Stringent and Relaxed Control of Phospholipid Metabolism in Escherichia coli*

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SUMMARY

Stringent cells of Escherichia coli (rel*) cease growth and protein, RNA, and lipid synthesis when deprived of a required amino acid; relaxed cells (rel−) cease growth and protein synthesis but continue lipid synthesis when deprived of a required amino acid, as assayed by [14C]acetate incorporation. When phospholipid synthesis was assayed by using 32P, some net synthesis does occur, but the majority of the incorporation is a consequence of rapid breakdown and resynthesis (turnover) of phosphatidylethanolamine, normally a stable component of the cell envelope.

When growing bacteria are deprived of a required amino acid they stop growing and quit making protein and stable RNA. The requirement for adequate supplies of amino acids for protein synthesis is apparent; however, the biochemical basis of the relationship between amino acid starvation and stable RNA synthesis is not understood (1). Organisms which are relaxed (rel−) (i.e. with a genetic lesion in the ribonucleic acid control (RC) gene) in contrast to wild type or stringent (Tel+) organisms seem to be a marked turnover of phosphatidylethanolamine, the major, normally stable phospholipid in these organisms, in amino acid-starved, relaxed cells.

A primary difference in phospholipid metabolism between stringent and relaxed organisms seems to be a marked turnover of phosphatidylethanolamine, the major, normally stable phospholipid in these organisms, in amino acid-starved, relaxed cells.

MATERIALS AND METHODS

Organisms and Materials—E. coli CP-78 (rel+, arg−, his−, thi−, leu−, thr−) and E. coli CP-79 (rel−, arg−, his−, thi−, leu−, thr−) were obtained from Dr. G. Edlin. E. coli PA-1 (rel+, thi−, arg+) and E. coli PA-2 (rel−, thi−, arg−) were obtained from Miss L. Meade. All are K-12 strains and are believed to be isogenic except for a small region of the chromosome between Minute 53 and 54 on the Taylor-Trotter map which includes the rel locus (18).

All isotopes were obtained from New England Nuclear Corp. Lipid standards were obtained as a gift from Dr. J. E. Cronan or from Supelco, Inc. Silica Gel G was obtained from Brinkmann Instrument Co.; silica gel-loaded paper (Whatman 58-1) was from Reeve Angel Co. The chloroform was of United States Patent grade. All other chemicals were reagent grade. All solvents were dried over molecular sieve (Linde 4A) before use.

Growth Conditions—In experiments where radioactive phosphate was not employed, the cells were grown in phosphate-rich Medium E (19). Glucose and, in one set of experiments, succinate and ribose were used as the carbon source by sterilizing each separately and adding to a final concentration of 0.2, 0.4, and 0.6%, respectively. Amino acids were added to a final concentration of 10−4 M, and thiamine was added to a final concentration of 0.5 µg per ml. The phosphate-poor medium (Tris-maleate buffer) used when the cells were to be labeled with radioactive phosphate was identical with phosphate-rich Medium E, except that the phosphates are replaced by 5.80 g of maleic acid, 6.06 g of 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), and 0.746 g of KCl adjusted to pH 7.4 (19). All experiments are carried out at 37° in 25 to 35 ml of medium in 250-ml Erylenmeyer flasks in a New Brunswick rotary incubator shaker at 180 rpm. Growth was measured by following the turbidity at 650 nm with a Bausch and Lomb Spectronet 600.
satisfactorily. Amino acid starvation was induced by re-suspending the culture in medium lacking only the required amino acid. Radioactive isotopes employed were added to the culture after the nutritional components but before the cells.

**Assay of Phospholipids**—A modification of the Bligh and Dyer (20) extraction procedure as suggested by Ames (21) for *E. coli* was employed. The cells were extracted for 30 min at room temperature, and the chloroform layers obtained after separation of the phases were evaporated to dryness under a stream of nitrogen. The lipid was taken up in a small volume of chloroform-methanol (2:1) and stored at 5°.

Commercial silica gel-loaded paper was used for the separation of phospholipids. The paper was cut in strips (21 x 31 cm), and their ends were stapled together to form cylinders. For activation of the paper, the chromatograms were first developed in dry acetone. The chromatographs were removed from the acetone tank, and the lipid sample was immediately applied and then developed in a solvent system (22) containing chloroform, methanol, and water (65:25:4) at room temperature for 1½ to 2 hours. The separations were good and quite reproducible (Fig. 1).

Phospholipids were identified by chromatography with commercial standards and with the phospholipids of defined composition from *E. coli* strain K-12 (21, 23). Radioactive lipids were located by autoradiography using Kodak No-Screen x-ray film. About 5000 cpm of 32P can easily be detected after a 24-hour exposure to the film. Quantitative estimation of radioactivity was obtained by cutting out the specific area from the paper chromatogram and assaying for radioactivity in a liquid scintillation counter. Our recoveries were 99% when the spots were cut from the silica gel-loaded paper and counted directly. Total CHCl₃-CH₂OH-soluble phospholipid phosphate was determined by the method of Ames by using the Mg(NO₃)₂ ashing procedure (24).

**Assay of Phospholipid Synthesis and Turnover**—Incorporation of [14C]acetate into lipid was measured by the method of Sokawa et al. (14). We also used the extraction procedure described below (21) with equivalent results.

Incorporation of 32P into phospholipids was assayed as follows. Cells grown in phosphate-rich medium with all of the nutritional requirements for normal growth were harvested in the log phase of growth by centrifugation at room temperature. The cell pellet was washed with phosphate-free medium at 37° and centrifuged again. The cells were resuspended in medium at 37° to which phosphate (0.3 mM final concentration) and the

Fig. 1. Fractionation of the phospholipids of *Escherichia coli* strains PA-1 and PA-2 on silica gel-loaded paper. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; X, unknown 32P-labeled lipid(s).
necessary nutritional requirements were added. Carrier-free "P; was added (0.1 µCi per ml), and the cultures were incubated at 37°C with shaking. Aliquots (4 ml) were removed at various time intervals, extracted by the procedure described earlier (21), and analyzed for "P content.

The "P specific activity of the medium was determined for every experiment. A portion (10 µl) of the medium and 990 µl of water were added to a counting vial. The samples were then assayed for radioactivity. The phosphate concentration of the medium was 0.3 mm.

The rate of turnover of phospholipids was assayed by a dilution technique. Cells were first fully labeled by growing in the presence of "P; (0.1 µCi per ml) for 7 to 12 hours. The cells were resuspended in medium at 37°C containing 0.3 mM unlabeled phosphate, the necessary carbon source, and vitamins, with or without the required amino acid. The loss of radioactivity from the labeled lipid pool was followed by removing 10 ml aliquots at various time intervals, extracting and analyzing as previously described.

The rate of breakdown and renewal of phospholipids could be most accurately determined by using a dual labeling technique with "P; and "P. These experiments were carried out identically to the dilution experiment. The cells were first fully labeled with "P; (0.07 µCi per ml) and then resuspended in medium containing 3 mM phosphate and a suitable concentration of "P. (The "P specific activity was adjusted so that the net incorporation of radioactivity into growing cells was comparable to that in growing cells. This adjustment was necessary to get optimal assay of "P in the presence of "P.)

Radioassay—Radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer, model 3320. Aqueous solutions were counted in 10 ml of Triton-X counting solution (333 ml of Triton-X-100, 667 ml of scintillation grade toluene, 0.1 g of 1,4 bis(5 phenyloxazolyl)benzena (POPPO), and 5.5 g of 2,5-diphenyloxazole (PPO) (25) containing a total of 1 ml of water. Chloroform extracts were counted by removing an aliquot and pipetting directly into an empty scintillation vial. The samples were evaporated to dryness under a stream of nitrogen and counted in 10 ml of Triton-X counting solution.

Suitable gain settings for assaying "P and "P were chosen in the Triton-X counting solution, using an Engberg plot as a guide (26). An efficiency of 98% for "P and 24% for "P could be obtained in one channel. In a second channel, 2% "P efficiency and 76% "P efficiency were obtained by suitable adjustment of the window settings.

"P was assayed in 1:1 (v/v) chloroform-methanol (the window settings were 1000 to 0 at 45% gain) with an efficiency of 55%. Since the efficiency for "P in this solution was approximately 0% (27), we could check the H3PO4 supplied for "P. We observed less than 5% "P dpm in the H3PO4 used in the dual label experiments.

Other Assays—Protein biosynthesis was assayed by the method of Byfield and Scherbaum (28) by using 0.02 µCi of [G-3H]leucine per ml. RNA biosynthesis was determined the same way by using 0.1 µCi of [6-3H]uracil per ml, but in the presence of 10 µg of carrier uracil per ml.

RESULTS

RNA, Protein, and Lipid Biosynthesis in Relaxed and Stringent Cells—The incorporation of "P into lipid in relaxed and stringent cells—["C]Acetate can be incorporated into a variety of metabolites in E. coli, and the rate of that incorporation may be a complex function of the growth conditions. However, if the relaxed control were a general control of phospholipid biosynthesis one should be able to observe relaxed and stringent control of "P incorporation into phospholipid. This general observation is correct for at least two different pairs of relaxed and stringent organisms (Figs. 5 and 6). In these figures the incorporation of "P into lipid is normalized to a constant cell mass, emphasizing the rate of phospholipid synthesis in the relaxed cells; the incorporation is nearly equal to that of exponentially growing cells. Essentially identical results are obtained when "P incorporation is assayed using succinate (Fig. 7) or ribose as the carbon and energy source, although the total incorporation was less than when the cells are labeled in the presence of glucose as carbon and energy source.

Phospholipid Turnover in Relaxed and Stringent Cells—The incorporation into phospholipid may be a result of synthesis leading to a net increase in phospholipid content per unit of cell mass or, if the phospholipids are degraded as rapidly as they are formed, "P incorporation may take place with no net increase in the phospholipid content of the cells.

Perhaps the most direct way to measure a net increase in phospholipid content is to assay total extractable lipid phosphate directly. The results of such a determination on cells grown and sampled just as described for experiments employing ["C]acetate or "P; are shown in Fig. 8. These results are comparable to those obtained with ["C]acetate (Fig. 4) and support the stringent and relaxed control of phospholipid. If these data were represented as total lipid phosphate per unit of cell mass, relaxed cells, when starved for a required amino acid, in contrast to stringent cells would be found to increase in total phospholipid content per unit cell mass. The net increase in phospholipid in relaxed amino acid-deprived cells was much less than anticipated from "P incorporation data (Figs. 5 and 6). Thus the incorporation of "P into the lipids of stringent amino acid-deprived cells must be a consequence of turnover since there is no net increase of total phospholipid per unit cell mass.

The degradation of phospholipids can be assayed directly by observing the loss of "P from the phospholipids of cells that are fully labeled from growth on "P; and resuspended in unlabeled phosphate-containing medium. We found in these experiments, carried out just as before, that the phospholipids of relaxed deprived cells are apparently degraded at a much more rapid rate.
Fig. 2 (left). Protein synthesis. Assayed as [3H]isoleucine incorporation into acid-insoluble material in relaxed (CP-79) cells in the presence (+Leu, ●) and in the absence (−Leu, ○) of the required amino acid, leucine.

Fig. 3 (center). RNA synthesis. Assayed as [3H]uracil incorporation into acid-insoluble material in stringent (CP-78) cells in the presence (+Leu, ■) and absence (−Leu, □) of the required amino acid, leucine, and in relaxed (CP-79) cells in the presence (+Leu, ●) and absence (−Leu, ○) of the required amino acid, leucine.

Fig. 4 (right). Lipid synthesis. Assayed by the incorporation of [14C]acetate into CHCl₃-CH₃OH-soluble material in stringent (CP-78) cells in the presence (+Leu, ■) and absence (−Leu, □) of the required amino acid, leucine, and in relaxed (CP-79) cells in the presence (+Leu, ●) and absence (−Leu, ○) of the required amino acid, leucine.

Fig. 5 (left). Phospholipid synthesis. Assayed by incorporation of 32P, into CHCl₃-CH₃OH-soluble material relative to the culture absorbance at 620 nm at the time the sample was taken in stringent (CP-78) cells in the absence (−Leu, □) of the required amino acid, leucine, and in relaxed (CP-79) cells in the presence (+Leu, ●) and in the absence (−Leu, ○) of the required amino acid, leucine.

Fig. 6 (left). Phospholipid synthesis. Assayed by 32P incorporation into CHCl₃-CH₃OH-soluble material in a second set of stringent (PA-2) cells in the absence (−Arg, □) of the required amino acid, arginine, and in relaxed (PA-1) cells in the presence (+Arg, ●) and in the absence (−Arg, ○) of the required amino acid, arginine.

Fig. 7 (center). Phospholipid synthesis in succinate-grown cells. 32PO₄ incorporation into CHCl₃-CH₃OH-soluble material is determined for stringent (CP-78) cells in the absence (−Leu, □) of the required amino acid, leucine, and in relaxed (CP-79) cells in the presence (+Leu, ●) and in the absence (−Leu, ○) of the required amino acid, leucine.

Fig. 8 (right). Phospholipid content. Assayed as total phosphate in the ashed CHCl₃-CH₃OH-extractable lipid from stringent (CP-78) cells grown in the presence (+Leu, ■) and absence (−Leu, □) of the required amino acid, leucine, and in relaxed (CP-79) cells grown in the presence (+Leu, ●) and absence (−Leu, ○) of the required amino acid, leucine.
than relaxed exponentially growing cells or stringent deprived cells.

A dual label experiment with cells grown under conditions identical with the above compared the extent of synthesis and degradation simultaneously. The cells were grown for 10 or more generations in medium containing $^{33}$P, of known specific activity, thus ensuring that the phospholipid phosphodiester phosphate should be of essentially the same specific activity as the medium.

We fractionated these fully labeled phospholipids on silica gel-loaded paper. The distribution of $^{33}$P was as follows: 13.0% in phosphatidylglycerol, 69.2% in phosphatidylethanolamine, 8.6% in cardiolipin, and 8.9% in unknown Compound X (Fig. 1). There were no significant differences in the lipid composition from PA-1 and PA-2. These values are in agreement with values obtained previously with E. coli K-12 strains (21, 22).

The cells fully labeled with $^{33}$P were carefully washed at 37° and resuspended at 37° in medium containing $^{33}$P, of known specific activity. The rate of phospholipid breakdown (loss of $^{33}$P) and phospholipid synthesis ($^{33}$P incorporation) was observed simultaneously by sampling at suitable time intervals, extracting, and fractionating on silica gel-loaded paper. Data from such an experiment on stringent and relaxed cells, in the presence and absence of a required amino acid, are given in Figs. 9 to 17.

Relaxed cells, when deprived of a required amino acid degrade phosphatidylethanolamine at a very rapid rate (loss of $^{33}$P in Fig. 9) compared with growing cells (Fig. 10) or stringent cells deprived of a required amino acid (Fig. 11). It is also apparent ($^{33}$P + $^{32}$P, Fig. 9) that in relaxed cells starved for the required amino acid, the rate of $^{33}$P uptake only slightly exceeds the rate of $^{32}$P breakdown in phosphatidylethanolamine. This observation is very striking in view of the known stability of phosphatidylethanolamine in growing cells under a variety of conditions (19, 29-31).

Phosphatidylglycerol was also broken down at a rapid rate in the relaxed cells deprived of an essential amino acid (Fig. 12) but this behavior is relatively not much different from growing cells (Fig. 13) or stringent amino acid deprived cells (Fig. 14).

When either relaxed or stringent cells were resuspended in the absence of a required amino acid, nearly half of the radioactivity of cardiolipin was lost (Figs. 15 to 17). The remaining portion seems to be relatively stable. Cardiolipin was actively resynthesized in the relaxed amino acid-deprived cells (Fig. 15).

Since the procedures used involve chloroform-methanol extraction of both cells and medium at each time point, the changes indicated above all involve conversion of phospholipid phosphodiester phosphate into non-lipid, water-soluble forms and not simply the loss of phosphate-containing lipid from the cells into the medium.

**DISCUSSION**

Sokawa et al. (14) first reported stringent and relaxed control of lipid synthesis in E. coli. More recently, results were presented (17) indicating equivalent rates of incorporation of [14C]-acetate into lipid in stringent and relaxed cells whether they are deprived or supplemented with a required amino acid, i.e. stringent and relaxed control of lipid synthesis was contraindicated.

In more recent work, the latter group has found that a simultaneous downshift in aeration was imposed on the culture at the same time that these cells were deprived of the required amino acid. When the aeration (shake-rate) was kept constant (adequate for maximal growth) the results reported by Sokawa et al. (14) were obtained. We have verified these observations in our laboratory, observing a marked decrease in the rate of [14C]acetate into lipid when the agitation of a rapidly shaken culture is greatly slowed at the same time that the [14C]acetate is added. However, we find no major effect of aeration or the lack of it on the relative.

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Fig. 12 (left). Phosphatidylglycerol synthesis (32P) and breakdown (32P) and the sum of 32P and 33P lipid in relaxed (PA-1) cells in the absence of the required amino acid, arginine.

Fig. 13 (center). Phosphatidylglycerol synthesis (32P) and breakdown (32P) and the sum of 32P and 33P lipid in relaxed (PA-2) cells growing exponentially in the presence of the required amino acid, arginine.

Fig. 14 (right). Phosphatidylglycerol synthesis (32P) and breakdown (32P) and the sum of 32P and 33P lipid in (PA-1) cells in the absence of the required amino acid, arginine.

Fig. 15 (left). Cardiolipin synthesis (32P) and breakdown (32P) and the sum of 32P and 33P lipid in relaxed (PA-1) cells in the absence of the required amino acid, arginine.

Fig. 16 (center). Cardiolipin synthesis (32P) and breakdown (32P) and the sum of 32P and 33P lipid in relaxed (PA-1) cells growing exponentially in the presence of the required amino acid, arginine.

Fig. 17 (right). Cardiolipin synthesis (32P) and breakdown (32P) and the sum of 32P and 33P lipid in (PA-2) cells in the absence of the required amino acid, arginine.

Figures 12 to 17 illustrate the incorporation of radioactive precursors into phospholipids of E. coli strains PA-1 and PA-2. The results indicate that the synthesis of phospholipids is controlled by the presence or absence of the required amino acid, arginine, in the growth medium.

Unpublished experiments.

Acetyl-CoA is synthesized endogenously by these organisms from the carbon source, and the intracellular specific activity of the acetyl-CoA actually used for lipid biosynthesis must depend on the relative amount of intracellular [14C]acetyl-CoA and endogenous acetyl-CoA. It seems possible that different conditions of growth (or nongrowth) might alter this specific activity. Thus, we employed radioactive phosphate in our subsequent experiments since the above arguments about the intracellular specific activity seem less relevant for phosphate as a precursor of phospholipids (the cell must normally obtain all of its phosphorous from the medium). As we will subsequently indicate, essentially equivalent results (i.e. some net accumulation of phospholipid in relaxed cells deprived of a required amino acid) can be obtained by using exogenous [14C]acetate and radioisotopic P1 (when turnover is taken into consideration) and when net phospholipid synthesis is assayed directly by determining changes in lipid phosphate colorimetrically after ashing.
The minimal rate of $[^{14}C]$acetate incorporation into lipid seen in stringent amino acid-starved cells may reflect a small amount of turnover or exchange (see below), since direct measurement of phospholipid accumulation by assaying for phospholipid phosphate afterashing shows no accumulation in the stringent cells under these conditions. A comparison of the relative magnitude of phospholipid accumulation with the magnitude of incorporation of radioisotopically labeled uracil into stable RNA in relaxed cells deprived of a required amino acid indicates a larger accumulation of RNA than of phospholipid. Stringent control of the accumulation of either metabolite is equally effective.

The marked incorporation of $[^{32}P]$ into the phospholipids predominantly represents turnover with only some net synthesis in relaxed cells deprived of a required amino acid, as shown by several lines of evidence. (a) The relative amount of $[^{32}P]$ incorporated into phospholipid was much greater than the amount of $[^{14}C]$acetate incorporated into lipid. (b) Quantitative assay of the lipid phosphate indicated no accumulation of phospholipid per unit of cell mass except in the relaxed amino acid-deprived cells; the lipid phosphate accumulation in the latter cells was of the same relative order of magnitude as the $[^{14}C]$acetate accumulation. (c) Synthesis and turnover of the individual phospholipids from stringent and relaxed cells were simultaneously determined in a dual label experiment. Phosphatidylethanolamine in relaxed cells fully labeled from $[^{32}P]$; and deprived of a required amino acid loses $[^{32}P]$ at nearly the same rate as exogenous $[^{32}P]$ is incorporated. Little turnover is observed for this phospholipid in stringent, amino acid-deprived cells or in growing cells. Phosphatidylylycerol synthesis and breakdown occurs to the same extent in relaxed, amino acid-deprived cells and in growing cells. In stringent cells, phosphatidylylycerol is broken down and resynthesized but it does not accumulate. Nearly one-half of the initial label in cardiolipin was lost in the first 30 min when either relaxed or stringent cells were deprived of a required amino acid. Cardiolipin was actively resynthesized in relaxed, amino acid-deprived cells.

Since phosphatidylethanolamine makes up 69% of the total phospholipid, the observed changes in over-all phospholipid metabolism in relaxed, amino acid-deprived cells probably largely reflect the turnover of phosphatidylethanolamine. This phospholipid is normally rather stable (19, 29-31) compared with cardiolipin or phosphatidylylycerol (19, 21, 29-31). Synthesis and breakdown of phosphatidylethanolamine in the absence of a required amino acid then appears to be a direct consequence of a lesion in the rel gene as is RNA accumulation (1-4). The precise sequence of enzymatic events leading to the loss of phosphate from any of the phospholipids, indeed the biological significance of such a process to the bacterium, is still an open question (32-34).

Two recent proposals have been made which would permit one rel gene product to effect a number of metabolic processes. If the stringent cell no longer transported a source of carbon and energy into the cell (13), all energy-requiring processes would cease. We have shown that amino acid deprivation of relaxed and stringent cells has essentially identical effects on phospholipid metabolism, whether the carbon and energy source is glucose, succinate, or ribose. Ribose and glucose are transported by energy-dependent, constitutive systems (35), whereas succinate apparently enters via a facilitated diffusion (36). If stringent control were an effect on transport, it would have to affect all three of the above transport systems in a similar manner.

A decreased rate of glucose phosphorylation was consistent with the idea of energy limitation (37), but close examination of the nucleotide content of the cell as a general indicator of energy level was not entirely consistent with that idea (9-12). Instead, an unusual nucleotide, guanosine tetraphosphate, was discovered (38), proposed as an inhibitor of RNA synthesis (39) and perhaps as the general metabolic inhibitor, i.e. the rel gene product. Recent work is now inconsistent with guanosine tetraphosphate being the rel gene product (40-44), although it may play some role in the stringent and relaxed control phenomenon.

Among the processes under stringent and relaxed control, lipid biosynthesis (45, 46), transport (13), and DNA synthesis (47) are all processes known to proceed in intimate association with the cell envelope. One might even argue for the inclusion of DNA-dependent RNA transcription in this category, given the large gaps in our knowledge of the mechanisms for the transcription of stable RNA or mRNA, or both, and the known association of DNA with the membrane (48-50). A major perturbation of the structure of the cell envelope which might arise from the rapid breakdown and resynthesis of the major structural phospholipid, phosphatidylethanolamine, might have a marked effect on some or all of these processes.

We probed possible alterations in the cell envelope by electron microscopy in collaboration with Dr. J. M. Shively and Dr. J. W. Greenawalt. After glutaraldehyde and osmium tetroxide fixation, we observed the same cell wall thickening for amino acid-deprived cells whether they were relaxed or stringent. Similar changes were seen earlier by C. Morgan as given in (6) for relaxed, amino acid-deprived cells, but pictures of stringent, amino acid-deprived cells were not shown for comparison. Streptococcus faecalis cells also exhibit cell wall thickening under conditions of unbalanced growth (51).

A more conservative hypothesis would relate stable RNA and phospholipid metabolism through a common function of the rel gene. We recall that phosphatidylethanolamine is the preferred substrate for the synthesis of the cyclopropane fatty acids (52); the origin of the methylene group is methionine (53). One hypothesis relates the stability of this phospholipid in stationary phase to the presence of newly synthesized cyclopropane fatty acids (23, 54). We are intrigued in this connection by reports that the RNA which accumulates in relaxed cells starved for a required amino acid is much less methylated than usual (55-57). Thus a disturbance in one-carbon metabolism mediated by the rel gene may be a possible cause for the phosphatidylethanolamine turnover and possibly the other manifestations of relaxed and stringent control. We will test this new hypothesis in our laboratory shortly.

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