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# Regulation of Macromolecular Biosynthesis in a Mutant of *Escherichia coli* Defective in Membrane Phospholipid Biosynthesis

**(temperature-sensitive glycerol 3-phosphate acyltransferase/protein/active transport)**

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**ABSTRACT** **Nucleic acid and protein synthesis were studied in temperature-sensitive mutants defective in phospholipid synthesis. The defect is due to a single mutation in glycerol 3-phosphate acyltransferase (EC 2.3.1.15). The results show that at the restrictive temperature not only does phospholipid synthesis cease, but DNA, RNA, and protein synthesis also cease. Active transport continues, however, indicating that the cells do not become leaky or lose their energy supply. These results suggest that phospholipid synthesis is coupled to DNA, RNA, and protein synthesis.**

Since lipid synthesis, and consequently membrane synthesis, is necessary for growth of cells, it is reasonable that cells would have regulatory mechanisms to couple the synthesis of lipids with the synthesis of macromolecules in order to maintain balanced cell growth. The availability of mutants in lipid biosynthesis has helped to elucidate the contribution of phospholipids to membrane structure and function (1). In particular, one apparent result was that protein synthesis and cell growth could continue for a significant amount of time in the absence of lipid synthesis. When an unsaturated fatty-acid auxotroph of *Escherichia coli* was deprived of unsaturated fatty acids, growth, as well as DNA, RNA, and protein synthesis, continued for several hours (2–5). Similar results were obtained when 3-decynoyl-*N*-acetylcysteamine was used to inhibit unsaturated fatty-acid synthesis (4, 6, 7). *Bacillus subtilis* (8–10), *Staphylococcus aureus* (11), and *E. coli* (12) glycerol auxotrophs have been studied. When these mutants were deprived of glycerol, net phospholipid synthesis stopped immediately. However, macromolecular synthesis and cell growth continued for some time.

Recently this laboratory has described a temperature-sensitive mutant of *E. coli*, CV15, that does not grow or make phospholipid at the restrictive temperature (13). The defect is due to a single mutation in glycerol 3-phosphate acyltransferase (EC 2.3.1.15), which catalyzes the first reaction unique to phospholipid biosynthesis. In this paper, detailed studies of the synthesis of phospholipid, DNA, RNA, and protein are reported for mutants with a thermolabile glycerol 3-phosphate acyltransferase. The results show that DNA, RNA, and protein synthesis do not continue at the restrictive temperature; the shutoff of the synthesis of these macromolecules parallels the shutoff of phospholipid synthesis. Active transport continues, however, indicating that the cells do not become leaky or lose their energy supply.

## MATERIALS AND METHODS

*Bacterial Strains, Media, and Growth Conditions.* CV15 and its parent, strain 8, were described previously (13). Mutant 15X1 is a recombinant of X478 obtained by transduction with phage $P_1$ grown on CV15 (14); this mutant, which was provided by Dr. J. E. Cronan, Jr., contains the temperature-sensitive glycerol 3-phosphate acyltransferase of CV15. Both mutants grow normally at 27°, but stop growing above 37°.

Strain 8 and CV15 were grown in medium 56-LP (low phosphate) (15) supplemented with 0.1% vitamin-free casein hydrolysate and 1% pyruvate at pH 7.6. X478 and 15X1 were grown on medium 56-LP supplemented with 0.4% glucose, 1 μg/ml of thiamine, and 20 μg/ml each of adenine, leucine, isoleucine, valine, proline, methionine, lysine, and tryptophan. In the experiments, parallel cultures of a mutant and its parent were grown at 27°. At about 75 Klett units, cultures were shifted to another water-bath shaker set at the restrictive temperature. Growth was followed with a Klett–Summerson colorimeter with a number 54 filter.

*Rates of Synthesis of DNA, RNA, and Protein.* For measurement of the rates of DNA and RNA synthesis (16, 17), 1.0-ml aliquots of culture were added to test tubes at the same temperature, containing 2 μCi of [*methyl*-$^3$H]thymidine (520 Ci/mol) and 0.1 μCi of [2-$^{14}$C]uracil (26 Ci/mol). For rates of protein synthesis, 1.0-ml aliquots were added to tubes containing 10 μCi of [4,5-$^3$H]leucine (50 Ci/mmol). After 3 min, 1.0 ml of 10% trichloroacetic acid was added. Samples were filtered through Millipore filters, washed with 5% cold trichloroacetic acid containing 10 mM leucine, and counted. Blanks were determined by addition of aliquots of the culture to a tube containing the radioactive precursors and trichloroacetic acid.

Rates of synthesis of DNA, RNA, and phospholipid were also measured with [$^{32}$P]phosphate as a common precursor. Aliquots of the culture (0.75 ml) were added to 17 μCi of [$^{32}$P]phosphate (carrier-free). After 5 min, 3 ml of chloroform–methanol 1:2 was added. Nucleic acids are insoluble in this mixture, and they are found in the pellet. The supernatants were used for determination of phospholipid. The pellets were suspended in cold 0.5 N NaOH. For determination of the total nucleic acid, 0.5 N cold HCl was added immediately to an aliquot to neutralize the solution, and trichloroacetic acid was added to 10% final concentration. Samples were filtered, washed, and counted. A second aliquot was incubated overnight at 37° to hydrolyze the RNA, and the samples were treated as above for determination of the amount of DNA. The results were similar when [$^3$H]thymidine and [$^{14}$C]uracil were used to label the nucleic acids and the samples treated by either the [$^{32}$P]phosphate procedure or the usual procedure where trichloroacetic acid was added directly to the cells.

Abbreviations: TMG, methyl-1-thio-β-D-galactopyranoside; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

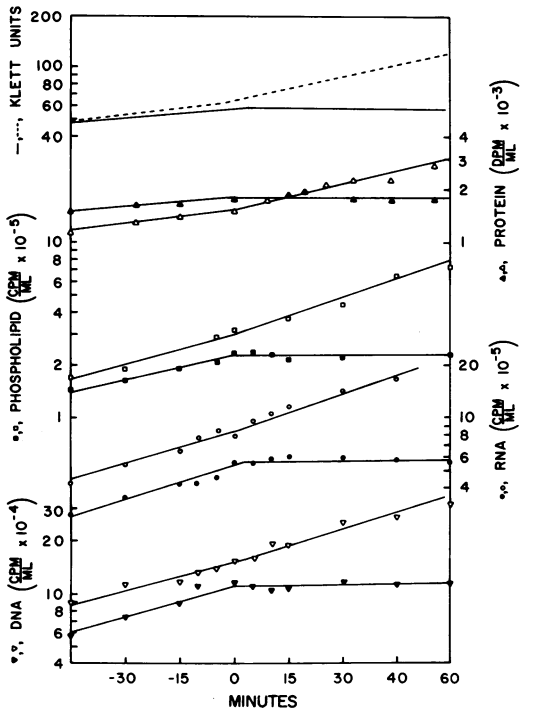


FIG. 1. Growth and net synthesis in strain 8 (*open symbols*) and CV15 (*closed symbols*). Cultures growing at 27° were shifted to 40° at zero time. Growth and phospholipid, DNA, and RNA synthesis were measured in one culture containing $^{32}PO_4$.

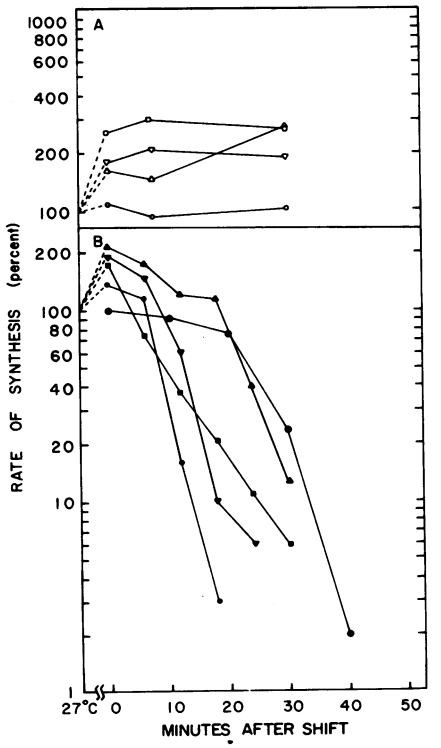


FIG. 2. Rates of synthesis in strain 8 (*A*) and CV15 (*B*). An aliquot of each culture was taken at 27° for analysis, and then the cultures were shifted to 39°. Minutes after shift refers to the time aliquots were taken for analysis. The rates of synthesis (dpm incorporated in pulse per ml) of phospholipid (□,■), DNA (▽,▼), RNA (○,●), and protein (△,▲) were measured with [$^{32}$P]-phosphate, [$^{3}$H]thymidine, [$^{14}$C]uracil, and [$^{3}$H]leucine, respectively. The number of viable colonies (⊗) was measured by plating dilutions of the culture at 27°.

*Rate of Synthesis of Phospholipid.* The rate of phospholipid synthesis was measured in a similar manner, except 0.8-ml aliquots of cultures were added to tubes containing 300 μCi of [$^{32}$P]phosphate (carrier-free). They were incubated for 5 min. Then 3 ml of chloroform–methanol 1:2 was added, and the phospholipids were extracted (18, 19) and counted.

*Net Synthesis of DNA, RNA, Protein, and Phospholipid.* For measurement of the net synthesis of DNA, RNA, and phospholipid, [$^{32}$P]phosphate was added to the growing culture (7 μCi/ml) for two generations before the shift to the restrictive temperature. For determination of the net amount of protein synthesized [*4,5*-$^{3}$H]leucine was added to a separate culture (0.1 μCi/ml). Aliquots were analyzed as above.

*Uptake of Methyl-1-thio-β-D-galactopyranoside (TMG), Glycerol 3-phosphate, and Phosphate.* Rates of uptake were measured by addition of 0.5-ml aliquots of culture to tubes containing 0.1 μCi of [$^{14}$C]TMG (0.2 mM final concentration) (20), 0.3 μCi of [$^{32}$P]phosphate (0.3 mM final concentration), or 0.01 μCi of [L-$^{14}$C]glycerol 3-phosphate (1.0 μM DL-glycerol 3-phosphate, final concentration) (21). Samples were filtered through Millipore filters, washed with 10 ml of 56-LP, and counted. In the experiments with TMG, the cells were induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for two generations at 27°, washed, and resuspended in medium without IPTG. After 15 min at 27°, the cells were shifted to the restrictive temperature. A value of 0.6 μl of cell water per ml of culture at 100 Klett units was used in calculating intracellular concentrations (20).

*Chemicals.* All radioactive compounds were obtained from New England Nuclear Corp. Organic compounds were obtained from Sigma Chemical Co.

## RESULTS

CV15 is a temperature-sensitive mutant that grows at a normal rate at 27°, but it stops growing and shuts off phospholipid synthesis when it is shifted to a temperature greater than 37° (13). When [$^{32}$P]phosphate was used to measure the net synthesis of DNA, RNA, and phospholipid and [$^{3}$H]-leucine was used to measure the net synthesis of protein, the increases in these cellular constituents in CV15 paralleled increases in its parent, strain 8, at 27° (Fig. 1). However, when the cultures were shifted to the restrictive temperature, synthesis of all these cell components continued at a slightly increased rate in strain 8, while synthesis in CV15 quickly stopped. The turbidity of the culture and the amount of DNA, RNA, protein, and phospholipid in CV15 at the restrictive temperature remained constant, indicating that lysis and degradation were not occurring.

In order to get a better idea of the rate of shutoff of macromolecular biosyntheses, pulse experiments were done with appropriate radioactive precursors. Fig. 2 shows the results when [$^{32}$P]phosphate, [$^{3}$H]thymidine, [$^{14}$C]uracil, and [$^{3}$H]-leucine were used to measure the rates of synthesis of phospholipid, DNA, RNA, and protein, respectively. Pulses for phospholipid synthesis were performed for 5 min, while the pulses for the other processes were performed for 3 min. When CV15 and strain 8 were shifted to the restrictive temperature, the rates of synthesis increased in strain 8 (Fig. 2*A*), while in CV15 rates of synthesis declined rapidly after an initial increase (Fig. 2*B*). There was not a large difference between the decline in the rates of synthesis of phospholipid, DNA, RNA, and protein. There was a slower loss in the number of

viable colonies in CV15, as measured by plating aliquots of the culture at the permissive temperature.

In order to eliminate the possibility that the shutoff of these biosynthetic processes was due to a unique property of CV15 unrelated to glycerol 3-phosphate acyltransferase or that CV15 contains multiple temperature-sensitive mutations, similar experiments were done with other mutants containing a thermolabile glycerol 3-phosphate acyltransferase. The results were similar for the mutant 15X1, a recombinant of X478 containing the thermolabile acyltransferase of CV15 (Fig. 3). For two other mutants, CV2 and CV31 (22), derived from strain 8 by the same isolation procedure used for CV15, synthesis of phospholipid, RNA, DNA, and protein was also shut off at the restrictive temperature (data not shown).

The time required for shutoff of the various biosynthetic processes after temperature shift was very sensitive to the final temperature. At 39° the shutoff in CV15 was almost complete in 30 min. At 42° the shutoff was almost immediate, and it was difficult to demonstrate precursor incorporation into macromolecules even at the initial point after temperature shift. On the other hand, at 37° growth continued, although at a reduced rate, and synthesis of phospholipid RNA, DNA, and protein also continued at reduced rates.

To avoid a problem that might arise by the use of different precursors, the rates of synthesis of phospholipid, RNA, and DNA were measured simultaneously by the use of [$^{32}$P]phosphate. The results again showed an increase in rates of biosynthesis for strain 8 after temperature shift, while in CV15 there was an initial increase and then a parallel decline in synthesis for all three processes (Fig. 4).

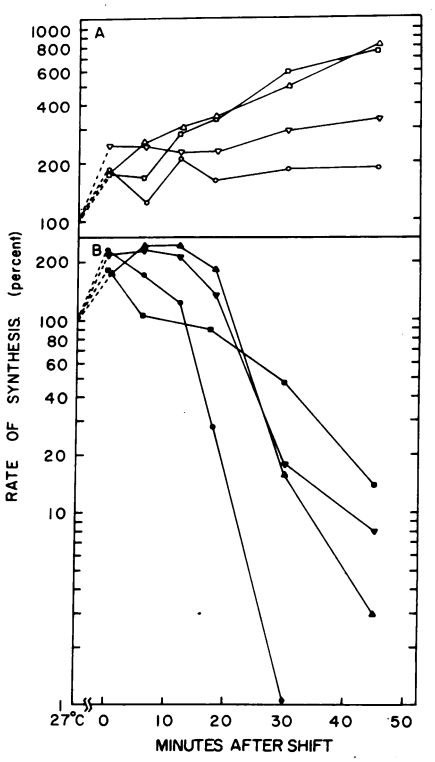


FIG. 3. Rates of synthesis in X478 (*A*) and 15X1 (*B*). An aliquot of each culture was taken at 27° for analysis, and then the cultures were shifted to 38°. The rates of synthesis of phospholipid (□,■), DNA (▽,▼), RNA (○,●), and protein (△,▲) were measured as in Fig. 2.

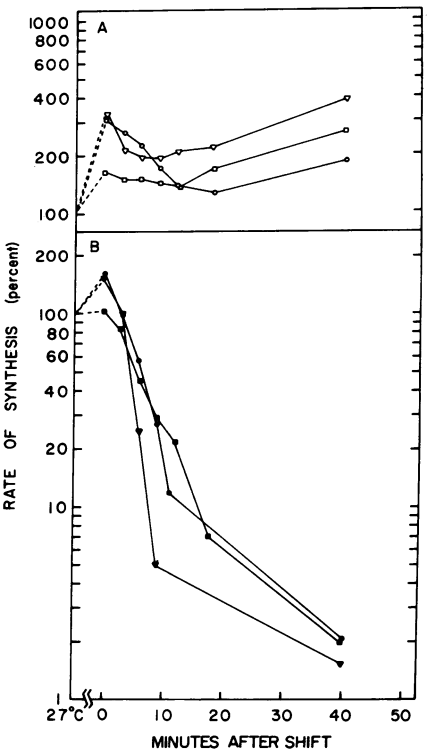


FIG. 4. Rates of synthesis in strain 8 (*A*) and CV15 (*B*). An aliquot of each culture was taken at 27° for analysis, and then the cultures were shifted to 39°. Minutes after shift refers to the time aliquots were taken for analysis. The rates of synthesis (dpm incorporated in pulse per ml) of phospholipid (□,■), DNA (▽,▼), and RNA (○,●) were measured with [$^{32}$P]phosphate.

The shutoff of biosynthesis in CV15 at the restrictive temperature cannot be explained by the inability of the cells to take up exogenous radioactive precursors after the shift to the restrictive temperature. The uptake of [$^{32}$P]phosphate by CV15 is shown in Table 1. There was an increase in uptake when the cells were shifted to the restrictive temperature, and afterwards the uptake was fairly constant for at least 60 min. During a 0.5-min pulse in growing cells, the [$^{32}$P]phosphate taken up remained mostly as inorganic phosphate, with some metabolized to organophosphate esters and a small amount converted to nucleic acid or phospholipid (23).

The uptake of glycerol 3-phosphate and TMG were also studied. Transport at 39.5° was very rapid, and by 1.0 min the intracellular concentration was reaching a plateau. Consequently, only the data for uptake during 0.5 min are shown (Table 1). Strain 8 was originally used for a study of glycerol 3-phosphate transport and utilization (21, 24). It lacks the catabolic glycerol 3-phosphate dehydrogenase and alkaline phosphatase and is constitutive for glycerol 3-phosphate transport. Consequently, glycerol 3-phosphate cannot be used for growth, and it is used only for phospholipid synthesis. Its utilization is negligible in comparison to the amount taken up by the cells during a short interval. The amount of glycerol 3-phosphate taken up in CV15 increased after the temperature shift, but thereafter it remained essentially constant for at least 60 min (Table 1). The energy dependence was illustrated by addition of azide, which inhibited the accumulation of glycerol 3-phosphate. Phospholipid synthesis, as measured with a pulse of [$^{14}$C]glycerol 3-phosphate of longer duration and higher specific activity, was almost completely shut off 15 min after the temperature shift.

TABLE 1. *Uptake of [$^{32}P$]phosphate, [$^{14}C$]glycerol 3-phosphate, and [$^{14}C$]TMG by CV15 after a shift to 39.5°*

| Min after shift | [$^{32}P$]phosphate uptake in 0.5 min (dpm/ml) | [$^{14}C$]glycerol 3-phosphate: Uptake in 0.5 min (dpm/ml) | [$^{14}C$]glycerol 3-phosphate: Conversion to phospholipid‡ (%) | [$^{14}C$]TMG uptake in 0.3 min (dpm/ml) |
|---|---|---|---|---|
| 27°* | 23,734† | 5,664† | 100 | 15,522† |
| 0 | 41,848 | 5,706 | 87 | 21,306 |
| 10 | 53,566 | — | — | — |
| 15 | — | 11,380 | 14 | 27,892 |
| 20 | 46,020 | — | — | — |
| 30 | 31,898 | 9,310 | 5 | 16,060 |
| 45 | 46,430 | 12,524 | 3 | 13,882 |
| 60 | 47,686 | 9,652 | 2 | — |
| Azide§ | — | 300 | — | 660 |

* An aliquot of the culture was taken immediately before the shift to the restrictive temperature. Uptake of [$^{32}P$]phosphate, [$^{14}C$]glycerol 3-phosphate, and [$^{14}C$]TMG were measured in separate experiments. Uptake for cells was measured at their growth temperature.

† The intracellular concentrations of [$^{32}P$]phosphate, [$^{14}C$]-glycerol 3-phosphate, and [$^{14}C$]TMG represent about a 13-, 550-, and 52-fold increase over the initial concentrations in the medium, respectively.

‡ [$^{14}C$]Glycerol 3-phosphate incorporated into phospholipid was measured during a 3-min pulse with [$^{14}C$]glycerol 3-phosphate of higher specific activity. The 100% value represented about 70,000 dpm/ml of culture.

§ An aliquot of the culture was incubated for 15 min with 50 mM sodium azide before uptake was measured.

—, Not done.

The ability of CV15 to transport TMG, a nonmetabolizable β-galactoside, was studied for 45 min after the shift to the restrictive temperature (Table 1). The results were similar to those obtained with phosphate and glycerol 3-phosphate uptake, with the exception that 30 min after the temperature shift the amount of TMG taken up by CV15 was apparently decreasing. Uptake at 39.5° decreased after synthesis of nucleic acid, protein, and phospholipid stopped. TMG uptake was still quite active at 45 min, showing that the cells did not lose the ability to generate energy as they did when azide was added.

## DISCUSSION

When the net synthesis of macromolecules was measured in CV15, a temperature-sensitive mutant defective in phospholipid biosynthesis, the results clearly showed that not only phospholipid, but also DNA, RNA, and protein synthesis stopped at the restrictive temperature (Fig. 1). The cells did not lyse, and no degradation was apparent for 60 min. The rates of synthesis of phospholipid, DNA, RNA, and protein decreased in parallel over the same time interval (Figs. 2–4), and the rate of the decline was very sensitive to changes of temperature. These biosynthetic processes stopped so rapidly at higher temperatures, such as 42°, that accurate biosynthetic rates could not be measured. This observation led to the erroneous conclusion in an earlier report that macromolecular synthesis continued at 42° (13). In the present experiments, at 39° the shutoff of these biosynthetic processes could be observed.

The rates measured in these pulse experiments are not intended to represent true rates of synthesis, but rather to give a relative idea of the amount of biosynthesis in a given pathway. There are several problems in interpretation of the data, especially in the comparison of the declines in the rate of synthesis of the different macromolecules. For example, conditions of the pulse were chosen so that the incorporation of the radioactive precursor was roughly linear with time at 27°. In the mutant, synthesis declined rapidly at the restrictive temperature, and during the relatively long pulses the rate may not have been linear. The amount of exogenous radioactive precursor incorporated into protein or nucleic acid relative to the amount used from endogenous synthesis might also have varied with the growth rate (25). In measurement of the rates of synthesis of RNA with [$^{14}C$]uracil and DNA with [$^{3}H$]thymidine a difficulty might arise regarding the selective transport and use of these precursors. To avoid this, an experiment was done with [$^{32}P$]phosphate to simultaneously measure the rates of synthesis of DNA, RNA, and phospholipid. The results again showed that in CV15 at the restrictive temperature there was a parallel decline in the rates of synthesis. The shutoff was not accounted for by a lack of transport because the cells retained their ability to transport and accumulate phosphate, glycerol 3-phosphate, and TMG long after macromolecular synthesis completely stopped. This result eliminated the possibility that the cells became leaky or lost their energy supply at the restrictive temperature.

To be able to conclude that there is tight coupling or regulation between phospholipid synthesis and macromolecular synthesis, it is critical that there are not multiple mutations responsible for this phenotype. There are several lines of evidence demonstrating that there is only one mutation and that it causes a defect in glycerol 3-phosphate acyltransferase. First, CV15 possesses a glycerol 3-phosphate acyltransferase that is more thermolabile *in vitro* than that of strain 8 (13). Second, the reversion rate for CV15 is consistent with a single mutation (13). Third, 15X1, a transductant, presumably containing a single mutation from CV15, displays the same phenotype as CV15 (Fig. 3). The same phenotype is also displayed by CV2 and CV31, independent mutants obtained in the same isolation procedure as CV15. Fourth, two other mutants obtained by a similar isolation procedure, as well as CV2, CV15, and CV31, all map as a single mutation at the same genetic locus (14), and they all possess a thermolabile glycerol 3-phosphate acyltransferase (14, 22).

Glycerol or unsaturated fatty-acid auxotrophs behave differently from these temperature-sensitive phospholipid mutants. When they are deprived of their growth requirement, they continue to make protein and nucleic acid for some time. The most obvious explanation is that a regulatory mechanism operates at the level of *de novo* phospholipid synthesis. In the auxotrophs, phospholipid synthesis can occur and, consequently, regulation or coupling is not apparent. When an unsaturated fatty-acid auxotroph is starved for unsaturated fatty acids, saturated fatty acids are still made, resulting in the continued synthesis of phospholipids containing only saturated fatty acids (26). When glycerol auxotrophs are deprived of glycerol, net phospholipid synthesis is stopped immediately, but there is some phospho-

lipid metabolism and a small amount of *de novo* synthesis (11, 12, 27–29). At the restrictive temperature, glycerol 3-phosphate acyltransferase of CV15 is inactivated and *de novo* phospholipid synthesis cannot occur. It is possible that at the restrictive temperature the substrates of glycerol 3-phosphate acyltransferase accumulate and inhibit macromolecular synthesis. Accumulation of glycerol 3-phosphate can inhibit growth of *E. coli* mutants lacking the catabolic glycerol 3-phosphate dehydrogenase, such as CV15, and the inhibition can be overcome by glucose (30). This does not appear to be the explanation because the results seen in CV15 were also seen in 15X1, which has this dehydrogenase and which was grown on glucose. In addition, glycerol 3-phosphate regulates its own synthesis by inhibiting the anabolic glycerol 3-phosphate dehydrogenase and, therefore, it should not accumulate (31). If fatty-acid synthesis is not regulated, free fatty acids, their coenzyme A, or acyl carrier protein derivatives could accumulate. Although this explanation cannot be ruled out, it seems unlikely that it could account for the results seen here. When *E. coli* glycerol auxotrophs are deprived of glycerol, free fatty acids do not accumulate. However, in gram-positive glycerol auxotrophs fatty acids do accumulate, but macromolecular synthesis continues (29).

There are numerous mechanisms whereby the absence of phospholipid synthesis could effect DNA, RNA, and protein synthesis. One primary effect would be to stop membrane growth, which could directly effect macromolecular synthesis. Jacob, Brenner, and Cuzin (32) postulated that membrane synthesis is necessary for DNA replication (for a current discussion see ref. 33). It has also been proposed that protein is synthesized on membrane-bound ribosomes (34, 35). Consequently, these processes could be mediated by cell membranes. Glycerol 3-phosphate acyltransferase is a membrane-bound enzyme, and it is responsive to the lipid composition of the membrane (36, 37). When it is denatured, a "cooperative" change could take place in the membrane that could effect a number of processes (see ref. 38).

Alternatively, one process could be mediated by another. For example, if RNA synthesis were stopped in the absence of phospholipid synthesis, the messenger RNA present would be quickly degraded, and protein synthesis would stop. Also, if RNA synthesis were necessary as a primer for DNA synthesis (39, 40), DNA synthesis would also stop.

Another possibility is that all processes are mediated by an inhibitor such as ppGpp, which accumulates when stringent cells are starved for an essential amino acid (41, 42). Under these conditions, RNA synthesis is inhibited, as well as several other processes, including phospholipid synthesis (43). The present results do not appear to be related to the *rel* gene locus because there was no difference between CV15, which is a $rel^-$ strain, and 15X1, which is a $rel^+$ strain.

Finally, the intriguing possibility should be mentioned that glycerol 3-phosphate acyltransferase might contain a thermolabile subunit(s) in CV15 that is shared by proteins in other pathways. This result has been found in protein biosynthesis for the elongation factors EF-Tu and EF-Ts, which are also part of the bacteriophage $Q_\beta$ replicase (44).

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