Stringent Control of Fatty Acid Synthesis in *Escherichia coli*

POSSIBLE REGULATION OF ACETYL COENZYME A CARBOXYLASE BY ppGpp*

(Received for publication, July 5, 1973)

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SUMMARY

The effect of amino acid starvation on the rate of fatty acid synthesis was examined in stringent (CP 78, rel+) and relaxed (CP 79, rel−) isogenic strains of *Escherichia coli* (leu*, his*, arg*, thr*, thi*). Rates of incorporation of [U-14C]glucose, [1-14C]acetate, and H2O into chloroform-methanol-soluble lipids (>95% phospholipid) were instantly reduced 50 to 60% by leucine starvation of stringent, but not relaxed, cells. That this depressed rate of lipid labeling was not due to leucine starvation-induced diversion of labeled fatty acyls into chloroform-methanol-insoluble form (e.g. lipopolysaccharide, etc.) is indicated by the fact that the rate of labeling from [1-14C]acetate of the lipid extract of saponified cells was decreased to the same extent by leucine deprivation. With leucine-deprived rel− cells, the rate of [1-14C]acetate incorporation into phosphatidylethanolamine and cardiolipin was more drastically curtailed than into phosphatidylglycerol, while the incorporation pattern in rel− cells (± leucine) was similar to that of nonstarved rel+ cells. An elevated turnover rate of fatty acyl groups due to amino acid starvation cannot account for the decreased rate of lipid labeling by various precursors, since no detectable loss of 14C-activity from fatty acyls labeled during growth or leucine deprivation occurs during subsequent growth or leucine starvation in the presence of unlabelled precursor. Only minor amounts of labeled lipid are secreted by stringent or relaxed cells grown in the presence of [1-14C]acetate; leucine deficiency has no significant effect on the rate of labeling of these extracellular lipids. These results lead to the conclusion that fatty acid synthesis per se is subject to stringent control being partially suppressed, i.e. 50 to 60%, by amino acid starvation in rel+ strains of *E. coli*.

Amino acid starvation of stringent, but not relaxed, strains of *E. coli* is known to activate ribosomal synthesis of ppGpp and pppGpp. The resultant accumulation of (pp)ppGpp appears to mediate stringent control of fatty acid synthesis by blocking the initial committed step of the pathway, i.e. the carboxylation of acetyl-CoA. Of the two catalytic components of the acetyl-CoA carboxylase system, namely biotin carboxylase and carboxyltransferase, only the latter component is inhibited by physiological concentrations (up to 4 mM) of ppGpp. Two carboxyltransferase-catalyzed reactions—(a) carboxyl transfer from malonyl-CoA to the model acceptor, d-biotin methyl ester, and (b) carboxyl carrier protein-dependent malonyl-CoA-[1-14C]acetyl-CoA exchange—are inhibited 50 to 60% at saturating concentrations (1.0 to 1.2 mM) of (pp)ppGpp. Inhibition is specific in that GTP, GDP, GMP, ATP, ADP, AMP, and cyclic adenosine 3':5'-monophosphate have no effect at comparable concentrations.

The binding of Mn2+ by ppGpp was investigated by electron paramagnetic resonance to evaluate the possible metal scavenging effect of the guanosine nucleotide on the metal-dependent reactions (i.e. biotin and acetyl-CoA carboxylation) catalyzed by the biotin carboxylase component of acetyl-CoA carboxylase. Kd values determined at pH 6.8 for the dissociation of MnppGpp from the binary (Mn∗ppGpp) and ternary (Mn∗ppGpp) complexes are 1.1 μM and 65 μM, respectively, the former being tighter and the latter an order of magnitude weaker than MnppGpp dissociation from Mn-ATP. Whereas the biotin carboxylase-catalyzed reaction per se (carboxylation of free d-biotin) is not inhibited, 2.5 mM Mn-ppGpp inhibits both acetyl-CoA carboxylation and malonyl-CoA-[1-14C]acetyl-CoA exchange 50 to 60%. These results suggest that stringent control of fatty acid synthesis in *E. coli* is mediated through the inhibitory action of (pp)ppGpp on the carboxyltransferase component of the acetyl-CoA carboxylase system.

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A number of reports (1, 2) have implicated the product of the relaxed† (rel) locus in the control of lipid synthesis in *Escherichia coli*. Evidence supporting this view comes solely from comparisons of the rates of incorporation of [14C]acetate or 32Pi into the chloroform-methanol-extractable lipids of rel+ and rel− strains during growth and amino acid starvation. Since the turnover of the fatty acyl moieties of these lipids was not investigated, it was not established whether fatty acid synthesis per se is affected by amino acid deprivation. Moreover, the possibility...

† The rel gene of *E. coli*, sometimes referred to as the RC locus, is responsible for the stringent response, i.e. the curtailment of accumulation of stable RNA species upon starvation for a required amino acid (3).
that stringent control is exerted at the level of acetate transport and activation, rather than at the level of fatty acid synthesis, has not been excluded. Although an effect of the stringent control mechanism on acetate transport has not been established, various other transport processes are inhibited (4–8). However, it is known that acetate transport is affected by other factors, e.g., the presence or absence of glucose in the medium (9). In addition, it has been our experience that the rate of \(^{14}C\)acetate incorporation into the lipids of \(E.\ coli\), in the presence of glucose as the major carbon source, accounts for <15% of the total rate of lipid synthesis, presumably due to the dilution of the \(^{14}C\)acetate-CoA pool by unlabeled precursors or rate-limiting acetate transport. Furthermore, it has been reported that the reduction in the rate of \(^{14}C\)acetate incorporation into the lipids of a stringent (rel\(^{-}\)) strain during amino acid starvation is dependent upon the degree of aeration of the culture (2, 10, 11). For these reasons, the relationship of the stringent control mechanism to the regulation of fatty acid synthesis in \(E.\ coli\) was re-examined. Beyond this, we have found that guanosine 5'-diphosphate-3'-diphosphate and guanosine 5'-triphosphate-3'-diphosphate, which appear to mediate the stringent response (12–14), specifically inhibit acetyl-CoA carboxylase at physiological guanosine nucleotide concentrations. In \(E.\ coli\) the acetyl-CoA carboxylase system is composed of three easily resolved protein components, biotin carboxylase which catalyzes the second half-reaction (Reaction 2), and carboxyl carrier protein which contains the covalently bound biotin prosthetic group (15–19).

\[
\text{ATP} + \text{HCO}_3^- + \text{CCP-biotin}^2 \xrightleftharpoons{\text{BC}} \text{Me}^{+}
\]
\[
-\text{CCP-biotin-CO}_2^- + \text{ADP} + \text{P}_1
\]
\[
\text{CCP-biotin-CO}_2^- + \text{acetyl-CoA} \xrightleftharpoons{\text{CT}} \text{malonyl-CoA} + \text{CCP-biotin}
\]

Net: \[
\text{ATP} + \text{HCO}_3^- + \text{acetyl-CoA} \xrightarrow{\text{BC, CT, CCP-biotin}} \text{Me}^{+}
\]
\[
-\text{malonyl-CoA} + \text{ADP} + \text{P}_1
\]

This investigation shows that acetyl-CoA carboxylation is inhibited by physiological concentrations of (p)pppGpp\(^3\) and that the site of action of the nucleotides is the carboxyltransferase reaction. A preliminary report of this work has been presented (20).

**EXPERIMENTAL PROCEDURE**

*Reagents-Enzymes, nucleotides, and other cofactors were purchased from Boehringer, P-L Biochemicals, and Sigma, and radioactive materials were from New England Nuclear. All other reagents were the best grade available. ppGpp and pppGpp were generously provided by Dr. M. Cashel (National Institute of Child Health and Human Development, National Institutes of Health) and Dr. R. Byrne (Johns Hopkins Uni-

\(^3\) The abbreviations used are: ppGpp, guanosine 5'-diphosphate-3'-diphosphate; pppGpp, guanosine 5'-triphosphate-3'-diphosphate; 5'-phosphate substitutions are written to the left of the nucleoside, and 3'-phosphate substitutions to the right as suggested by Cashel et al. (12). (pppGpp, ppGpp and pppGpp collectively; BC, CT, and CCP, the biotin carboxylase, carboxyltransferase, and carboxyl carrier protein components, respectively, of the \(E.\ coli\) acetyl-CoA carboxylase system.

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Determination of Total Fatty Acyl Groups Excreted into Medium. The cell filtrates were concentrated to dryness in a rotary evaporator; when [1-14C]acetate was present, the residue was re-suspended in 5 ml of 0.1 M acetic acid and taken to dryness several times. The residue was re-suspended in 2 ml of 2.3 M alcoholic KOH (ethanol-water, 1:1, v/v), transferred into a conical tube and the flask was washed twice with 2 ml of alcoholic KOH. The residue and washes were hydrolyzed, and fatty acids were extracted with petroleum ether and counted as described above.

Determination of Turnover of Labeled Fatty Acyl Groups during Growth or Leucine Deprivation. To cells entering log phase growth was added 0.017 mM [1-14C]acetate (15 μCi per μmole). After growing to an optical density of 1.0, the labeled cells were harvested by filtration and washed repeatedly on the filter with the appropriate medium containing unlabeled acetate. The washed cells were re-suspended to an optical density of 1.0 in unlabeled complete or leucine-deficient media and the fate of the labeled fatty acyl groups followed during a subsequent 1-hour incubation at 37° under vigorous aeration. Aliquots of cells withdrawn at various time intervals were worked up as before, and radioactivity in the chloroform-methanol extract of cells, in the total fatty acyls of cells, and in the total fatty acyls in material excreted into the growth medium was determined as described above.

To label cells during leucine starvation, growth was allowed to proceed to an optical density of 1.0 unit on complete medium. After harvesting and washing with medium devoid of leucine, the cells were re-suspended in the same medium to an optical density of 0.5 unit. [1-14C]Acetate was added (0.075 mM, 15 μCi per μmole), and the cells were incubated for 1 hour at 37° with constant aeration. During this period there was no demonstrable decrease in viable cell count. After labeling under conditions of leucine starvation, the cells were harvested by filtration, washed with medium devoid of leucine but containing unlabeled acetate, and re-suspended in unlabeled complete or leucine-deficient media to an optical density of 1.0 unit. The fate of fatty acids labeled in the starvation period was followed during the subsequent growth or leucine starvation as described above.

Distribution of Labeled Fatty Acyl Residues in Chloroform-Methanol-extractable Lipids. Cells washed with the appropriate medium were re-suspended to an optical density of 1.0 in either complete medium or medium lacking leucine. [1-14C]Acetate was added (0.141 mM, 1.6 μCi per ml) and the cells incubated with constant aeration at 37° for 30 min before extraction with chloroform-methanol. The extracts were brought to dryness under reduced pressure, redissolved in chloroform, and chromatographed on Whatman silica gel loaded paper SG-81. For two-dimensional chromatography, the first solvent system was petroleum ether-diethyl ether-acetic acid (90:10:1, v/v/v), and the second solvent was chloroform-methanol-acetic acid (65:25:8, v/v/v) (25). The latter system was also used for one-dimensional chromatography. Lipid spots were identified by autoradiography using appropriate standards. The chromatograms were cut into strips, counted, histograms constructed, and total radioactivity in each lipid class calculated.

Measurement of Mn2+ Binding by ppGpp. Measurements were performed at 30° in 48 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 6.8, by electron paramagnetic resonance spectroscopy at 910 MHz essentially according to Cohn and Townsend (26). The volume of the sample was 0.05 ml, and the temperature was controlled to within ±0.5°.

Manganese ion was titrated with ppGpp, and free manganese was determined at all six peaks of the Mn2+ spectrum by comparing the height of each peak to the height of Mn2+ standards. For each experiment the average free Mn2+ concentration was calculated from the values of free Mn2+ obtained at each absorption maximum, and average values were used to determine the dissociation constants of the ppGpp complexes with Mn2+.

Results were fitted by the least squares method.

Enzyme Preparations and Assays. Biotin carboxylase, carboxy transferase, and carboxyl carrier protein were purified from threelfourths log phase E. coli B cells grown on enriched medium purchased from Grain Processing Corp. (Muscatine, Iowa). Biotin carboxylase and carboxytransferase were homogeneous preparations, and the carboxyl carrier protein was estimated to be >50% pure. All enzyme activities were determined at 30°. Biotin carboxylase was assayed radiochemically in a total volume of 0.05 ml as described by Dimroth et al. (15); however, 1 mM MnCl2 was used instead of 8 mM MgCl2. The specific activity of KH2CO3 was 20 x 106 cpm per μmole and the reaction was initiated by addition of ATP. The progress of the reaction followed at 1, 2, 3, and 4 min of incubation was linear.

Carboxytransferase activity was determined either spectrophotometrically in a total volume of 0.2 ml or radiochemically in 0.1 ml, pH 8.0, using biotin methyl ester as model acceptor substrate as described by Guchhait et al. (17, 27), but with the following modifications. The concentration of buffer was reduced to 20 mM, and the coupling enzymes employed in the spectrophotometric assay were freed of ammonium sulfate by dialysis. The progress of the reaction in the radiochemical assay was followed kinetically at 1, 2, 3, and 4 min. The reaction was initiated by addition of a mixture of substrates.

Biotinyl-CoA-[1-14C]acetyl-CoA exchange activity was measured at pH 6.5 in a volume of 0.05 ml (97)3. The specific activity of the [1-14C]acetyl-CoA employed was 5 x 106 cpm per μmole. The reaction initiated with a mixture of malonyl-CoA and [1-14C]acetyl-CoA was followed kinetically at 2, 4, 6, and 8 min of incubation.

Acetyl-CoA carboxylase activity was determined at pH 6.8 to 6.9 in a final volume of 0.05 ml containing: imidazole (pH 7) buffer, 50 mM; bovine serum albumin, 0.03 mg; GSH, 3 mM; KH2CO3, 10 mM (10 to 30 x 106 cpm per μmole); MnCl2, 1 mM; ATP, 1 mM; and enzyme (25 to 42 μg dialyzed ammonium sulfate fraction (28)), 60 to 100 μg. The reaction initiated by the addition of ATP was followed kinetically at 1, 2, 3, and 4 min.

Results

Effect of Leucine Starvation on Rates of Incorporation of [1-14C]-Acetate, [U-14C]Glucose, and H2O into Lipids of E. coli. To ascertain whether the apparent inhibitory effect of amino acid starvation on the labeling of lipids by [14C]acetate is exerted on fatty acid synthesis per se or upon acetate transport and activation, or both, the rate of [14C]acetate incorporation into the lipids of rel+ cells (CP 78) during leucine starvation was compared with the rates of incorporation of [U-14C]glucose and H2O. When [14C]glucose is used both as labeled precursor and sole carbon source, dilution of the [14C]acetyl-CoA pool by unlabeled precursors is precluded; consequently, the rate of lipid labeling from [14C]glucose should more closely approximate the in vivo rate than from [14C]acetate. Moreover, the use of H2O (29-31)

1 R. B. Guchhait, S. E. Polakis, J. Moss, P. Dimroth, and M. D. Lane, manuscript in preparation.
as precursor of fatty acids is not subject to the same limitations as [14C]acetate or glucose and probably provides the best estimate of the rate of fatty acid synthesis, since incorporation of tritium from H2O is independent of the source of acetyl-CoA and unaffected by transport processes. It was found (Fig. 1) that the reduced rate of incorporation of all three radioactive precursors into chloroform-methanol-extractable lipids of stringent cells caused by leucine deprivation is independent of the nature of the precursor. Essentially identical results were obtained using two concentrations of [1-14C]acetate, [U-14C]glucose, or H2O. In all instances a 50 to 60% reduction in rate of lipid labeling was observed when results were normalized to counts per min incorporated per optical density unit of cells. Normalization was necessary to account for the fact that cells in complete medium were growing logarithmically while the leucine-starved cells were not. During the course of the 60-min incubation in complete medium and leucine-deficient medium optical density at 450 nm increased approximately 100% and <10%, respectively. As anticipated (Figs. 2 and 3B), the rate of incorporation of H2O and [1-14C]acetate into the lipid fraction of relaxed cells (CP 79, rel-) either in complete medium or in the absence of a required amino acid were essentially identical. These results suggest that the effect of stringent control on the rate of incorporation of [14C]acetate into lipids is not exerted at the level of acetate transport or activation. Hence, [14C]acetate incorporation into the lipid fraction of cells is a valid measure of the stringent control of lipid labeling. However, before it can be concluded that this control mechanism operates at the level of fatty acid synthesis per se, the possibility that amino acid starvation affects fatty acyl turnover or excretion, or both, had to be excluded.

**Effect of Leucine Starvation on Rate of [1-14C]Acetate Incorporation into Total Cellular and Extracellular Fatty Acyl Groups**—It is well documented that E. coli cells excrete into the medium a lipopolysaccharide which may originate from the outer cell membrane (32-38). While the amount excreted into the medium is usually small in relation to total cellular fatty acids, it is

![Fig. 1](image1.png)  
**Fig. 1.** Incorporation of labeled precursors into the chloroform-methanol-soluble lipids of Escherichia coli (CP 78 Tel+). For details see "Experimental Procedure." ♦, complete medium; ○, medium minus leucine.

![Fig. 2](image2.png)  
**Fig. 2.** Incorporation of label from H2O into the chloroform-methanol-soluble lipids of Escherichia coli (CP 79 rel-). For details see "Experimental Procedure." ♦, complete medium; ○, medium minus leucine.

![Fig. 3](image3.png)  
**Fig. 3.** Effect of leucine starvation on the rate of lipid synthesis in stringent (CP 78 rel) and relaxed (CP 79 rel-) strains of *Escherichia coli*. For details see "Experimental Procedure." A, total fatty acyl residues in cells determined after saponification at 90° for 6 hours with 2.3 N alcoholic KOH. B, chloroform-methanol-soluble lipids of cells. C, extracellular fatty acyl residues determined after saponification (as in A) of the residue of the medium after removal of the cells. ♦, complete medium; ○, medium minus leucine. Note the difference in the ordinate scales of A or B and C.
A fraction of the fatty acyl groups synthesized by the cell is in the stringent strain (Tel+) of E. coli. For details see "Experimental Procedure." Cells labeled with [1-14C]acetate during growth (Panel A) or during leucine starvation (Panel B) were harvested and washed to remove the labeled substrate and then transferred to the incubation medium (complete or minus leucine) and the radioactivity per ml of medium was determined at different times during incubation. O, total fatty acyl residues determined as free fatty acids after saponification of the cells; □, chloroform-methanol-soluble lipids of cells; △, extracellular fatty acyl residues determined as free fatty acids after saponification of the medium.

While E. coli possesses an inducible system for β oxidation of fatty acids (39, 40), this pathway is strongly repressed by glucose (39), a component of the medium employed here; hence, this degradative system would not be expressed and, therefore, could not account for the reduced rate of labeling of fatty acyl groups observed during amino acid starvation. Moreover, Crowfoot et al. (41) have described a mutant of E. coli (fao-6) which is β oxidation− and yet it exhibits stringent control of fatty acid synthesis. This observation argues against the possibility that a small pool of fatty acyl CoA's is rapidly degraded, resulting in turnover of fatty acids before incorporation into phospholipids. Since the rate of incorporation of labeled precursors into total cellular and extracellular fatty acyl groups is

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Effect of Leucine Starvation on Turnover of Fatty Acids Synthesized during Growth or Starvation—Since the reduction in rate of incorporation of radioactive precursors into fatty acyls of the stringent strain (CP 78) during leucine starvation (Figs. 1 and 3) could not arise from a reduced rate of synthesis, the fate of fatty acids synthesized in stringent and relaxed cells during growth and starvation was examined. It is evident from Fig. 4A that fatty acids of rel+ cells, labeled with [14C]acetate during growth on complete medium, showed no loss of label during subsequent incubation in unlabeled medium either in the presence or absence of leucine. Not only did the 14C activity in total fatty acids remain constant, but label in the chloroform-methanol extract did as well. This result argues against transfer of fatty acyls from structures extractable with chloroform-methanol to substances (e.g. lipopolysaccharide) insoluble in this solvent. Moreover, only an insignificant amount of radioactivity was detected under any circumstance in fatty acyls of substances excreted into the medium (<500 cpm of a total of 33,000 cpm in lipids). Similarly, there was essentially no turnover of 14C-fatty acids in the lipids of the rel− strain (CP 79) labeled during growth (Fig. 4A, lower panels). Virtually identical results were obtained in correlative experiments (Fig. 4B) in which the lipids of both rel+ and rel− cells were labeled with [14C]acetate during leucine starvation; no detectable loss of 14C label in fatty acyl groups or the chloroform-methanol extract occurred during subsequent incubation of the cells in complete or leucine-deficient medium. Again insignificant amounts of radioactive fatty acyl groups were excreted into the medium (Fig. 4B).

The amount of 14C activity incorporated into fatty acyls of material excreted into the medium was insignificant in all cases (Fig. 3C) and not materially altered by leucine starvation. It can be concluded, therefore, that amino acid deprivation causes a marked reduction in the rate of labeling of fatty acyls per ae in the stringent strain (rel+) of E. coli.

Turnover as used in this paper is defined as the conversion of fatty acyl groups into a form not extractable with petroleum ether after saponification and acidification.
curtained by amino acid deficiency and yet fatty acyl turnover is not altered, one is led to the conclusion that fatty acid synthesis per se is blocked by amino acid starvation.

In contrast to the stability of the fatty acyl residues of E. coli phospholipids (this work, 42), the phosphoryl group turns over at an appreciable rate and, in addition, the extent of turnover varies among different classes of phospholipids. Thus, substantial turnover of phosphatidylglycerol and cardiolipin has been observed during growth and starvation of both stringent and relaxed strains (2, 43, 44). On the other hand, phosphatidylethanolamine has been shown to be exceedingly stable under most conditions. Turnover of the phosphoryl group of the latter phospholipid has only been observed during amino acid starvation of relaxed strains (2) and in shifting from growth at 37° to growth at 10° (42). It is interesting that in neither case has turnover of fatty acids been demonstrated (this work, 42).

Effect of Leucine Starvation on Distribution of Radioactivity from [1-14C]Acetate in Lipids of Stringent and Relaxed Strains of E. coli—An investigation of [1-14C]acetate incorporation into the chloroform-methanol extractable lipids of rel+ or rel- strains of E. coli grown on complete or leucine-deficient media showed that >95% of the label appeared in phospholipids (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin). Two-dimensional thin layer chromatography and radioautography revealed virtually no fatty acids, in agreement with the findings of Mindich (45). The results summarized in Table I show no substantial difference in the distribution of 14C activity incorporated into the three major classes of phospholipids in rel+ or rel- cells growing in complete medium or rel- cells deprived of leucine. The possible exception to this may be cardiolipin, which comprised ~3% of the label in rel+ and rel- cells grown on complete medium, but 6% in leucine-starved rel- cells. Aside from the latter difference, the relative insensitivity of relaxed cells to amino acid starvation is similar both with respect to labeling pattern and rate of fatty acid synthesis. However, leucine deprivation of rel+ cells caused a large relative decrease in the percentage of label incorporated into phosphatidylethanolamine and a corresponding increase in labeling of phosphatidylglycerol; the synthesis of cardiolipin under these circumstances appears to be abolished. Considering the fact that total fatty acyl, and presumably phospholipid, synthesis was reduced by about 50% by leucine starvation, net phosphatidylethanolamine synthesis must have been curtailed to a much greater extent (70 to 75%) than net phosphatidylglycerol synthesis (25 to 30%). Moreover, net de novo cardiolipin synthesis appears to be completely blocked by leucine deprivation of rel+ cells.

Guanosine 5’diphosphate-3’diphosphate (ppGpp) as Possible Mediator of Stringent Control of Fatty Acid Synthesis at the Level of Acetyl-CoA Carboxylase—The in vivo labeling experiments with rel+ and rel- strains described in the preceding sections indicate that stringent control is exercised at the level of fatty acid synthesis in E. coli. These findings raise two important questions. (a) At what site(s) in the fatty acid synthetic pathway is stringent control exerted? (b) What factor(s) mediate this control? Since acetyl-CoA carboxylase catalyzes the committed step of fatty acid synthesis in E. coli and since there is precedent for its regulation in animal cells, this enzyme was considered a likely candidate as a target of stringent control. In this connection, Gallant and Cashel (12) have demonstrated the appearance of two unusual nucleotides ppGpp and pppGpp in stringent, but not relaxed, strains of E. coli during amino acid starvation. The kinetics of formation of these nucleotides upon removal of a required amino acid and of their disappearance upon resupplementation (13, 14) is compatible with their suggested role as mediators of the stringent response. Furthermore, these nucleotides have been shown to inhibit key enzymatic activities of various pathways subject to stringent control (46–51). Hence, we considered the possibility that ppGpp or pppGpp, or both, might act as negative effectors on the catalytic components of E. coli acetyl-CoA carboxylase.

Interestingly, it was found that the carboxyltransferase component, one of the two resolved catalytic elements of the E. coli acetyl-CoA carboxylase system, is inhibited by ppGpp and pppGpp. As illustrated in Fig. 5, both carboxyltransferase-catalyzed transcarboxylation (Reaction 4) and malonyl-CoA-[1-14C]acetyl-CoA exchange, which measures the second half-reaction (Reaction 2) in acetyl-CoA carboxylation, are inhibited by ppGpp. pppGpp, which also blocks the model carboxyl transfer reaction (Fig. 5), was not available in sufficient quantity to test its effect on the exchange reaction.

Malonyl-CoA + d-biotin methyl ester \[\rightleftharpoons\] \[\text{V-N.}\]

Malonyl-CoA-[1-14C]acetyl-CoA exchange is inhibited to the same extent by ppGpp whether tested with crude unresolved acetyl-CoA carboxylase preparations or with a reconstituted system of homogeneous carboxyltransferase and carboxyl carrier protein (Fig. 5, lower panel). Importantly, maximal inhibition of both carboxyltransferase-catalyzed reactions, i.e. 50 to 60%, is achieved at concentrations of ppGpp which correspond to those generated in vivo (up to 4 mM) during amino acid starvation of rel+ cells (50). Moreover, 50 to 60% inhibition at saturating ppGpp concentration agrees well with the extent to which fatty acid synthesis is curtailed in vivo by amino acid deprivation (Figs. 1 to 3).

It should also be noted that inhibition of the carboxyltransferase reaction by ppGpp is specific; none of the other nucleotides tested, including 5’-GTP, 5’-GDP, 5’-GMP, 5’-ATP, 5’-ADP, 5’-AMP, and cyclic adenosine 3’:5’-monophosphate, were inhibitory at a concentration where the ppGpp effect is maximal (see Table II). Furthermore, it appears that the two inhibitory nucleotides, ppGpp and pppGpp, bind at a common site on the

### Table I

**Distribution of label from [1-14C]acetate into the major phospholipid classes of E. coli**

<table>
<thead>
<tr>
<th>Strain and condition</th>
<th>[14C]Acetate incorporation into</th>
<th>PE</th>
<th>PG</th>
<th>CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP78 (rel+) complete</td>
<td>59.2</td>
<td>30.0</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>CP78 (rel+) minus leucine</td>
<td>50.3</td>
<td>48.4</td>
<td>&lt;0.5</td>
<td></td>
</tr>
<tr>
<td>CP79 (rel-) complete</td>
<td>58.2</td>
<td>37.7</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>CP79 (rel-) minus leucine</td>
<td>55.2</td>
<td>30.3</td>
<td>6.9</td>
<td></td>
</tr>
</tbody>
</table>

Malonyl-CoA-[1-14C]acetyl-CoA exchange is inhibited to the same extent by ppGpp whether tested with crude unresolved acetyl-CoA carboxylase preparations or with a reconstituted system of homogeneous carboxyltransferase and carboxyl carrier protein (Fig. 5, lower panel). Importantly, maximal inhibition of both carboxyltransferase-catalyzed transcarboxylation (Reaction 4) and malonyl-CoA-[1-14C]acetyl-CoA exchange, which measures the second half-reaction (Reaction 2) in acetyl-CoA carboxylation, are inhibited by ppGpp. pppGpp, which also blocks the model carboxyl transfer reaction (Fig. 5), was not available in sufficient quantity to test its effect on the exchange reaction.

Malonyl-CoA + d-biotin methyl ester \[\rightleftharpoons\] \[\text{V-N.}\]
carboxyltransferase. At a concentration of 0.4 mM, each nucleotide inhibits the model reaction by about 20%, and their effect, in a reaction mixture containing 0.4 mM of each nucleotide, is additive (Table III). However, in a reaction mixture containing a saturating concentration of either ppGpp (1.0 mM) or ppGpp (1.24 mM), the effect is not additive; in fact, the addition of either nucleotide, in a mixture already containing the other at saturating concentration, is virtually without effect (Table III).

Finally, the effect of ppGpp on the reactions dependent upon the biotin carboxylase component, including the carboxylation of free d-biotin and the carboxylation of acetyl-CoA, was tested. Both of these reactions are quite sensitive to divalent cation to ATP ratios; maximal activities at pH 6.8 to 6.9 are achieved with Mn⁴⁺:ATP = 1:1, Mg⁴⁺:ATP = 2:5:1, and Co⁴⁺:ATP = 2:5:1, the Vₘₐₓ obtained with any of these divalent cations being the same. However, activity declines, although not steeply, on either side of the optimal metal to ATP ratio. Since free ATP is also inhibitory in both the biotin carboxylase and carboxyltransferase activities, meaningful inhibition studies with ppGpp could only be conducted with levels of ATP, Mn²⁺, and ppGpp such that the concentration of free \( \text{Mg}^{2+} \) did not increase significantly and the ratio of \( \text{Mg}^{2+} : \text{ATP} \) did not change reaction; 0.15 milliunit of biotin carboxylase in the radioactive assay of biotin carboxylase. The numbers in parentheses are the rates of the control assays in nanomoles per min.

**Table II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Carboxyltransferase-catalyzed</th>
<th>Biotin carboxylase-catalyzed</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100 (5.3)</td>
<td>100 (0.15)</td>
<td>100</td>
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<tr>
<td>1.2 mm ppGpp</td>
<td>40</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>1.2 mm pppGpp</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>1.2 mm GTP</td>
<td>90</td>
<td>90</td>
<td>90</td>
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<tr>
<td>1.2 mm GDP</td>
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<tr>
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<td>111</td>
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<tr>
<td>1.2 mm AMP</td>
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<td>101</td>
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<tr>
<td>1.2 mm cAMP</td>
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<td>113</td>
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<tr>
<td>2.5 mm ppGpp-Mn</td>
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<tr>
<td>2.5 mm EDTA-Mn</td>
<td>131</td>
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<td>131</td>
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<tr>
<td>2.5 mm GTP-Mn</td>
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<td>2.5 mm GDP-Mn</td>
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<tr>
<td>2.5 mm ATP-Mn</td>
<td>101</td>
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**Table III**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Carboxyltransferase-catalyzed</th>
<th>Biotin carboxylase-catalyzed</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
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<tr>
<td>None (control)</td>
<td>4.9</td>
<td>4.9</td>
<td>0</td>
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<tr>
<td>0.4 mm ppGpp</td>
<td>4.0</td>
<td>4.0</td>
<td>17</td>
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<tr>
<td>0.4 mm pppGpp</td>
<td>3.7</td>
<td>3.7</td>
<td>23</td>
</tr>
<tr>
<td>0.4 mm ppGpp + 0.4 mm pppGpp</td>
<td>3.0</td>
<td>3.0</td>
<td>34</td>
</tr>
<tr>
<td>1.2 mm ppGpp</td>
<td>2.3</td>
<td>2.3</td>
<td>52</td>
</tr>
<tr>
<td>1.0 mm pppGpp</td>
<td>2.6</td>
<td>2.6</td>
<td>49</td>
</tr>
<tr>
<td>1.2 mm ppGpp + 1.0 mm pppGpp</td>
<td>2.1</td>
<td>2.1</td>
<td>58</td>
</tr>
<tr>
<td>2.2 mm ppGpp</td>
<td>2.2</td>
<td>2.2</td>
<td>56</td>
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</table>
deviate substantially from 1:1. This proviso necessitated the determination of the affinity of ppGpp for Mn²⁺. The dissociation constants for Mn²⁺ from the ternary (Mn⁺⁺)₂·ppGpp complex were assessed by titrating MnCl₂ with ppGpp and measuring the electron paramagnetic resonance of free Mn²⁺ at pH 6.8; the values obtained were 1.1 μM and ~65 μM. Since the K₆₅ for the Mn²⁺-ATP complex, 5.3 μM, determined under the same conditions, is more than an order of magnitude lower than that for the Mn²⁺ bound most weakly by ppGpp, in the inhibition studies sufficient Mn²⁺ was added to saturate ATP and the tight binding site of ppGpp. Thus in all experiments with the biotin carboxylase and acetyl-CoA carboxylase systems, the concentration of Mn²⁺ used was equal to the concentration of ATP plus ppGpp.

As illustrated in Table II, at a Mn²⁺·ppGpp concentration of 2.5 mM, which maximally inhibits malonyl-CoA-[¹⁴C]acetetyl-CoA exchange and acetyl-CoA carboxylation (Fig. 6), biotin carboxylase activity is not inhibited or is slightly increased. Consistent with the finding that free ppGpp at saturating concentration inhibits the carboxyltransferase-catalyzed model and exchange reactions 50 to 60% (Fig. 5 and Table II), saturating Mn²⁺·ppGpp blocks malonyl-CoA-[¹⁴C]acetetyl-CoA exchange and acetyl-CoA carboxylation to the same extent. However, the concentration of Mn²⁺·ppGpp required for maximal effect on the latter-mentioned reactions, about 2 mM and still within the physiological range, was twice as high as the concentration of free ppGpp needed for 50% inhibition of carboxyltransferase. This difference is most likely due to a lower affinity of the enzyme for Mn²⁺·ppGpp. The inhibition cannot be attributed to the free ppGpp in the mixture because its calculated concentration is minuscule. Direct measurement by electron paramagnetic resonance of free Mn²⁺¹ in reaction mixtures containing 2.5 mM ppGpp shows its concentration to be <0.1 mM.

Because of the differences in the concentration of nucleotide required for inhibition of acetyl-CoA carboxylation by Mn²⁺·ppGpp and of carboxyl transfer by free ppGpp, the effects of Mn²⁺·ppGpp on both reactions were compared in the same experiment. As shown in Fig. 6, the pattern of inhibition by Mn²⁺·ppGpp of malonyl-CoA-[¹⁴C]acetetyl-CoA exchange, catalyzed by either crude acetyl-CoA carboxylase preparations or by purified enzymes in a reconstituted system, was quantitatively similar to the pattern observed for acetyl-CoA carboxylation. Moreover, 50% inhibition of either reaction was achieved with the same concentration of Mn²⁺·ppGpp (about 2 mM). Furthermore, as shown in Table II, the inhibition of the exchange reaction is specific. All of the nucleotide chelates of Mn²⁺, as well as Mn²⁺-EDTA, tested at a concentration of 2.5 mM were not inhibitory. It is interesting that biotin carboxylase is activated to a small extent by Mn²⁺·ppGpp (Table II). This effect is not specific, however, because, as shown in the same table, Mn²⁺-EDTA exhibited the same degree of activation. In addition, acetyl-CoA carboxylase is also influenced to the same extent by the chelate (data not shown). The activation phenomenon is most likely of no physiological significance and may simply constitute a manifestation of the degree of accessi-

bility of the metal to the enzyme, the former being more accessible when present in the less ionic chelated form.

On the basis of the results presented in this paper, it appears that the inhibition of the over-all reaction by ppGpp is the result of the specific inhibition of the carboxyltransferase component of the acetyl-CoA carboxylase. Unfortunately, it is not possible to test the specificity of the inhibition of the over-all carboxylase reaction directly, because various nucleotides are known to be competitive inhibitors of the biotin carboxylase component of the complex. Thus, Mn⁺⁺·ADP, Mn⁺⁺·GDP, and Mn⁺⁺·GTP are all competitive inhibitors of biotin carboxylase with respect to Mn²⁺-ATP.

**DISCUSSION**

The results presented in this paper provide definitive proof that stringent control in *E. coli* is exercised on fatty acid synthesis rather than on precursor transport (acetate and glucose) or fatty acyl turnover. In addition, our results suggest that this control is mediated by (p)ppGpp and is exerted at the level of acetyl-CoA carboxylase. The intracellular concentration of ppGpp plus pppGpp rises following amino acid starvation from 0.1 to 0.4 mM to 2 to 4 mM (50). Our results indicate (Fig. 6) that maximal inhibition of acetyl-CoA carboxylase is manifested at these physiological (p)ppGpp concentrations, and, in particular, that the focus of action is the carboxyltransferase reaction (Fig. 5, Tables II and III). Furthermore, we have shown (Table II) that the inhibition is specific, as other nucleotides at comparable concentrations do not inhibit the carboxyltransferase-catalyzed reactions.

In the discussion to follow, attention will be focused on the broader aspects of this control mechanism. Fine control of biosynthetic pathways, i.e. control at the level of catalysis per se, is generally exerted at an early step in the metabolic sequence. In most cases this step is relatively irreversible, thus committing the product of the reaction to the pathway. Biosynthetic processes leading to the storage of reserve sources of energy are frequently regulated by feed-forward activation. The fatty acid biosynthetic pathway in animal tissues fulfills such a role, and existing evidence (52, 53) strongly suggests that this pathway is regulated by citrate activation of the committed step catalyzed by acetyl-CoA carboxylase. The rationale for this mechanism suggests that the accumulation of citrate in the cytoplasmic compartment serves to indicate that acetyl-CoA, reducing power, and energy in the form of ATP are all in abundance for the biosynthesis of fatty acids.

In contrast to animal systems, where fatty acids are used principally as a reserve source of energy, in *E. coli* fatty acids are primarily incorporated into phospholipids, which in turn are used for structural purposes in membranes. Hence, regulation of fatty acid synthesis in this organism would be expected to reflect the requirement for phospholipids in growth and cell proliferation. The elegant work of Maaløe and Kjeldgaard (54) on the control of macromolecular biosynthesis has established fundamental quantitative relationships between the rate of growth and such parameters as cell mass, number of nuclei, DNA, RNA, and protein content per cell. Their results suggest that, in a state of balanced growth, the rates of synthesis of the various cell components should be constant. Furthermore, during shift-up or shift-down experiments, it appears (54) that a new steady state is established and that this new state is also characterized by constant, but different, rates of synthesis of macromolecular cell constituents. Consequently, it is likely that a control mechanism is operative which coordinates the rates of synthesis of the macromolecules and components of the cell membrane to the rate of growth. This, in turn, implies the existence of one or more growth modulator(s), and (p)ppGpp may be one of these. The kinetics of appearance and disappearance of this nucleotide during amino acid starvation and refedding (12-14), its accumulation during shift-down experiments or carbon-source starvation (13, 14, 55), as well as the fact that (p)ppGpp concentration is inversely proportional to the RNA
content of the cell (14) and growth rate (54) lend support to this assumption. Finally, the recent interesting experiments of Haseltine et al. (56) and Haseltine and Block (57) have established that (p)ppGpp is formed on the ribosome by pyrophosphoryl transfer from ATP to GDP or GTP as an idling step in protein synthesis. The reaction requires both the 30 S and 50 S ribosomal subunits, the G translocation factor, the stringent factor—found only in stringent cells—messenger RNA, and an unchanged transfer RNA in the acceptor site of the ribosome. These observations also suggest that the mode of formation of (p)ppGpp is intimately connected to the cessation of protein synthesis and, thus, to inhibition of growth. Inhibition by (p)ppGpp of acetyl-CoA carboxylase, an enzyme which should supply malonyl-CoA at a rate proportional to cell proliferation, is a rational mechanism for controlling fatty acid synthesis.

In addition to an inhibition of fatty acid synthesis by amino acid starvation of stringent cells, the results presented in Table I suggest a preferential incorporation of radioactivity into the phosphatidylglycerol fraction. As pointed out under "Results," it appears that net phosphatidylethanolamine synthesis is curtailed to a greater extent than phosphatidylglycerol synthesis. This apparent block on phosphatidylethanolamine synthesis may be related to the yet unexplained observation of Etz et al. (38) that the "committed" step of phosphatidylethanolamine synthesis is catalyzed by an enzyme, phosphatidylinerine synthase, which is tightly bound to ribosomes. In this connection, it should be re-emphasized that the synthesis of (p)ppGpp, the apparent mediator(s) of the stringent control of lipid synthesis, is also synthesized on the ribosome.

Available data on the stringent control of fatty acid synthesis (this work, 1, 2) indicate that, per cell, fatty acid synthesis is reduced by only 50 to 60%, while protein synthesis ceases completely and RNA synthesis appears to be inhibited to a greater extent (13, 14). The reason for this continued, although reduced, level of fatty acid synthesis in the absence of growth is not known; however, it is conceivable that successful transitions during alterations in nutritional state may require changes in the membrane composition and, therefore, continued net phospholipid synthesis. Examples of such changes are the increased formation of cyclopropane fatty acids at the end of log phase growth (59, 60), although this change is not vital to cell survival, and the changes in the fatty acid composition of cell membranes when cells are shifted from high to low temperature (42). That changes in the membrane composition may be important factors for cell survival is suggested by the fact that viability decreases soon after cessation of growth of unsaturated fatty acid auxotrophs of E. coli deprived of oleate (61, 62), after transfer to nonpermissive temperatures of a mutant with a temperature-sensitive glycerol 3-phosphate acyltransferase (63), and after transfer of an unsaturated fatty acid auxotroph grown at 27°C with elaidate to 27°C in the absence of oleate (64-66). In addition, it is possible that amino acid starvation induces the synthesis of certain membrane enzymes and proteins and that this induction requires net phospholipid synthesis. Both, induction of enzymes during starvation (67, 68) and a requirement for net phospholipid synthesis for induction (69-72), have been observed experimentally.

The results of Silbert et al. (73, 74) and Esfahani et al. (64, 65) suggest that supplementation with fatty acids results, at least in certain strains, is a curtailment of the synthesis of saturated and unsaturated fatty acids. Supplementation with unsaturated fatty acids (64, 60, v5) preferentially decreased the incorporation of [14C]acetate into unsaturated fatty acids; however, supple-

mentation with saturated fatty acids (74), while reducing incorporation of [14C]acetate into saturated fatty acids preferentially, also resulted in reduced incorporation of label into the unsaturated fraction. The authors suggest that a primary control mechanism is operative at the level of glycerol 3-phosphate acyltransferase which determines the fatty acyl composition of the phospholipid. In addition, the results of Wakil and his colleagues (65) suggest that cells unable to degrade long chain (C16) unsaturated fatty acids compensate by adjusting both the chain length and the quantity of the saturated fatty acids synthesized. It is not yet clear whether the over all curtailment of fatty acid biosynthesis in the presence of exogeneous fatty acids is the result of feedback inhibition of the biosynthetic pathway by accumulating products, e.g., acyl-CoA, or of repression of synthesis of the enzymes involved.

It is evident that the phospholipid-synthesizing systems of E. coli exhibit extreme versatility and adaptability and this, no doubt, is of survival value to the organism. The formation of a functionally efficient and stable membrane for a given set of conditions appears to be vital, and a multiregulatory system both at the level of synthesis and degradation is probably required to achieve this.

The observations described in this paper suggest that the inhibition of acetyl-CoA carboxylase by ppGpp is one of the regulatory mechanisms operating at the biosynthetic level, by which the cell regulates the supply of malonyl-CoA and the rate of phospholipid synthesis for growth.

Acknowledgments—We thank Mr. Eberhard Zwergel for outstanding technical assistance, Dr. Albert Mildvan for carrying out the electron paramagnetic resonance measurements, Drs. M. Cashel and R. Byrne for generously providing us with ppGpp and pppGpp, and Dr. N. Fiil for supplying the strains of E. coli used in these experiments.

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Stringent Control of Fatty Acid Synthesis in *Escherichia coli* : POSSIBLE REGULATION OF ACETYL COENZYME A CARBOXYLASE BY ppGpp

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