shown). Further, amino acid starvation of relaxed cells in the presence of butanol failed to induce an increase in ppGpp, but rather resulted in a rapid decrease (Fig. 9A). Similar pretreatment of stringent cells with butanol did not impair their ability to react to amino acid starvation (Fig. 9B). The results in Fig. 9 also suggested that the ability of relaxed cells to accumulate ppGpp was related in some way to the capacity for protein synthesis, since both amino acid starvation and chloramphenicol treatment antagonized the butanol-induced increase in ppGpp.

Kinetic Response to Butanol—It has been suggested that alterations in the level of ppGpp in relaxed cells are controlled by changes in its degradation rate and that this is related to the ATP content of the cells (DeBoer et al., 1976). In the present study no consistent relationship between the level of ATP and ppGpp was discerned in cells treated with alcohols.

In some experiments a slow decline in ATP was observed, while in others, the rise in ppGpp occurred concomitant with a rise in ATP. However, the decay of ppGpp in butanol-treated relaxed cells in the presence of chloramphenicol (Fig. 9A) was somewhat slower than had been anticipated. Thus, both stringent and relaxed strains were assayed for the effect of butanol on the rate of ppGpp degradation.

Fig. 10 shows the disappearance of ppGpp in treated and untreated cultures after further synthesis was halted by the addition of chlorotetracycline, and Table I shows the kinetic parameters calculated from such data. Although butanol exerted only a moderate effect on ppGpp degradation in stringent cells, it significantly depressed the degradation of ppGpp in relaxed cells. At early times after butanol addition, however (Fig. 10C), the decreased rate of degradation seen in relaxed cells did not appear to completely account for the rise in the ppGpp pool observed.
This suggested the existence of a variable ppGpp synthetic activity in relaxed cells. In an attempt to examine the components of relaxed cells involved in the response of ppGpp to butanol, the in vitro temperature sensitivity of the enzyme(s) involved in the ppGpp response to isoleucine limitation and butanol challenge was examined. If these responses depend upon different enzymatic activities, different temperature sensitivities might be expected. As demonstrated in Table II, in CP 78, stringent factor activity, as assayed by the response to isoleucine limitation, was maintained for at least 20 min after the shift from 37 to 45°C. Likewise, some response to butanol was maintained. However, CP 79 lost all responsiveness to butanol at 45°C. The strains also differed in the effect of temperature on the basal level of ppGpp. The level of ppGpp was elevated in relaxed CP 79. The relaxed cells appeared to have lost the ability to produce ppGpp at the high temperature.

**DISCUSSION**

During alcohol-induced downshifts the change in the rate of RNA accumulation was coupled to the change in the growth rate. In general, this decrease was inversely related to the pool of ppGpp observed under these conditions. It appears that the treated cultures were experiencing a condition similar to a nutrient limitation of the growth rate rather than a strict starvation. Thus, it is not surprising that some of the responses were less dramatic than seen upon complete withdrawal of a required nutrient. However, a partial uncoupling of RNA accumulation and ppGpp level was observed in the experiment presented in Fig. 7. This suggests that the alcohols affect RNA synthesis through more than one mechanism.

The response of ppGpp leads to ppGpp metabolism to the presence of low concentrations of alcohols in the culture medium was complex. The pattern of response was dependent not only on the genetic character of the relA locus but also on the molecular size of alcohol employed. Although there were differences in the degree of response, which may suggest a progressive alteration in the mode of action, the alcohols employed could be operationally divided into two groups, with the transition between propanol and butanol. The lower molecular weight alcohols affected the cultures in a manner that the cells perceived as roughly equivalent to an amino acid limitation. The response of ppGpp to butanol and amyl alcohol, however, was similar to that observed when the cells are subjected to a limitation for carbon source.

Because the alcohols are slightly hydrophobic it might be expected that an important aspect of their action on the cells would be an alteration in the cell membrane. In fact, Ingram (1976) has shown that straight chain alcohols alter the fatty acid composition of the membrane. In this respect, too, the smaller alcohols act differently from the larger ones. Such changes in membrane composition may alter nutrient uptake or cause leakage of ppGpp through alcohol-damaged cell membranes. This latter possibility was eliminated, at least in the case of butanol, by testing the extracellular fluid for the presence of ppGpp after alcohol treatment.

Alcohol-induced changes in nutrient uptake appeared to influence ppGpp metabolism. The smaller three alcohols in particular inhibited the uptake of the required amino acids and thus elevated ppGpp in stringent cells. These alcohols may also influence the synthesis of ppGpp in a more direct fashion. For instance, Gorini et al. (1967) have shown that methanol and ethanol, in particular, alter the structure of the ribosome making misreading more common. Perhaps such structural alterations affect the stringent factor. Further, Sy et al. (1973) have demonstrated that methanol can directly stimulate stringent factor activity in vitro.

Although increasing the extracellular content of the required amino acids failed to inhibit the butanol-induced increase in ppGpp, it seems likely that larger alcohols also influence nutrient uptake and metabolism. Indirect evidence for an effect upon amino acid availability is the fact that the increase in ppGpp in stringent cells was greater than that in relaxed cells exposed to these alcohols (compare panels C and F of Figs. 4 and 5). This is apparently due to a substantial stimulation of ppGpp synthesis in the stringent cells (Table I). We have also observed that the extent of ppGpp accumulation induced by these alcohols was moderated slightly by glucose, suggesting that the more hydrophobic alcohols have generalized metabolic effects.

The unexpectedly strong ppGpp response of relaxed cells to butanol and amyl alcohols provides a unique opportunity to examine the factors involved in ppGpp production in these cells. Consistent with the work of other investigators (Gallant et al., 1972; Friesen et al., 1975) our kinetic analysis of the response to butanol leads to the conclusion that the major controlling element is the rate of ppGpp degradation (Table I, Fig. 10). However, under the conditions employed in this study, there does appear to be an elevation of the rate of ppGpp synthesis at early times after alcohol perturbation (Table I). Since CP 79 harbors a defective stringent factor, this result argues for the existence of an auxiliary ppGpp synthetic mechanism in relaxed cells. Recently, elegant work
by Friesen et al. (1978) on the construction of viable relaxed strains whose stringent factor harbors a nonsense mutation point. In contrast, these results demonstrated that in relaxed cells, unlike stringent cells, the failure of relaxed cells to respond to butanol at high temperature suggests that either an enzyme directly involved in ppGpp production or the mechanism by which butanol gives rise to a stimulation of this activity are more sensitive to temperature than the stringent factor system.

Relaxed cell production of ppGpp is also sensitive to the state of the protein synthetic machinery. In this study (Figs. 9 and 10) and other studies (Gallant et al., 1972) protein synthetic inhibitors have been shown to be potent antagonists of the accumulation of ppGpp in relaxed cells. Further, Fig. 9 demonstrated that in relaxed cells, unlike stringent cells, inhibition of protein synthesis by starvation for a single amino acid antagonized ppGpp accumulation. In contrast, Friesen et al. (1975) reported that in growth upshift experiments, the addition of amino acids to cultures of relaxed cells resulted in an abrupt decline in ppGpp. We have observed a similar inhibition by amino acids of \( ^{32}P \) incorporation into ppGpp by relaxed cells permeabilized according to the method of Raue and Cashel (1975) (not shown). The presence of glucose in the medium during these tests makes it unlikely that the reported results are due simply to the utilization of the added amino acids as a carbon source. The extent and rapidity of the decline in ppGpp observed argues for a substantial, if temporary, decline in the synthesis of ppGpp by these cells.

These results can be explained if one of the conditions which influences the production of ppGpp in relaxed cells is the overall level of tRNA charging. The signal for an increase in the basal synthetic rate of ppGpp would be a decrease in the overall ratio of charged to uncharged tRNA. Unlike the stringent factor system which appears to act primarily as a trigger for ppGpp production when one amino acid becomes sufficiently limiting, this system would undergo mild fluctuations in activity in response to general shifts in amino acid availability and, to an extent, in the availability of energy for the charging reaction. Further, the synthesis would be antagonized by any condition which produced a temporary excess of charged tRNA such as the abrupt inhibition of protein synthesis by chloramphenicol or during an upshift induced by the addition of amino acids.

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ppGpp Metabolism in *E. coli*
Figure 1-5 Effects of various alcohols on growth, RNA accumulation, pygDp and pygDp levels of a stringent and relaxed isogenic pair of E. coli strains.

A culture of CP 78 or CP 79 was split into two equal portions. One portion was added to one portion to a final optical density of 0.22. A second was utilized for the determination of growth (Fig. 1). At the time designated by the arrow (zero time), the indicated alcohol was added to equal aliquots of both labelled and unlabelled cultures. The final alcohol concentration used were ethanol, 2%, ethanol, 2%, propional, 1.5%, butanol, 1.5% and pentanol, 1.5%. The cultures were incubated for 1 hour after the addition of the alcohol and then labeled for 1 hour. The determination of growth (panel B, D), RNA accumulation levels (panel E) and pygDp and pygDp levels (panel C, F) were measured as indicated in Materials and Methods. Panel C represents the response of stringent cells, CP 78, and those at the right (C, D, F) the response of the relaxed cell, CP 79. Note the difference in the scale used to express the optig spot level for stringent and relaxed cells: panel A, panel B, panel C, panel D, panel E, panel F.