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METABOLIC CONSEQUENCES OF THE REL GENE IN ESCHERICHIA COLI

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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To Robert Virginia, Bobby, Sherby, Bruce, Alberta, Judith, Cecelia, Jacob, Ophelia, Cuba, Harry, Becky, Baha, Peggy, Gwen, Fitzgerald, Tawfiq and all my brothers and sisters everywhere.

Abstract

METABOLIC CONSEQUENCES OF THE REL GENE IN ESCHERICHIA COLI

by

Linda Meade

Advisor: Professor Burton Tropp

Two pairs of strains that differ in the rel-1 locus were compared for their ability to synthesize protein, RNA, and lipid in the presence or absence of an essential amino acid. In the absence of the essential amino acid, protein synthesis ceased in both relaxed control (rel^-) and stringent control (rel^+) cells, and RNA synthesis ceased only in the relaxed cells. Lipid synthesis continued at an approximately equal rate in the presence or absence of the essential amino acid in both relaxed and stringent cells. Analysis of the lipid extracts showed a decrease in cardiolipin in relaxed cells deprived of the essential amino acid. Otherwise, the distribution of the lipid was not affected by the absence of the essential amino acid in either the relaxed or stringent cells and was quite similar in both types of cells. The fatty acid distribution also appeared unaffected. These results are consistent with the view that the control of RNA synthesis cannot be simply at the level of nucleoside triphosphate availability.

The synthesis of RNA, protein and total lipids in two strains of E. coli isogenic except for the rel gene was assayed at two different speeds of agitation by the incorporation of radioactive substrates. Total lipid synthesis is not regulated by the rel gene at either speed. The distribution of phospholipids and the pattern of total lipid synthesis, however, are affected by the shaking speeds. During a shift down in shaking speed, incorporation of ^{14}C-2-acetate into the neutral lipid fraction from each strain increases, and addition of valine causes no inhibition of lipid synthesis. The characteristic phenotypic responses of RNA and protein syntheses to amino acid deprivation were manifested in both strains at either speed.

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INTRODUCTION

1. The stringent and relaxed phenomena

In 1952, an investigation by Sands and Roberts of protein and nucleic acid syntheses in an Escherichia coli auxotroph requiring tryptophan and histidine revealed the existence of a regulatory connection between ribonucleic acid and protein syntheses. When a culture of the auxotroph was deprived of either essential amino acid, net protein synthesis virtually ceased, and net RNA synthesis was drastically reduced (1). Since neither of the amino acids removed was a precursor of nucleic acids, they reasoned that this inhibition of RNA synthesis must be a consequence of the cessation of protein synthesis. Strains exhibiting this dependence of RNA synthesis on protein synthesis were subsequently called stringent (2).

In 1954 Borek, Ryan and Rockenbach reported preliminary results of an investigation of the enhanced resistance to ultraviolet radiation exhibited by lysogenic organisms deprived of an essential amino acid. A methionine auxotroph of E. coli accumulated large quantities of RNA during methionine starvation (3). This mutant, K12 W6, methionine $^-$ biotin $^+$, evidently arose as a spontaneous mutation of E. coli 58-161 methionine $^-$ biotin $^-$. This phenomenon of continued RNA synthesis and accumulation in the absence of net protein synthesis, brought on by the removal of an essential amino acid, has been termed relaxed control (2). E. coli W6 was used extensively in early studies of genetic recombination. Several Hfr strains also used in these studies were derived from this strain and possess the relaxed character. Addition of chloramphenicol, an inhibitor of peptide bond synthesis (4), and thus

of protein synthesis, to starved stringent cells reverses the inhibition of RNA synthesis and accumulation. This chloramphenicol effect strongly resembles the behavior of starved relaxed cells (5).

Amino acid starvation can be induced by depriving an auxotroph of an essential amino acid, by adding an amino acid analogue which interferes with synthesis of a common amino acid to a culture prototrophic for that amino acid and by addition of valine to K12 strains of E. coli. Expression of the stringent or relaxed phenotype is not dependent upon the absence of any particular amino acid, since the phenotype is observed in multiple auxotrophs, regardless of which essential amino acid is absent. The magnitude of the stringent response is dependent upon the bacterial genotype (6) and the number of amino acids removed (2). A ten to twenty-fold decrease in RNA accumulation is usually observed in starved stringent cells.

2. Genetic studies of the loci for the rel gene and for stable RNA

A single genetic locus (2), the rel gene, mapping between minutes 53 and 54 of the E. coli genome (8), is responsible for the amino acid regulation of RNA synthesis. Bacterial strains with mutations at this locus possess the rel^- genotype and exhibit relaxed control of RNA synthesis, as opposed to the stringent control maintained by the wild-type allele. The genotype of the mutant allele is designated rel^-, and the phenotype is designated RC^{rel} or relaxed (8). The genotype of the wild-type allele is rel^+, and the phenotype is RC^{str} or stringent (8). The rel^- mutation is recessive (9). Partial diploids with genotypes of $rel^-/F'rel^+$ and of $rel^+/F'rel^-$ are phenotypically stringent (9). Introduction of an F' carrying the $rel\text{-}1^-$ gene from W6 into other relaxed

mutants does not produce the RC^{str} phenotype by complementation (6,10).

An additional E. coli mutant which is able to accumulate RNA in the absence of net protein synthesis, but which maps at a locus distinctly different from the classical rel-1 gene, has recently been partially characterized (11). The temperature sensitive mutation isolated by Atherly and Suchanek maps near minute 34, and results in an altered phenylalanyl tRNA synthetase (11).

Current investigations suggest that the genes coding for stable RNA (rRNA, tRNA) may form a polycistronic operon. Consideration of the molecular weights of the E. coli genome, 16S rRNA and 23S rRNA, and of the extent of hybridization of rRNA to DNA suggests that a maximum of six genes for 16S rRNA (12), six genes for 23S rRNA (12) and six genes for 5S RNA (13) are present on the E. coli chromosome. The 16S rRNA genes map very closely to one another (14) and together with the 5S gene(s), may be clustered in a single chromosomal region (13). When RNA chain initiation is inhibited by rifampicin, the labeling patterns of the stable RNA isolated indicated that the genes for 16S, 23S and 5S RNA may form three cistrons of a single operon (13). When initiation is synchronized by restoration of the essential amino acid to a starved strain, the 16S rRNA appears first before the 23S RNA (15). The order of transcription after a single initiation by RNA polymerase might then be 16S, 23S, 5S (14, 15). The primary transcription product of the rRNA genes has not been identified, although slightly larger precursors of 16S and 23S are known (16).

3. Effects of the rel gene on stable RNA

During starvation for an essential amino acid, the synthesis of stable RNA, (rRNA, tRNA) virtually ceases in stringent strains within one minute (17). Starved relaxed strains exhibit a 25% decrease in their rates of overall RNA synthesis, and can still accumulate stable RNA at near normal rates during the first hour of starvation (18). During starvation of relaxed strains, the rRNA which accumulates differs from the 16S and 23S rRNA found in mature ribosomes in its sedimentation characteristics and in its elution pattern from methylated albumin-kieselguhr columns (19). This rRNA can be converted to mature rRNA without undergoing extensive degradation (19). The rRNA which accumulates during methionine starvation is unmethylated (20). The rRNA which accumulates during lysine (19), histidine (21), and arginine (19,22) starvation is methylated to 50% of normal levels (18). The 16S rRNA synthesized during arginine starvation contains 28% of its unstarved level of methyl groups, and the 23S contains 62% of its unstarved level (23). The effect of submethylation on the biological activity of the rRNA has not been conclusively defined. Methionine starvation could lead to unmethylated rRNA by preventing formation of S-adenosyl-methionine, the rRNA methylating agent. The cause of formation of submethylated RNA during deprivation of arginine, lysine and histidine is still obscure.

The rRNA from relaxed strains accumulates as ribonucleoprotein particles called relaxed particles. These particles sediment on sucrose density gradients at 14-16S in 10^{-4} M Mg^{+2} (23) and at 20, 30 and 45S in 10^{-2} M Mg^{+2} (24,25). The former Mg^{+2} concentration, although more

physiological, seems to permit extensive degradation of larger relaxed particles (23). The particles are extremely sensitive to pancreatic RNase (24). They contain considerable amounts of protein which may result in part from contamination during isolation by protein derived from ribosomes (25), since nonribosomal proteins can also absorb non-specifically to free nascent rRNA (26). The particles can be converted into mature ribosomes without extensive degradation of RNA or dissociation of RNA and protein, if protein synthesis be restored (27).

The synthesis of tRNA is also drastically inhibited in stringent strains within one minute of starvation (17). Unmethylated tRNA is synthesized during methionine starvation (20). Investigation of the effect of the lack of methylation on the charging or transfer of amino acids, and on codon recognition has been complicated by difficulties in purifying the unmethylated tRNA. Contradictory results have been obtained (28-32) and further experimentation is needed. Elution profiles from MAK columns indicate that all tRNA of a methionine starved relaxed strain may not be submethylated. Methionine starvation of E. coli W6 yielded submethylated leucyl-tRNA, but valyl-, glutamyl- and lysyl- tRNA appeared fully methylated (33).

Intracellular levels of charged tRNA, measured as the fraction of total amino acid acceptance capacity protected from periodate oxidation, (34,35) are maintained in all tRNAs of leucine-starved stringent and relaxed strains except leucyl-tRNA. In leucyl-tRNA, the level of charging decreases from 70-97% to 10-40% and is maintained at that level by protein turnover (34,35). There is no loss of specificity of amino acylation during starvation of either genotype for the essential amino acid (36).

4. Non-coordinacy of regulation of RNA synthesis

Although it is clear that the regulation achieved by the rel^+ allele drastically inhibits the synthesis and accumulation of methylated stable RNA, the extent of control over the synthesis of normally unmethylated (37) mRNA, has until recently, been less clear. Edlin and Broda have pointed out some of the errors inherent in early techniques of measurement of highly unstable mRNA (38). Direct hybridization studies of DNA and RNA, using a large excess of RNA, may not permit complete hybridization of all mRNA species present. Studies of synthesis of a single species of mRNA may involve effects peculiar to that particular operon. Radioactive pulses of relatively long duration may not permit detection of mRNA with shorter half-lives. Measurements of functional mRNA activity may exclude large quantities of inactive molecules (38). In spite of these difficulties, extensive evidence affirming the continued synthesis of mRNA in starved stringent cells has been obtained. The residual RNA made by stringent cells during amino acid starvation has been characterized as predominantly functional messenger by its decay kinetics (39,40), hybridization characteristics (41-43) and its specificity in the synthesis of alkaline phosphatase (44) ornithine transcarbamylase (45), β-galactosidase (46) and tryptophan synthetase A protein (42,47).

Lazzarini and Winslow measured rates and compositions of RNA synthesized and accumulated in starved stringent strains. By using rapid pulses of label and competitive hybridization, they avoided some of previously mentioned difficulties. They observed persistent and substantial rates of RNA synthesis, but an apparent preferential inhibition of RNA accumulation. Ribosomal RNA synthesis was reduced 90% and mRNA synthesis was reduced 60%

by starvation (48). Stamato and Pettijohn, using competitive hybridization between rRNA and DNA which had been selectively enriched with rRNA cistrons, found that the fraction of RNA synthesis which results in rRNA decreases 82% upon starvation of a stringent strain (49). Although stable RNA synthesis is inhibited more than mRNA synthesis, the observed inhibition of both classes suggests that the regulation of RNA synthesis possesses both coordinate and non-coordinate character.

5. Regulation of RNA synthesis during shift-down

The amount of intracellular RNA in bacteria is exponentially related to the growth rate (50). Following shift-down or transfer of bacteria from rich nutrient media to poor nutrient media, the growth rate is seriously inhibited. Shiftdown may be achieved by culturing bacteria in medium supplemented with all 20 common amino acids, and then transferring them to the same medium supplemented with only those amino acids essential for growth. It may also be achieved by replacing a carbon source supporting rapid growth (glucose) with one supporting slower growth (lactose) or by replacing a nitrogen source supporting rapid growth (ammonium chloride) with one supporting slower growth (tryptophan). Shiftdown in carbon or nitrogen source causes a rapid and drastic reduction in net RNA synthesis (51), and a gradual decline in net protein synthesis (50), in both relaxed and stringent strains. These changes persist until the cell's RNA to protein ratio stabilizes at a lower value, characteristic of cells growing in poor medium (52). Starvation for an essential amino acid, in contrast, causes an abrupt cessation of net protein synthesis (1). A shiftdown induced by removal of non-essential amino acids, however, affects relaxed and stringent strains differently.

Relaxed strains, unlike stringent strains, do not effectively depress RNA synthesis during amino acid shiftdown, and continue to accumulate RNA (51).

6. Hypotheses of the mechanism of control exerted by the rel gene

The isolation of phenotypically relaxed mutants of E. coli, mapping at loci distinctly different from the locus of the classical rel-1 gene, suggests that a series of reactions may be involved in in vivo RNA control (11). Major hypotheses seeking to explain the mechanism of amino acid control of RNA synthesis are:

Control by tRNA

Control by ribosomes or a ribosomal component

Control by polyamines

Control by nucleotides or their derivatives

CONTROL BY tRNA

Stent and Brenner proposed in 1961 that RNA synthesis is inhibited by the uncharged tRNA present when a required amino acid is absent (2). Uncharged tRNA was proposed to be a repressor of RNA polymerase, and to become inactive when charged with its corresponding amino acid. But subsequent in vitro studies of the inhibition of RNA polymerase by charged and uncharged tRNA showed that although uncharged tRNA was more inhibitory than charged tRNA (53), there were no differences attributable to the rel gene between the tRNA, RNA polymerase or DNA templates isolated from relaxed and stringent cells (54). Levels of acylation of different tRNAs from the same cells varied. Relaxed and stringent cells cultured under the same conditions possess similar levels of charging of most classes of tRNA (34). The extent of charging showed no correlation with the rate of RNA synthesis (35). Starvation for an amino acid does not completely deacylate the corresponding tRNA, as residual levels of leucyl-, histidyl-, arginyl-, isoleucyl-, and methionyl- tRNA have been observed during starvation for leucine (35,55), histidine, arginine and threonine (55) and methionine (56) respectively.

During leucine starvation, 6-9% of the total leucyl-tRNA acceptor capacity of a stringent leucine auxotroph appears to be complexed to some metabolite other than an amino acid. This complex is not observed in leucine-starved relaxed strains. A specific species of leucyl-tRNA is involved in the formation of this complex (55). This species of tRNA is preferentially absent from the ribosomes during exponential growth (57). The complex can be destroyed by a crude enzyme preparation (55).

Yegian and Stent suggest that the metabolite may be a peptide, an N-acyl amino acid derivative, a nucleotide, or an exchangeable terminal tRNA nucleoside (55).

Amino acid control of RNA synthesis is not dependent upon the total level of free amino acids (58,59), as conclusively shown by Fangman and Neidhardt, or of uncharged tRNA (60). A parafluorophenylalanine-resistant E. coli mutant with an altered phenylalanyl-tRNA synthetase, unable to activate and thus incorporate parafluorophenylalanine (58) cannot achieve net synthesis of RNA or protein in the absence of phenylalanine, although the other 19 common amino acids and the analogue were present (59). Stringent temperature-sensitive valyl-tRNA synthetase mutants, which cannot charge valyl-tRNA at the restrictive temperature are also unable to accumulate RNA at that temperature. Relaxed strains of this mutant are unable to charge valyl-tRNA, but are able to accumulate RNA at the restrictive temperature. Thus stringent amino acid control appears to require the presence of a full complement of charged tRNAs (60).

Control of RNA synthesis may reside in a translational step involved with or subsequent to the charging reaction. Freundlich and Williams have investigated the charging reaction in a stringent valine auxotroph of E. coli (61). Using two analogues of valine: DL-alpha-aminobutyrate, and DL-threo-beta-chlorobutyrate, they confirmed the earlier work showing that valine must be attached to its specific tRNA to support RNA synthesis in stringent cells (60). The former analogue can be activated by valyl-tRNA synthetase to L-alpha-aminobutyryl-AMP, but cannot be transferred to tRNA, and does not support RNA or protein synthesis in the starved stringent

valine auxotroph. The latter analogue can be activated and transferred to tRNA, and can support RNA synthesis in the absence of protein synthesis. Trimethoprim, an inhibitor of protein synthesis, blocks the synthesis of 10-formyltetrahydrofolate (62) and thus inhibits the formation of N-formyl-methionyl-tRNA (63), the primary initiator of protein synthesis in E. coli. Trimethoprim added to minimal media cultures supplemented with purines, pyrmidines and amino acids inhibits RNA synthesis in stringent but not in relaxed strains (64). This result indicates that RNA synthesis in stringent strains depends either on some reaction following charging of amino acids to tRNA, as formylation occurs after methionine is charged to its specific tRNA, or on the synthesis of N-formyl-methionyl-tRNA and thus the initiation of protein synthesis (63,64). The results also indicate that relaxed cells do not require N-formylmethionyl-tRNA, and presumably any initiation of protein synthesis to synthesize RNA.

The rel$^-$ mutation may cause a translational defect. Stringent arginine auxotrophs exhibit derepressed ornithine transcarbamylase synthesis in the absence of arginine, but relaxed cells, isogenic except for the rel gene, produce practically no enzyme (45). The relaxed strains can be shown to contain an abundance of mRNA specific for the enzyme, but seem unable to translate it in the absence of arginine (45). Similar translational defects have been reported in relaxed cells for the synthesis of β-galactosidase in the absence of glucose and of histidine (9), alkaline phosphatase in the presence of valine (44), D-serine deaminase during partial amino acid starvation (45), and tryptophan synthetase in the absence of tryptophan (42). This difference in rates of enzyme synthesis is not a result of repression of mRNA synthesis. Specific enzymes can probably be synthesized during starvation of stringent cells from amino acid from protein degrad-

ation, since protein synthesis continues at 5% of the normal rate, presumably as a result of protein turnover. Hall and Gallant prepared by transduction a pair of strains, isogenic except for the rel gene, which were genetically derepressed and constitutive for alkaline phosphatase synthesis. The observed translational defect persisted in relaxed strains even in the absence of a functional phosphatase repressor (44).

Sokawa, Sokawa, and Kaziro have also obtained data supporting a relationship between an alteration in the protein synthesizing machinery and the relaxed phenotype (47). Induction of the synthesis of tryptophan synthetase as a result of amino acid shiftdown exhibited a long lag in relaxed strains, but was only briefly depressed in stringent strains (47). Since synthesis of mRNA for the tryptophan operon is known to occur to the same extent in starved relaxed and stringent cells (41, 42, 47), they also conclude that the defect must be translational.

Hall and Gallant propose that either relaxed cells do not translate their mRNA, or they translate it to produce altered inactive enzymes. They measured the quantities and activities of β-galactosidase inducible in relaxed and stringent cells in the presence and absence of an essential amino acid (65). In the presence of the amino acid relaxed and stringent strains synthesize similar amounts of enzymes with similar activities. During arginine starvation, the relaxed strain synthesized one-third as much β-galactosidase as the stringent strain, and the enzyme was less than one-third as active. β-Galactosidase was assayed by mixing an aliquot of an induced culture with O-nitrophenylgalactoside, and measuring the change in absorbency at 420 nm. The active β-galactosidase made by starved relaxed cells has an increased thermolability (65). The nature of the defect, however, is still obscure.

CONTROL BY RIBOSOMES OR A RIBOSOMAL COMPONENT

Morris and DeMoss have proposed that free ribosomes, or some ribosomal component inhibits the synthesis of stable RNA or ribosomes (66). The hypothesis is based upon the observation that the inhibition of RNA synthesis in an amino acid starved stringent auxotroph was accompanied by a dissociation of polysomes. Addition of the essential amino acid caused an immediate restoration of normal levels of RNA and protein synthesis, and a slower restoration of polysome levels. Addition of chloramphenicol to the amino acid-starved cells immediately reversed the inhibition of RNA synthesis and the degradation of polysomes, without the occurrence of protein synthesis (66). However, polysomes in other stringent strains have been reported stable during amino acid starvation (67,78). This conflict in results has been resolved by Ron (69). She confirmed a earlier suggestion by Friesen (67) that the conflicting results might be due to differences in strains or in amino acids removed. She observed that the more frequently the amino acid appears in cellular protein, the less stable the polysomes during its removal.

Polysomes in stringent K-12 strains are thus unstable during leucine (66,69) or arginine (66,69,70) starvation but are stable during histidine (67,69) or threonine (69) starvation. Polysomes in relaxed cells possess an increased stability compared to stringent cells, even when starved for rare amino acids (69). This stability appears to be a direct consequence of the relaxed genotype, because transduction of the rel^- allele into a rel^+ recipient confers a greatly increased stability to the polysomes during starvation for common amino acids (71). Polysomes undergo continuous turnover in both stringent and relaxed cells, regardless of the amino acid removed (69). The decreased rate of polysome dissociation observed in

relaxed cells is of sufficient magnitude to account for their increased stability (69).

The rel$^-$ allele could influence polysome stability by causing an increase in synthesis of some ribosomal binding factor normally present in limiting concentrations, an increase in mRNA during amino acid starvation, or a modification in ribosome structure (72). Mahadik and Srinivasan recently isolated a protein associated with ribosomes which stimulates in vitro transcription of E. coli DNA by core polymerase 3-fold and by whole polymerase nearly 2-fold (73). The protein is found only in starved relaxed cells, and has been distinguished from sigma and M factors by sedimentation characteristics and stability at high ionic strengths (73). Relatively large amounts are required for stimulation, indicating that the protein is not catalytic. It contains no RNA, DNA or any detectable ribonuclease or deoxyribonuclease activity. Addition of rifampicin to the in vitro system before the factor blocks its stimulatory effect on RNA synthesis. Addition of rifampicin after initiation of RNA synthesis inhibits the stimulation 30%. The protein thus may be preferentially but not totally involved in chain initiation (73). Goodman, Manor and Rombauts have observed that during amino acid starvation, relaxed strains preferentially synthesize 3 ribosomal proteins at rates similar to those observed during exponential growth. Starved stringent strains synthesize a very small proportion of ribosomal proteins during starvation. Although some stringent ribosomal proteins appear to be preferentially labeled, they are synthesized in much smaller amounts than those proteins synthesized preferentially in relaxed strains (74).

Inhibition of RNA synthesis by rifampicin and the subsequent decrease in polysomes (75) indicates a requirement for RNA synthesis to maintain polysome stability. Glucose starvation of both relaxed and stringent strains causes polysomes to be degraded to 70S ribosomes with a half-life very similar to that of mRNA (75). Restoration of glucose results in the immediate reformation of polysomes from pre-existing ribosomes and newly synthesized RNA (76). This data suggests that the amount of free mRNA available may influence polysome stability. Nakada has shown that relaxed cells accumulate mRNA during amino acid starvation, but do not translate it (77, 78). This accumulated mRNA could contribute to the observed polysome stability in starved relaxed cells.

At present, however, there is no clear evidence that the breakdown of polysomes and the subsequent production of free ribosomes prevents RNA synthesis.

CONTROL BY POLYAMINES

Putrescine and spermidine, the two major polyamines in E. coli interact in several ways with different classes of RNA in vitro. Both polyamines can partially substitute for magnesium by stimulating the binding of aminoacyl-tRNA to ribosomes at suboptimal (for in vitro), but physiological magnesium concentrations (79). In these assays, spermidine was much more effective than putrescine (79). Both a mixture of putrescine and spermidine (80) and spermidine alone (81), stimulate the magnesium dependent formation of 70S ribosomes from subunits. Spermidine stimulates initiation and elongation of RNA chains in vitro during transcription of native calf thymus DNA by a crude E. coli RNA polymerase preparation (82). A more purified enzyme preparation, however, gave less stimulation (82). Low concentrations of spermidine inhibit enzymatic degradation of ribosomes (83). Spermidine is found associated with tRNA and rRNA when isolated from cells under mild conditions (84). A polyamine-tRNA complex has recently been implicated as the active acceptor of the amino acid from an enzyme-amino acid adenylate complex (85).

Raina and Cohen have observed that a correlation seems to exist between the incidence of unbalanced RNA synthesis and the accumulation of free spermidine (86). In a starved stringent auxotroph, intracellular RNA and spermidine accumulation cease, and intracellular putrescine increases. Chloramphenicol addition results in RNA accumulation, and a concurrent increase in free intracellular spermidine.

Addition of exogeneous spermidine to stringent bacteria also results in an increased accumulation of RNA, which is antagonized by putrescine. The finding that putrescine prevents the stimulation of RNA synthesis by spermidine led them to suggest that the ratio of putrescine to spermidine, rather than the concentration of spermidine alone, determines the cellular response (86).

Relaxed auxotrophs starved for the required amino acid exhibited an increase in free spermidine, which subsequently exerted feedback inhibition of putrescine biosynthesis; and continued RNA accumulation. The inhibition of putrescine biosynthesis is proposed to be a result of feedback inhibition by accumulated spermidine (87). Simultaneous starvation for arginine and uracil caused a cessation of RNA accumulation, but did not result in alterations in polyamine concentration. Net synthesis of spermidine continues at the same rate as before uracil starvation (87). This spermidine synthesized is acetylated and excreted. The above results also suggest that RNA accumulation is related to the intracellular spermidine concentration but the involvement of putrescine is less well-defined. Although stimulation of RNA synthesis by putrescine addition has been observed in an E. coli mutant whose polyamine synthesis is inhibited by arginine (88), this may only reflect the conversion of the putrescine to more active spermidine.

Abraham has suggested that polyamines can facilitate the removal of nascent RNA from a DNA-enzyme complex, by preventing random association of RNA to the complex, and that this effect is achieved by modifying the secondary structure of RNA (89). Support for this hypothesis comes from the observation that spermidine can dissociate a RNA - RNA polymerase complex, but not a DNA - DNA polymerase complex (90).

Ezekiel and Brockman (91) propose that spermidine decreases the rates of protein synthesis, and increases the rates of protein degradation, thereby increasing the levels of charged tRNA, and that this effect increases the rates of RNA synthesis. They observed that amino acid deprived E. coli show a stimulation in the rates of RNA and protein degradation upon addition of exogeneous spermidine. In unstarved cells,

spermidine inhibited protein synthesis (91). Relaxed cells, however, have considerably less protein turnover than do stringent strains (92). In addition, levels of charged tRNA, as discussed earlier, show no correlation to rates of RNA synthesis (35). The question of whether polyamines are a primary cause or a secondary effect of RNA control is as yet unresolved.

CONTROL BY NUCLEOTIDES OR THEIR DERIVATIVES

This hypothesis proposes that the cessation of RNA synthesis during starvation of a stringent strain is a result of limitation of nucleotide substrates for RNA polymerase. Since these substrates are also intimately involved with other metabolic pathways, the limitation of substrate would be proposed to be reflected in other metabolic pathways such as those of phospholipid biosynthesis.

Early measurements of the changes in intracellular levels of ribonucleoside triphosphate pools in relaxed and stringent E. coli, in response to amino acid starvation, yielded conflicting results. (93-99) These conflicts may have been due to differences in solvents used for extraction of nucleotides, or differences in culture conditions. Extraction of ribonucleoside triphosphates with formic acid was found to yield more accurate and reproducible results than extraction with trichloroacetic acid or perchloric acid (96, 97).

Subsequently amino acid starvation was shown to cause a rapid large decrease in GTP pools, a slower smaller decrease in ATP pools, and lesser decreases in UTP and CTP pools of starved stringent strains. Relaxed strains showed only slight variations in their ribonucleoside triphosphate pools during starvation (96). When RNA synthesis is inhibited directly by uracil starvation, a large increase in the pools of GTP and ATP is observed (96). The pool size would be expected to expand due to continued synthesis of the ribonucleoside triphosphates and their decreased utilization as RNA precursors. The higher concentrations of ribonucleoside triphosphates should then provide increased feedback inhibition of nucleotide biosynthesis, subsequently decreasing nucleotide pool levels and availability. However,

this blockage of RNA synthesis does not inhibit uptake of exogeneous label into ATP as expected.

In order to minimize metabolic effects resulting from amino acid starvation but independent of rel gene control, Edlin and Stent inhibited protein synthesis with trimethoprim, or by placing a temperature sensitive valyl-tRNA synthetase mutant at the restrictive temperature. In neither instance did they observe an obvious relationship between net RNA synthesis and ribonucleoside triphosphate pool levels in relaxed or stringent strains (98). However, in these experiments, the ribonucleotides were extracted with 5% TCA. This solvent, as mentioned earlier, gives less accurate and reproducible results than the formic acid, used by Cashel and Gallant (96).

The question of substrate limitation of RNA synthesis is approached from another perspective in Chapter 2 of this thesis.

Irr and Gallant observed that the phosphorylation of ribonucleosides and glucose is severely amino acid dependent, but independent of RNA synthesis (99). The incorporation of P_i into the intracellular pool of orthophosphate was only mildly amino acid-dependent. The reduction in the ability of crude extracts to form glucose-6-phosphate caused by amino acid starvation could be overcome by the addition of exogeneous ATP, suggesting that the rel gene may have a broader effect on cellular energy metabolism rather than just on RNA synthesis alone (99).

In 1969, a compound was discovered, the kinetics of whose appearance seemed to correlate with the rapidity, magnitude, and specificity of the relaxed response (100, 101). The compound was characterized as guanosine 5'-diphosphate, 3'- (or 2'-) diphosphate, ppGpp (102). Within seconds after amino acid starvation is created in stringent strains, there is a large increase in ppGpp levels often exceeding the pool levels of all

nucleotides except ATP, and an almost equimolar decrease in the GTP pool levels (45). These changes occur even before inhibition of RNA synthesis can be detected (101).

Cashel (103) assayed the in vitro interaction of ppGpp with RNA polymerase and reports that ppGpp inhibits the incorporation of UMP into RNA by about 40-50% in diverse assay conditions. His results also suggest that ppGpp preferentially inhibits the synthesis of RNA chains beginning with GTP. Although this inhibition is not complete, he suggests that the in vivo effect of ppGpp may be to modify the specificity of RNA polymerase to allow the observed partially noncoordinate synthesis of RNA. The polycistronic nature of the rRNA operon mentioned earlier (12-15) makes attractive speculation on the interaction of ppGpp with the 16S promotor. Stamato and Pettijohn, using competitive hybridization between rRNA and DNA fragments enriched with rRNA cistrons, find that rRNA synthesis appears to be partially blocked at the initiation step in starved stringent strains (49). The nucleotide ppGpp has been reported to inhibit a protein factor psi_r (104). This factor has been suggested as a positive regulator of E. coli rRNA genes mediating their preferential transcription during balanced growth and their repression during amino acid starvation (105). No synthesis of rRNA by purified RNA polymerase was observed in vitro (105). More recent research has not been able to reproduce these results (106). E. coli RNA polymerase holoenzyme has been found to synthesize 7-14% rRNA without psi_r factor (106). Psi_r factor does not alter the proportion of rRNA in the total product (106). Guanosine tetraphosphate does not inhibit rRNA synthesis in the presence or absence of psi factors (106).

Gallant and Harada noted that once RNA synthesis has been directly inhibited in stringent cells the GTP pool remains at about half its normal size, instead of expanding as expected (107). They, therefore, studied ppGpp as a possible inhibitor of GTP biosynthesis. ppGpp is a strongly competitive and enzymatically specific inhibitor of the first step of the GTP pathway, the conversion of inosine monophosphate to xanthine monophosphate by inosine monophosphate dehydrogenase. Measurement of intracellular ppGpp levels in starved stringent strains show that ppGpp is present in sufficient quantity to seriously inhibit the enzyme (45, 108). The failure of inosine monophosphate to accumulate on amino acid starvation suggests that a block may exist even before IMP in the purine nucleotide biosynthetic pathway.

Both relaxed and stringent cells in balanced growth contain ppGpp. During exponential growth, the level of ppGpp in stringent cell is three times higher than the level in relaxed cells. Starvation causes a 20-fold increase in ppGpp levels in stringent cells, but has no effect on the levels in relaxed cells (45). The appearance of ppGpp in relaxed strains indicates that it is not a specific inhibitor of RNA polymerase which appears only during unbalanced growth, and whose production is decreased by the relaxed allele.

Other physiological conditions have been shown to give rise to ppGpp (109). Glucose starvation and KCN addition give much higher levels of ppGpp in stringent cells than even amino acid starvation. These rapidly attained high levels gradually decrease over a period of 60 minutes, but so do the levels attained during valine addition. In the cases of starvation for sulfur, or nitrogen, ppGpp levels increase slowly to a higher level after 60 minutes than those due to amino acid starvation.

There is no increase in ppGpp in a relaxed strain under any of the above conditions. The levels of ppGpp during amino acid starvation and shift-down may be strain-dependent (110). The role of ppGpp in regulation of RNA synthesis therefore, is as yet undefined.

7. A proposed intracellular site of RNA synthesis

Morphological studies have demonstrated the attachment of DNA to membrane in E. coli (111). Sedimentation and isotopic studies also suggest that the replication point of DNA is membrane-bound in vivo (112-114). Recently several investigators have been able to isolate membrane-DNA-RNA and DNA-RNA-protein complexes from E. coli which may more fully represent the in vivo situation than in vitro systems created by addition of separate components (115-117). These complexes contain high molecular weight DNA, nascent RNA, and RNA polymerase. The highly organized complex isolated by Stonington and Pettijohn is capable of synthesizing RNA at 20% of the in vivo rate. Pulse-chase experiments reveal that the RNA associated with the complexes is more rapidly labeled in vivo than the total intracellular RNA (115-117). The precursors of rRNA in E. coli appear first on the membrane and cosediment with it even after DNase treatment (118). Thus, the membrane may be the site of RNA synthesis.

The cell envelope of E. coli, composed of the cytoplasmic membrane, together with the cell wall (119), is the only membraneous structure in E. coli. The outer membrane contains a high proportion of lipopolysaccharide, and a low proportion of cytochromes and phospholipid (120). The inner membrane is rich in cytochromes, electron transport and other enzymes, and phospholipids (120). Phospholipids comprise the bulk of the lipids of E. coli. The major classes of phospholipids in E. coli membranes are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. Small amounts of other phospholipids such as phosphatidylserine and phosphatidic acid are also present. Most of the phospholipid biosynthetic enzymes appear to be membrane-associated (121-125).

The apparent proximity of sites of lipid, protein, and RNA synthesis, and the involvement of nucleotides ATP and CTP in both lipid and RNA synthesis led to an investigation of the effects of the rel gene on lipid synthesis detailed in this thesis. Other metabolic processes have also been investigated in order to determine if they are also affected by expression of the rel gene. These pathways have usually been studied in the hope of supporting one or another of they hypotheses which have been proposed to account for the biochemical mechanism of expression of the relaxed phenomenon.

E. coli lipids require ATP and CTP for their biosynthesis. The synthesis of lipids in relaxed and stringent strains was therefore investigated to determine if the rel gene exerted its regulation by effecting limitation of these nucleotide substrates, for if the substrates were limiting for RNA synthesis, lipid synthesis should also be affected. Under certain conditions, lipid synthesis was not affected by the rel gene. Some of the results of these investigations have been published and are described in detail in Chapter 2 of this thesis. While the above research was in progress, Sokawa, Nakao, and Kaziro published results concluding that lipid synthesis was under the control of the rel gene (126,127). Chapter 3 of this thesis describes investigations leading to the resolution of this discrepancy.

The research upon which this thesis is based shows that the relaxed phenomenon in E. coli is not affected by limitation of ATP and CTP, that the mild inhibition of lipid synthesis noted under some conditions is not a primary and direct consequence of the rel gene, that lipid synthesis can be uncoupled from RNA synthesis in stringent cells deprived of

required amino acid, and that the sensitivity of total lipid synthesis to amino acid deprivation and the relative distribution of ^{14}C-2-acetate in the major phospholipids are affected by the growth rate.

I. LIPID SYNTHESIS AND THE REL GENE

A. Introduction

It has recently been proposed that the difference in the control of net RNA synthesis may be related to the availability of ribonucleoside triphosphates (96,128). Several different laboratories have attempted to directly analyze the intracellular nucleoside triphosphate pools and to show a correlation between pool size and RNA synthesis (93, 96-98, 107, 128). At first, the results appeared to be in conflict with one another. When RNA synthesis is inhibited increases, decreases, and no changes in pool levels were reported. The partial noncoordinacy of regulation of RNA synthesis (41,42,48,129,130), indicates that the control could not be a result of a limitation of ribonucleoside triphosphate availability, which would affect all classes of RNA similarly. However, the proposal that net lipid synthesis continues in relaxed cells deprived of amino acids, but not in stringent cells (126) would certainly be consistent with control by nucleotides or by some other intermediate common to both synthetic pathways. Two pairs of strains that differ in the rel locus for their ability to synthesize RNA, lipid and protein in the presence and absence of a required amino acid were compared. The lipids synthesized by one of the pairs cultured under these conditions have been characterized.

B. Materials and Methods

CHEMICALS

^{3}H-6-Uracil (3.1-Ci/mole) was purchased from Schwarz Bioresearch, Orangeburg, N. Y. ^{3}H-L- Isoleucine (1.0 mCi/0.087mg) was a product of New England Nuclear, Boston, Mass. ^{14}C-2-Acetate (39 microcuries/mg) was purchased from Mallinckrodt Nuclear, St. Louis, Missouri. The scintillation fluid used in all the experiments for monitoring ^{14}C and ^{3}H contained 16 grams of 2,5-diphenyloxazole (PPO) and 0.8 grams of 1,4-bis (2-(5-phenyloxazolyl)) benzene, (POPOP), per gallon of toluene, both purchased from Sigma, St. Louis, Missouri. The L-arginine and L-histidine were also products of Sigma Chemical Co. All other chemicals were of reagent grade.

BACTERIA, CULTURE AND ASSAY CONDITIONS

Escherichia coli PA1 (arg^{-}, thiamine^{-}, rel^{-}) and Escherichia coli PA2 (arg^{-}, thiamine^{-}, rel^{+}), a pair of strains isogenic as far as is known except for the rel gene, belong to the TLB_1 family of Escherichia coli K-12. They were generously provided by Dr. R. Lavallé. Escherichia coli Tb6(arg$_A^{+}$, his^{-}, rel^{-}) and Escherichia coli B77 (arg$_A^{ts}$, his^{-}, rel^{+}), a pair of strains, isogenic as far as is known except for the argA and rel loci, are derivatives of Escherichia coli B isolated by Dr. E. Ron. They were generously provided by Dr. B. Davis. The bacteria were cultured in standard Davis medium (131). This synthetic growth medium for PA1 and PA2 was supplemented with 0.5 mg/liter thiamine-HCl, and where indicated, with 100 mg/liter of L-arginine. The growth medium for Tb6 and B77 was supplemented with 100 mg/liter of L-arginine, and where indicated, with L-histidine, 50 mg/liter. A fully supplemented

overnight culture was diluted 25-fold into fresh medium. These final cultures were incubated, as were all the previous cultures, on a New Brunswick floor shaker at 37°. All cultures were incubated with agitation at 163 excursions per minute (epm). Growth was followed on a Klett-Summerson colorimeter at 660 nm. Sterile conditions were maintained from the inoculation of the broth until the turbidity reached 80-90 Klett units. The cells were then chilled in an ice bath and harvested at 4° in a Sorvall RC-2B Centrifuge, using a type SS-34 rotor, at 15,000 rpm for 5 minutes. The cells were washed in 75% of the volume of cold culture medium lacking the required amino acid. Each of the pellets was then resuspended in a volume of cold culture medium lacking the required amino acid, equivalent to 1.25 times the volume of the initial aliquot that yielded it.

ASSAY FOR PROTEIN SYNTHESIS

The culture medium was supplemented with DL - isoleucine, 200 micrograms/ml, and ^{3}H-L-isoleucine, 0.1 microcuries/ml (specific activity, 1.0 mCi/ 0.087mg.) and where indicated, with 100 mg/liter of L-arginine. The total volume was 1.0 ml. The cells were incubated in small test tubes at 37° in a Warner- Chilcott shaker bath, model 02156, with agitation equivalent to 168 excursions per minute (epm). Incorporation of label into protein in the presence or absence of L-arginine was determined by a slight modification of the procedure of Byfield and Scherbaum (132). At intervals, 0.10 ml of assay solution was spotted onto one inch square of Whatman No. 3 MM filter paper. The squares were immediately immersed in cold 5% trichloroacetic acid (TCA). All the squares from each timepoint were immersed in the same beaker of cold TCA. The acid was decanted ½

hour after the last disc from the last time point was collected, and the discs were washed three times for 20 minutes each with fresh aliquots of 5% trichloroacetic acid. The discs were next washed twice for 5 minutes each with acetone. Approximately 10 ml of 5% TCA and 5 ml of acetone were used for each square in each wash. The air-dried discs were counted in toluene scintillator fluid in the Beckman model LS-150 scintillation counter.

ASSAY FOR RNA SYNTHESIS

The culture medium was supplemented with uracil, 10 micrograms/ml, and ^{3}H-6-uracil, 0.1 microcuries/ml (specific activity, 3.1 Ci/ mole), and where indicated, 100 mg/liter of arginine. The total volume was 1.0 ml. The filter paper square method described for measuring protein synthesis was used.

LIPID SYNTHESIS

Two different procedures were used. The first method was esentially that of Sokawa et. al. (126). The culture medium was supplemented with potassium acetate, 10 micrograms/ml and ^{14}C-2-acetate, 0.02 microcuries/ml (specific activity 39 microcuries/mg), as well as 100 mg/liter of L-arginine where indicated. The samples were incubated in a Warner- Chilcott Shaker bath, Model 02156 at 168 excursions per minute (epm). The samples were dried in scintillator vials either by overnight evaporation, or by heating at temperatures below 50° C.

The second method was a more extensive modification of the procedure of Sokawa et. al. (126). Aliquots of 2 ml were removed from incubation at the appropriate times, mixed with chilled unlabeled carrier cells and centrifuged in the cold. After all the samples had been collected, the pellets were washed once with 2 ml of cold minimal medium. The washed

pellets were then extracted overnight with 4 ml of a 3:1 chloroform-methanol solution instead of the 1:1 chloroform-methanol solution used in the first method. The remainder of the procedure was identical to the first method. The radioactivity was determined by dissolving the residue in toluene scintillator fluid and counting it in a Beckman LS-150 scintillation counter.

LIPID ANALYSIS*

After the bacteria were incubated for 80 minutes, the lipids were isolated for analysis by scaling up the second method to 40 ml and omitting the addition of unlabeled carrier cells. Total lipid extracts were examined by thin layer chromatography on Silica Gel H (S. Merck, Darmstadt, Germany) with chloroform-methanol-water (65:25:4). The distribution of labeled compounds on the plates were determined by the zonal scanning technique of Snyder (133). In this procedure, the silica is mechanically scraped from the plates into counting vials, in 2-mm increments, starting below the origin and proceeding through the solvent front. A 1-ml sample of 10% glacial acetic acid in absolute ethanol and 15 ml of toluene-base scintillation fluid is added to each vial for counting. The distribution of ^{14}C was visualized by constructing a histogram of counts per minute with respect to the increment number. A duplicate chromatogram, run on some of the samples, was sprayed with sulfuric acid and heated to visualize the separate compounds.

Fatty acid methyl esters were prepared from the total lipids by transesterification with boron trichloride-methanol, or by saponification, extraction, and treatment with diazomethane. Results with the two methods

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* I am indebted to Dr. Paul Thomas of the Mayo Foundation for the detailed analysis of the lipids.

were comparable. The methyl esters were separated by gas chromatography at 180°, on a glass column. (6 ft. x $\frac{1}{4}$ inch) packed with 10% EGSS-X on 100 to 120 mesh Gas Chrom P (Applied Science Laboratories, Inc., State College, Pennsylvania). An argon diode, which does not bring about destruction of the sample, was used as the detector. No correction was made for a difference in conductivity of eicosanoic acid. Peak areas were measured with a CRS-104 digital integrator (Infotronics Corporation, Corporation, Houston, Texas). The combined weight of C_{16}, C_{17}, C_{18}, and C_{19} fatty acids was obtained through comparison with a known milligram weight of an internal standard. When radioactivity was to be measured, the effluent gas of the chromatograph was bubbled into a flowing stream of toluene base scintillation fluid and collected automatically in serial vials at 45 second intervals, as described by Dutton (134). The vials were counted in a scintillation counter and the counts plotted to provide a profile of the distribution of ^{14}C in the methyl esters.

C. Results

In the absence of the required amino acid, arginine, E. coli PA1 continues to synthesize RNA, but E. coli PA2 does not (Fig. 1). Both E. coli PA1 and PA2 stop making protein under these same conditions (Fig. 2). Lipid synthesis was not affected by the removal of arginine in E. coli PA1 or PA2 (Fig. 3). Similar results were obtained in the absence of histidine when E. coli Tb6 rel^- and B77 rel^+ were studied. (Fig. 4). Extraction of the lipids with either 3:1 or 1:1 chloroform-methanol yielded similar results regarding acetate incorporation. (Fig. 4)

Total fatty acids of E. coli PA1 and PA2, as measured by gas-liquid chromatography with an internal standard (Table I), show a definite increase for both strains during arginine starvation. Lipid turnover was also measured directly by resuspending (^{14}C-acetate)-prelabeled cells in cold media in the presence and absence of arginine. The loss of ^{14}C observed was 0-16% after 80 min of incubation at 37°. (Table II)

Thin-layer chromatography and sulfuric acid charring of the lipid extracts of E. coli PA1 and PA2 indicated four components, a neutral component migrating with the solvent front and three polar or phospholipid components migrating with an R_f of 0.3 to 0.65. A small amount of polar material remained at the origin. The neutral component, although visible with sulfuric acid, contained invariably less than 1% of the total ^{14}C and therefore was not listed with the others in Table III. The major phospholipid (R_f 0.4) coincided with phosphatidylethanolamine and gave

a positive reaction with ninhydrin. A minor component with R_f 0.65 coincided with cardiolipin obtained from mouse liver. The other minor component, R_f 0.3, was assumed to be phosphatidylglycerol. The distribution of radioactivity in these lipid classes is summarized in Table IV. With the possible exception of cardiolipin, the distribution is virtually identical for E. coli PA1 and PA2, whether the essential amino acid was present or absent.

The distribution of ^{14}C in the fatty acids, shown in Table IV, was considered identical within the error of the analysis, and was not significantly altered by the presence or absence of arginine in either E. coli PA1 or PA2.

D. Legends Tables and Graphs

LEGEND - TABLE I

Cells of strains PA1 and PA2, after being incubated on supplemented Davis medium to a turbidity of about 85 Klett $_{660}$ units, were divided into three equal portions (40 ml), centrifuged and washed. Two pellets of each strain were resuspended in 50 ml of medium supplemented with or lacking L-arginine as indicated. These cells were incubated at 37° for 80 min and centrifuged. During this period of time, the turbidity of the cells cultured in the presence of arginine increased by 30 Klett$_{660}$ units. Those cultured in the absence of arginine did not increase in turbidity. The pellets were then extracted with 10.0 ml of chloroform-methanol (3:1) containing 0.50 mg of eicosanoic (C_{20}) acid. Zero time controls were extracted in the same way without incubation. Methyl esters prepared after saponification of the lipid extracts were examined by gas-liquid chromatography, as described in the text, and the combined weight of C_{16}, C_{17}, C_{18}, and C_{19} fatty acids was calculated from the relative weight in milligrams of the C_{20} internal standard. The experiment was performed in duplicate.

TABLE I

Total fatty acids in strains PA1 and PA2 after incubation with and without arginine.

Strain	Zero time	After 80 min of incubation	
		Minus arginine	Plus arginine
	mg	mg	
PA1	0.88	1.33	1.10
	0.86	1.22	1.04
PA2	0.97	1.20	1.23
	1.04	1.17	1.35

TABLE II

Percentage of ^{14}C-2 Acetate Remaining In Total Lipid Extract

Genotype	Arginine	TIME 20'	40'	60'	80'
PA1	+	100	93	93	84
PA1	-	98	96	106	100
PA2	+	98	97	98	92
PA2	-	88	101	96	102

Cells of strains PA1 and PA2 cultured in supplemented medium were cultured to early log phase and chilled. One 30-ml aliquot and one 60-ml aliquot were removed from each culture. ^{14}C-2-acetate was added to each 30-ml aliquot. Cold acetate was added to the 60-ml aliquot and its growth was monitored to estimate the growth of the labeled culture. Both sets of aliquots were incubated at 37° until the larger unlabeled cultures reached mid-log phase. The smaller labeled cultures were then harvested and resuspended in medium containing cold acetate, and where indicated, L-arginine. These cultures were incubated at 37° and sampled periodically for the amount of radioactivity remaining in the lipid fraction as described in the text. Zero time aliquots were equivalent to 100%.

TABLE III

Distribution of ^{14}C from ^{14}C-2-acetate in total lipids.

Strain	Origin	Total ^{14}C recovered		
		Phosphatidyl-glycerol	Phosphatidyl-ethanolamine	Cardiolipin
	%	%	%	%
PA1, plus arginine	3	17	60	17
	3	17	57	18
PA2, plus arginine	2	18	56	20
	2	20	53	21
PA1, minus arginine	3	21	55	10
	4	19	63	8
PA2, minus arginine	6	18	56	17
	6	19	56	16

Cells of strains PA1 and PA2 cultured in supplemented medium were harvested, resuspended, and incubated for 80 min in media containing ^{14}C-2-acetate and arginine where indicated, as described in the text. After incubation, the total lipid extract was separated by thin layer chromatography, and the distribution of ^{14}C was determined by zonal scanning, as described in the text.

TABLE IV

Incorporation of ^{14}C into individual fatty acids from ^{14}C-2-acetate.

Strain	Experiment	Fatty Acid					
		14:0	16:0	16:1	17:Δ	18:1	19:Δ
PA1, plus arginine	1	3	40	30	4	20	2
	2	2	30	30	5	30	3
PA2, plus arginine	1	3	30	40	4	20	2
	2	2	30	30	4	30	4
PA1, minus arginine	1	3	30	30	2	30	2
	2	2	30	30	5	30	3
PA2, minus arginine	1	3	40	30	3	20	3
	2	2	40	20	5	30	3

Fatty acids synthesized from ^{14}C-2-acetate, as in Table II, were analyzed in duplicate in two separate experiments. The results presented are averages of the duplicates in each experiment. See the text for a detailed description of the methods.

LEGENDS

Figure 1:

RNA synthesis determined by ^{3}H-uracil incorporation by E. coli PA1 and PA2 in the presence or absence of arginine. See text for the details of the assay system. □-□ , PA1 plus arginine; ■-■ , PA2 plus arginine, ○-○ , PA1 minus arginine; ●-● , PA2 minus arginine.

Figure 2:

Protein synthesis determined by ^{3}H-isoleucine incorporation by E. coli in the presence or absence of arginine. See text for details of the assay system. □-□ , PA1 plus arginine; ■-■ , PA2 plus arginine; ○-○ , PA1 minus arginine; ●-● , PA2 minus arginine.

Figure 3:

Lipid synthesis determined by ^{14}C-acetate incorporation by E. coli PA1 and PA2 in the presence or absence of arginine. See text for the details of the assay system. □-□ , PA1 plus arginine; ■-■ , PA2 plus arginine; ○-○ , PA1 minus arginine; ●-● , PA2 minus arginine.

Figure 4:

Lipid synthesis determined by ^{14}C-acetate incorporation by E. coli Tb6 and B77 in the presence or absence of L-histidine. Cells of Tb6 and B77 were cultured in supplemented medium, harvested, resuspended, and incubated for 80 minutes in media containing ^{14}C-acetate, and where indicated, L-histidine. Aliquots were removed at the designated times, and the amount of radioactivity in counts per minute was measured, as described in the text. Results are shown for extraction of aliquots in 3:1 or 1:1 chloroform:methanol. ✕-✕ , PA1 plus histidine; ○-○ , PA1 minus histidine; △-△ , PA2 plus histidine; □-□ , PA2 minus histidine.

Figure 1

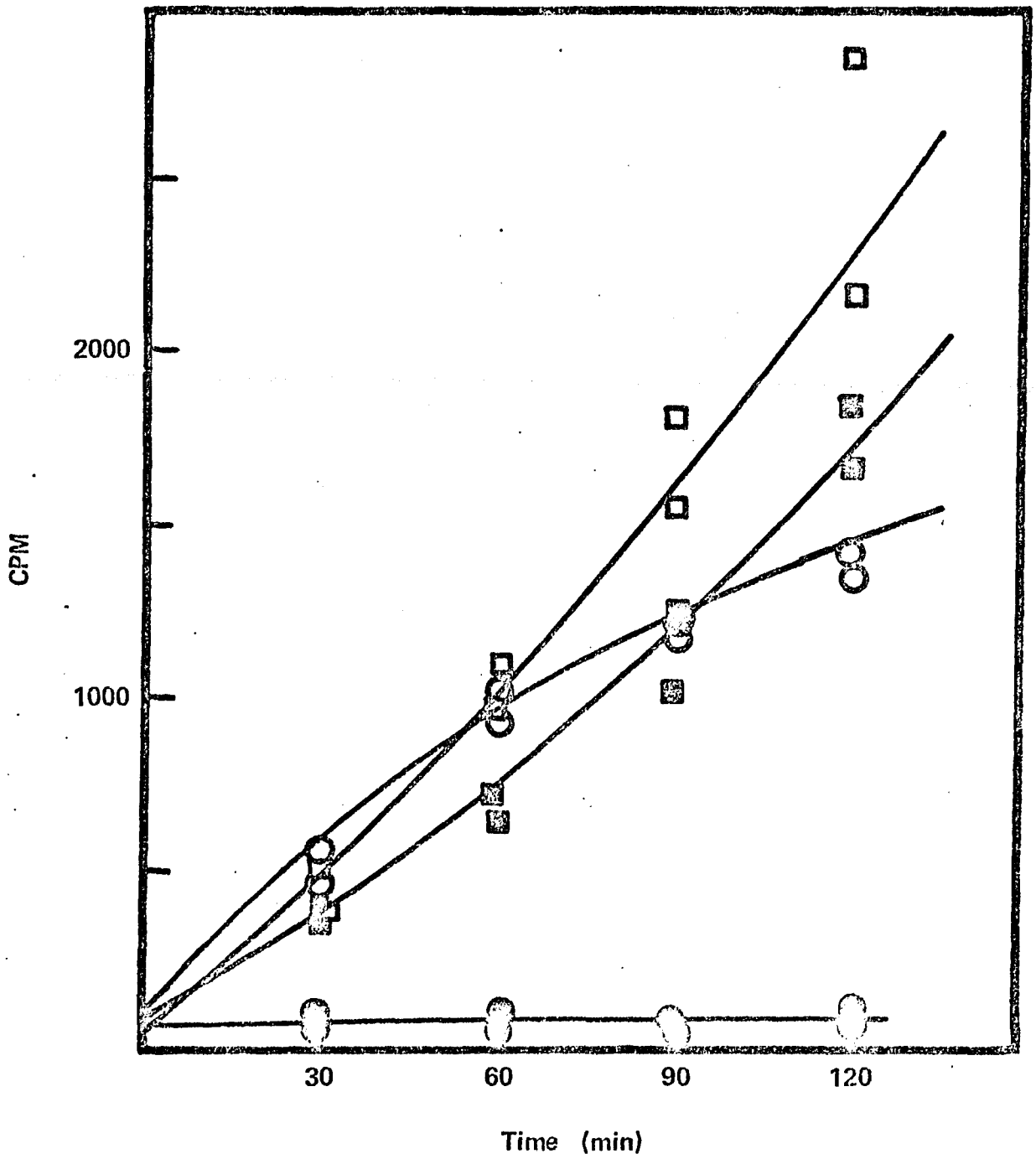


Figure 2

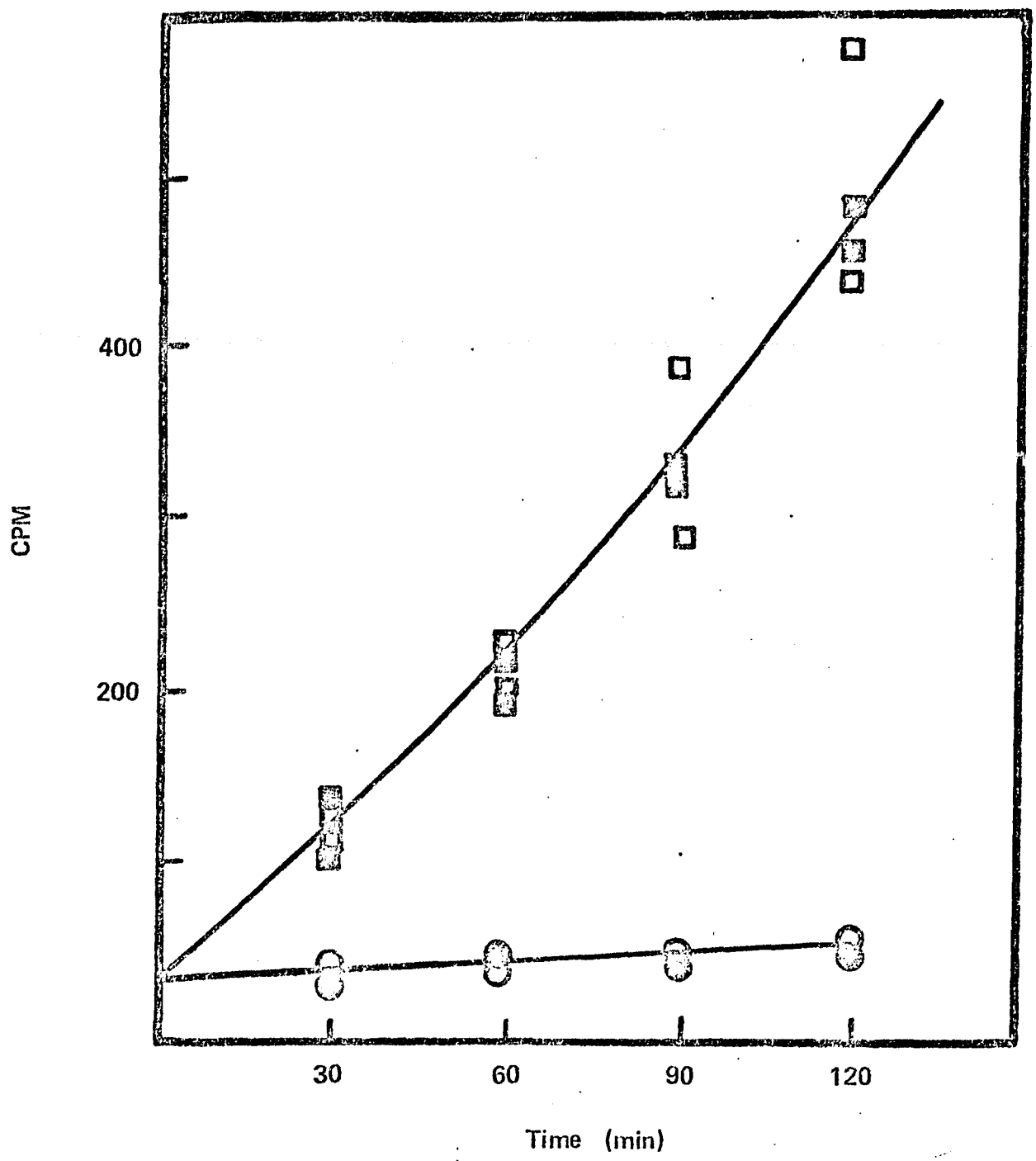


Figure 3

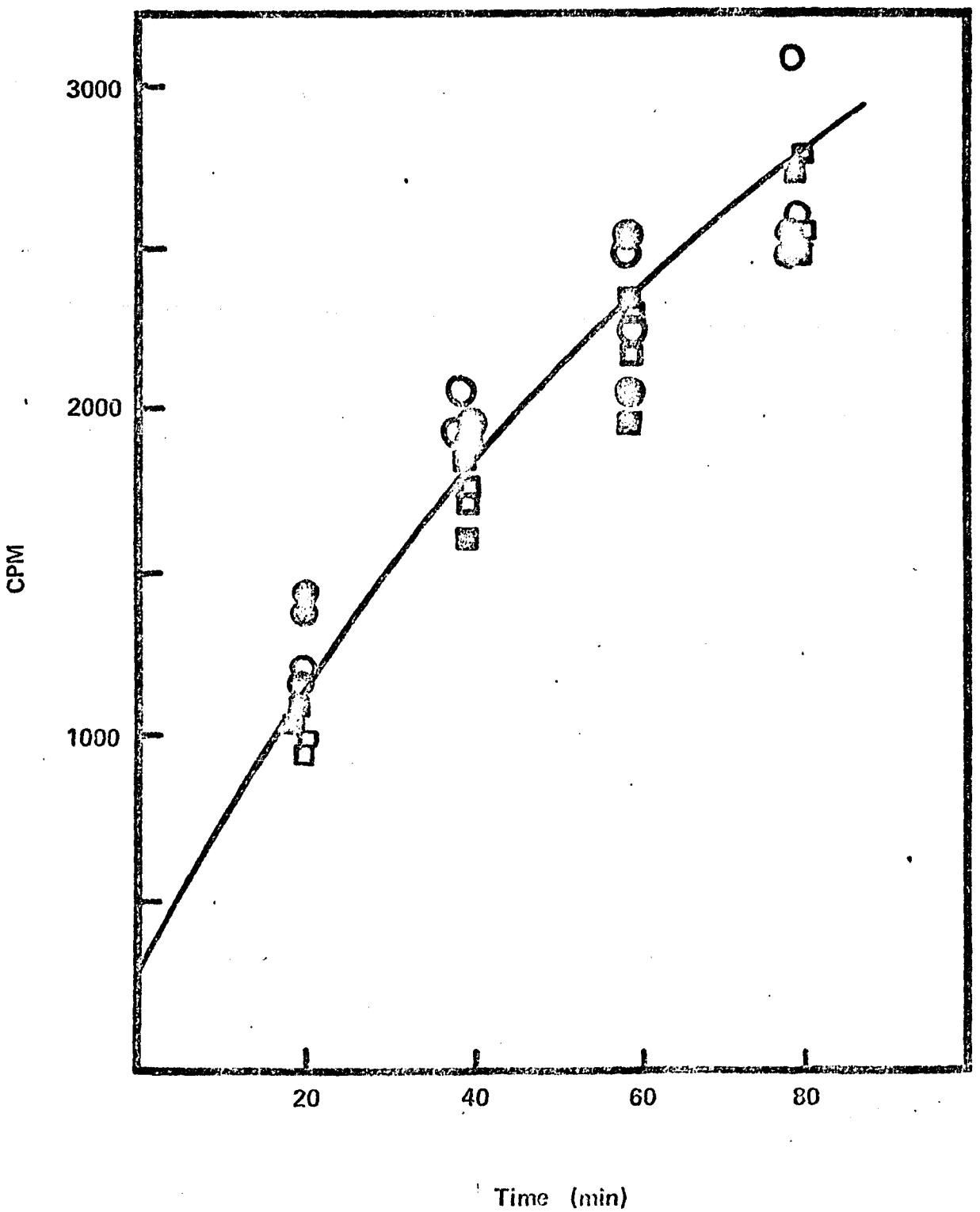
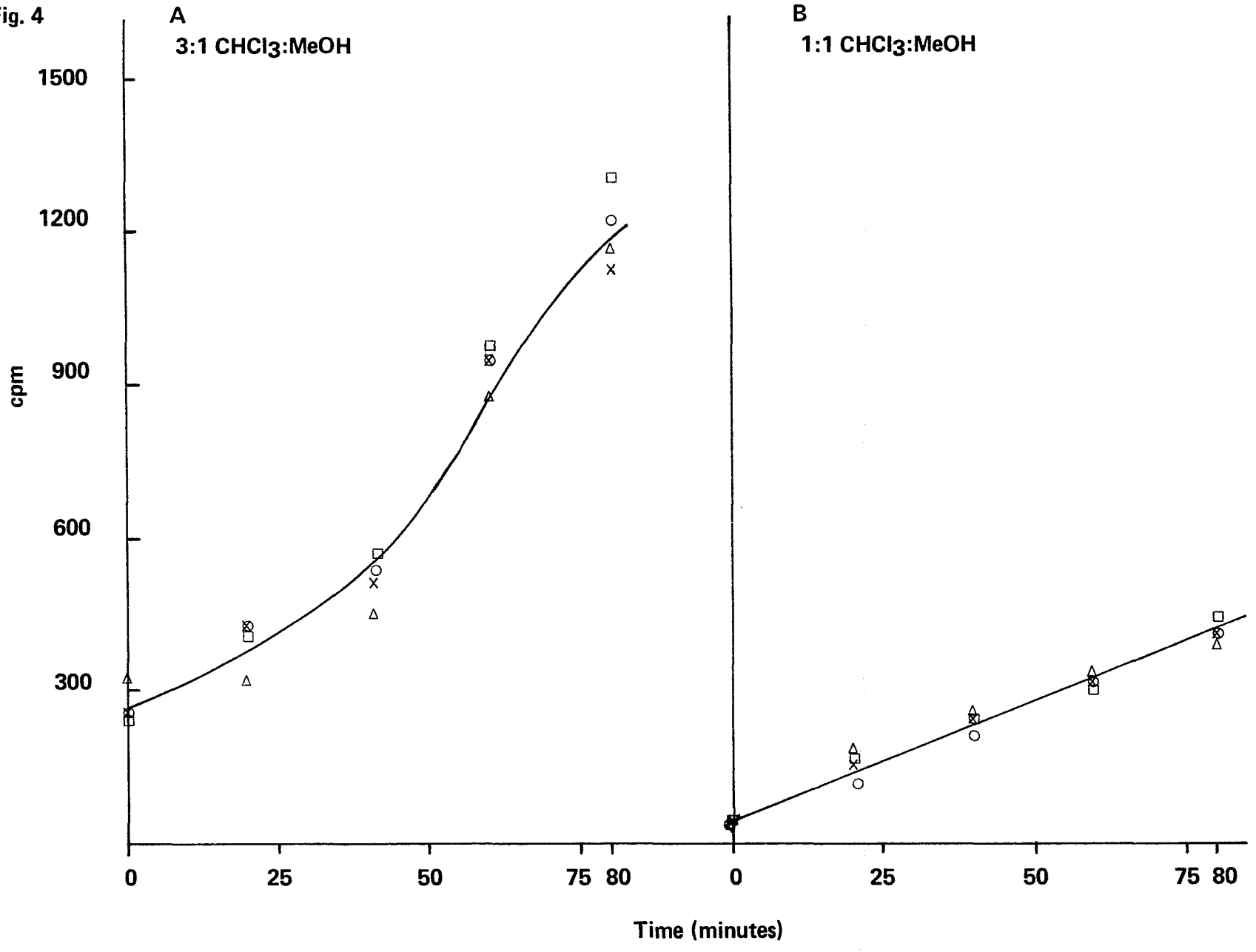


Fig. 4


A
3:1 CHCl3:MeOH
B
1:1 CHCl3:MeOH
1500
1200
900
600
300
cpm
0
25
50
75
80
0
25
50
75
80
Time (minutes)

E. Summary

These studies were designed to investigate whether the rel locus controls lipid synthesis as well as RNA synthesis. It is clear that RNA synthesis abruptly ceases upon the removal of a required amino acid in stringent cells but not in relaxed cells. Lipid synthesis continues in both types of cells in the presence or absence of arginine. The results concerning lipid synthesis are in disagreement with those of Sokawa, et. al. (126). However, in their studies, abrupt cessation of lipid synthesis of a magnitude comparable to that observed in RNA or protein synthesis was not observed. These workers dried their samples by heating under an infra red lamp which at first was thought to account for the differences observed in total lipid synthesis provided that some component of the lipid fraction was more volatile than that present in relaxed cells. The lipid analysis do not support this idea because the lipid made by stringent and relaxed cells in the absence of a required amino acid appear to be nearly the same. Decreased incorporation of label into cardiolipin for the relaxed cells during arginine starvation seems to be the only reproducible difference. There is no explanation for this deviation, and its significance is not apparent. Nunn and Tropp have also observed a similar decrease in the percentage of cardiolipin in chloramphenicol-treated cells (135). Although a relatively small increment in fatty acids was chemically determined, the largest increment in fatty acids we observed occurred in relaxed strains lacking arginine.

The observation that lipid synthesis continues in both stringent and relaxed cells in the absence of the required amino acid would seem to make it unlikely that the control of RNA synthesis is at the level of nucleoside

triphosphate synthesis. This is particularly true for ATP and CTP, which are necessary for the continued lipid synthesis observed. Starvation of a stringent cell for a required amino acid does not appear to affect the synthesis of cyclo propane fatty acids. This must mean that S-adenosyl-methionine continues to be synthesized in these cells. Measurements of intracellular ATP and CTP pool levels by the more accurate formic acid method also reveals that ATP and CTP still continue to be synthesized under conditions of amino acid starvation (96). RNA synthesis may be controlled by the combined effects of the concentration of several nucleotides, including the mono- and diphosphates, or be sensitive to the concentration of guanosine triphosphate (107). The independence of lipid synthesis from the rel locus is consistent with recent observations concerning partially non-coordinate control of RNA synthesis (41,42,48,129,130), and the reports that nucleoside triphosphates are not the limiting factor for RNA synthesis (97, 98).

II. THE EFFECTS OF A SHIFT-DOWN IN SPEED OF AGITATION DURING ASSAY ON PROTEIN, RIBONUCLEIC ACID, AND LIPID SYNTHESES

A. Introduction

In Chapter I, the possibility that the rel gene exercised its control at the level of nucleoside triphosphate limitation was examined. In particular, lipid biosynthesis was studied as a probe for nucleoside triphosphate availability in a pair of K 12 strains of E. coli, isogenic except for the rel locus. In the absence of an essential amino acid, net protein synthesis was inhibited to an equal extent in both rel^+ and rel^- strains. Net RNA synthesis ceased only in the rel^+ cells. Total lipid synthesis, as measured by the incorporation of labeled acetate into the chloroform-methanol soluble fraction, was not regulated by the rel gene. Except for a decrease in the relative proportion of cardiolipin synthesized in relaxed cells deprived of an essential amino acid, the distribution of phospholipids synthesized was not significantly affected by the rel gene.

These results were in disagreement with an earlier report of Sokawa, Nakao, and Kaziro (126), which proposes that during amino acid deprivation, total synthesis of lipids is subject to rel gene control. However, their data showed that the effect of amino acid starvation is much more pronounced for RNA synthesis than for lipid synthesis. This chapter describes investigations designated to elucidate the factors responsible for the different conclusions reported by Sokawa, Nakao, and Kaziro (126), and by this laboratory (136).

B. Materials and Methods

CHEMICALS:

^{3}H-6-Uracil (3.1 Ci/Mole), and ^{14}C-2-sodium acetate (39 mCi/Mole), were purchased from Schwarz/Mann, Orangeburg, N. Y.; ^{3}H-L-lysine (1.00mCi/0.06 mg) was purchased from New England Nuclear, Boston, Mass. L-Valine was purchased from Sigma Chemical Co., St. Louis, Mo. Supelcosil silica gel 12A and the "chromatographically pure" phospholipids, bacterial phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin were obtained from Supelco, Inc., Bellefonte Pa. The toluene-based scintillation fluid used in all experiments for monitoring ^{14}C and ^{3}H was the same as that previously described (136). All other chemicals were of reagent grade and all solvents were distilled before use.

BACTERIAL STRAINS AND CULTURE CONDITIONS:

Escherichia coli PA1 (rel-1$^-$, arg$^-$, B_1^-) and PA2 (rel-1$^+$, arg$^-$ B_1^-) belong to the TLB_1 family of *E. coli* K-12, and are isogenic except for the rel locus. The bacteria were a generous gift of Dr. H. Lavalle, The cells were cultured in synthetic medium formulated as described by Davis and Mingioli (131) with 0.5% glucose as the carbon source, and were supplemented with 0.5 micrograms /ml of thiamine-HCl, and with 100 micrograms/ml of L-arginine. All cultures were incubated at 37° in a New Brunswick Metabolyte water bath shaker, Model No. G77. "Rapid" shaking speeds are defined as 400 excursions per minute (epm), and "slow" shaking speeds are defined as 75 excursions per minute (epm). Stationary phase cultures in broth were diluted 250-fold into fully supplemented synthetic medium and grown to stationary phase at 400 epm (rapid agitation). These overnight cultures were then diluted 33-fold into fully supplemented fresh synthetic

medium and incubated under the same conditions. Cell growth was determined at 660 nm on a Klett-Summerson colorimeter. All isotope incorporation assays were initiated when the turbidity reached 60-70 Klett units.

In these experiments, the addition of 250 micrograms/ml of L-valine was used to deprive the cells of L-isoleucine, since valine induces amino acid starvation in E. coli K-12 strains by feedback inhibition of the first enzyme of the isoleucine biosynthetic pathway (137). This rapid addition of valine obviated the need for chilling and centrifuging the cells, thus maximizing the maintenance of their normal physiological state. All the cultures were agitated at 400 epm (rapid agitation) during all culturings prior to the incubation for assay. However, two different shaking speeds were used during the assay period. Rapid agitation involved no change in the shaking speed. Slow agitation involved a "shift-down" to a shaking speed of 75 epm. The doubling time at the rapid agitation speed is approximately 70 minutes for rel^+ cells and 60 minutes for rel^- cells. The doubling time at the slow agitation speed is approximately 135 minutes for rel^+ cells and 125 minutes for rel^- cells.

ASSAY FOR RNA AND PROTEIN SYNTHESIS

These assays were carried out in exactly the same manner as previously described (136), except that ^{3}H-L-lysine was used as a labeled substrate in the protein assay instead of ^{3}H-L-isoleucine. For RNA synthesis, the culture medium was supplemented with uracil (10 micrograms/ml), ^{3}H-6-uracil, (0.1 micrograms/ml, specific activity 3.1 Ci/Mole), and where indicated 250 micrograms/ml L-valine. For the protein assay, the culture medium was supplemented with L-lysine (10 micrograms/ml), ^{3}H-L-lysine, (0.1 microcuries/

ml, 1.00 microcuries/0.06 mg) and where indicated, 250 micrograms/ml L-valine. Incorporation of label was measured by the same procedure detailed previously (136). The cultures were incubated at 37°C and either 400 epm or 75 epm for 60-70 minutes.

ASSAY FOR LIPID SYNTHESIS

The synthesis of lipids was monitored by measuring the incorporation of ^{14}C-2-acetate. The culture medium was supplemented with potassium acetate (100 micrograms/ml) and acetate-2-^{14}C (0.02 microcuries/ml), and where indicated, with 250 micrograms/ml L-valine. The cultures were incubated at 400 epm or 75 epm for 60-70 minutes. During this period, 2 ml aliquots were removed at intervals, mixed with 2 ml of chilled unlabeled carrier cells centrifuged and stored at 3°. The suspension of carrier cells had been supplemented with 100 micrograms/ml of potassium acetate. After all the aliquots had been collected, the pellets were washed once with 2 ml of cold medium containing 250 micrograms/ml of valine. The washed pellets were extracted overnight at room temperature with 4 ml of a 3:1 chloroform-methanol solution. The extract was washed 3 times with 1 ml aliquots of distilled water, pipetted into scintillation counting vials, and dried by evaporation at room temperature. The radioactivity was determined by dissolving the dried extract in 10 ml of toluene scintillator fluid and counting in a Beckman LS-150 scintillation counter.

LIPID ANALYSIS

In order to perform analysis of the lipids, the method used for lipid assay was scaled up from 2 ml aliquots to 44-ml aliquots. The addition of unlabeled carrier cells was omitted. After extraction, the chloroform

was evaporated, and the extracts were dissolved in 1 ml of chloroform for subsequent fractionation of phospholipids. The phospholipids were resolved by thin-layer chromatography as previously described (135). Two ml of 10:90 glacial acetic acid: 95% ethanol followed by 10 ml of toluene scintillation fluid were added to each vial for counting. The various fractions were identified by co-chromatography with known standards. The R_f values observed for phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were the same as those previously reported (136).

C. Results

Valine induces starvation for isoleucine by repressing the first enzyme of the isoleucine biosynthetic pathway (137). PA1 rel$^-$ and PA2 rel$^+$ cells were cultured to log phase at 400 epm, and then assayed in the presence or absence of valine at 400 epm or at 75 epm. Under these conditions, agitation at 400 epm during assay involved no shift in shaking speeds. Agitation at 75 epm during assay, however, involved a "shift-down" in shaking speeds.

PROTEIN SYNTHESIS

Neither PA1 (rel$^-$) nor PA2(rel$^+$) exhibits significant net synthesis of protein in the presence of valine, as measured by the incorporation of tritiated L-lysine into cold trichloracetic acid-precipitable material. (Fig. 1A and 1B) This characteristic observation is independent of shaking speeds. In the absence of valine, the rate of incorporation of L-lysine is not affected by the rel gene, but decreases approximately 50% when the cells are incubated at 75 epm, rather than at 400 epm. (Fig. 1A,1B)

RNA SYNTHESIS

The PA2 rel$^+$ strain was unable to accumulate RNA at either shaking speed in the presence of valine, as measured by the incorporation of ^{3}H-6-uracil into cold trichloracetic acid-precipitable material. (Fig. 2A,2B) The PA1 rel$^-$ strain exhibited the relaxed response characteristic of its genotype at either speed, and continued to accumulate RNA in the presence or absence of valine. In the absence of valine, at both speeds, PA2 rel$^+$ incorporates ^{3}H-6-uracil at slightly lower rates than PA1 rel$^-$. The rates

of accumulation of RNA in PA1 rel$^-$, PA2 rel$^+$, and PA2 rel$^+$ plus valine at 75 epm are approximately 50% of the rates at 400 epm.

LIPID SYNTHESIS

During incubation at 400 epm, valine decreases lipid synthesis in PA1 rel$^-$ by 28%. (Fig. 3A) Under the same conditions, the rate of lipid synthesis in PA2 rel$^+$ is reduced by 38% by the addition of valine. Significant lipid synthesis thus occurs in both strains in the presence of valine, and the degree of inhibition is similar in both strains. These results are most similar to those obtained by Sokawa, Nakao and Kaziro in the isogenic pair CP78 and CP79 (126).

Sokawa, Nakao, and Kaziro (126) reported that starvation of E. coli W677 rel$^+$ for leucine and threonine, or of CP78 rel$^+$ for leucine, arginine, threonine, histidine, or all four amino acids simultaneously, reduces the rate of incorporation of ^{14}C-acetate approximately 55% (126). Starvation of 58-161 rel$^-$ for methionine, or of CP79 rel$^-$ for leucine, arginine, or histidine, or for leucinine, arginine, histidine and threonine reduced the rate of incorporation of ^{14}C- acetate approximately 30% (126.) Threonine starvation of CP79 rel$^-$ had less effect, decreasing net acetate incorporation by only 10% (126).

During incubation at 75 epm, the incorporation of ^{14}C-2-acetate into PA1 rel$^-$ and into PA2 rel$^+$ is not inhibited by valine addition, and the amount of incorporation into PA1 rel$^-$ is equivalent to the amount incorporated into PA2 rel$^+$. (Fig 3B) Lipid synthesis thus is the only one of the three biosynthetic pathways monitored which shows a significant change induced by a drastic change in speed of agitation during the incubation assay.

The distribution of radioactivity in the major phospholipids is summarized in Table 1. The values shown are averages of reproducible multiple determinations of distributions of samples from separate experiments. The distribution of radioactivity in the phosphatidylethanolamine fraction, agitated at 75 epm during assay, was slightly lower than reported in our earlier paper (136), or observed when the bacteria were assayed at 400 epm. This result may be due to an exaggeration in the difference between shaking speeds during culturing and assay, the difference in the method of instituting amino acid starvation, or both.

D. Legends, Tables and Graphs

Figure 1:

Protein synthesis determined by ^{3}H-L-lysine incorporation by E. coli PA1 and PA2 in the presence and absence of valine. See text for details of the assay system. x-x, PA1, o-o, PA1 _ valine; Δ-Δ , PA2; □-□ PA2 + valine; 1a, agitation at 400 epm during assay; 1B, agitation at 75 epm during assay.

Figure 2:

RNA synthesis determined by ^{3}H-6-uracil incorporation by E. coli PA1 and PA2 in thepresence and absence of valine. See text for details of the assay system. x - x, PA1; o - o, PA1 + valine; Δ-Δ , PA2; □-□ PA2 + valine, 2 a, agitation at 400 epm during assay; 2B, agitation at 75 epm during assay.

Figure 3:

Net synthesis of total lipids determined by ^{14}C-acetate incorporation by E. coli PA1 and PA2 in the presence and absence of valine. See text for the details of the assay system. x - x, PA1; o - o, PA1 + valine Δ-Δ , PA2; □-□ , PA2 + valine, 3A, agitation at 400 epm during assay, 3b, agitation at 75 epm during assay.

Fig. 1

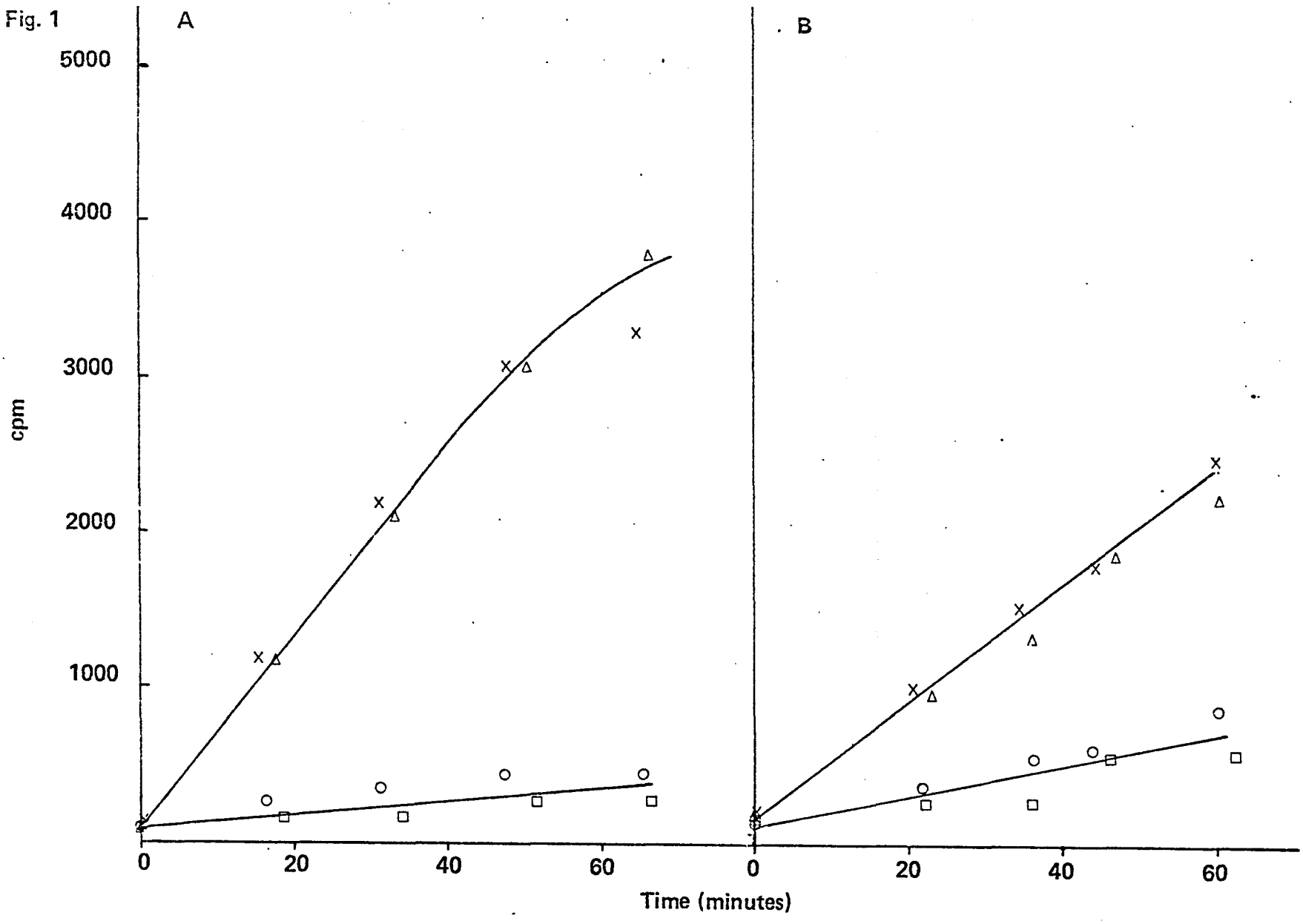

A
B
5000
4000
3000
2000
1000
cpm
0
20
40
60
0
20
40
60
Time (minutes)

Fig. 2

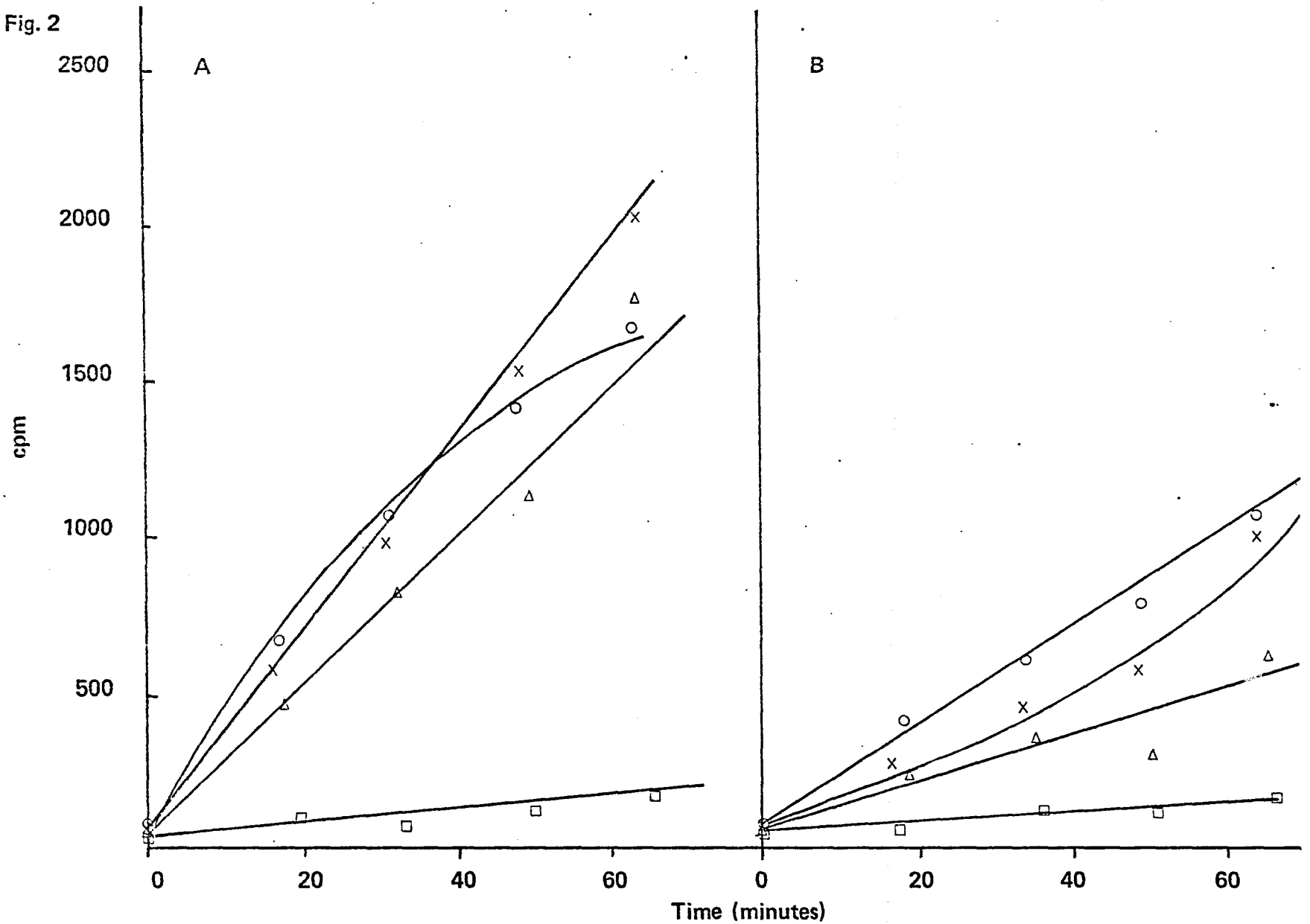


Fig. 3A

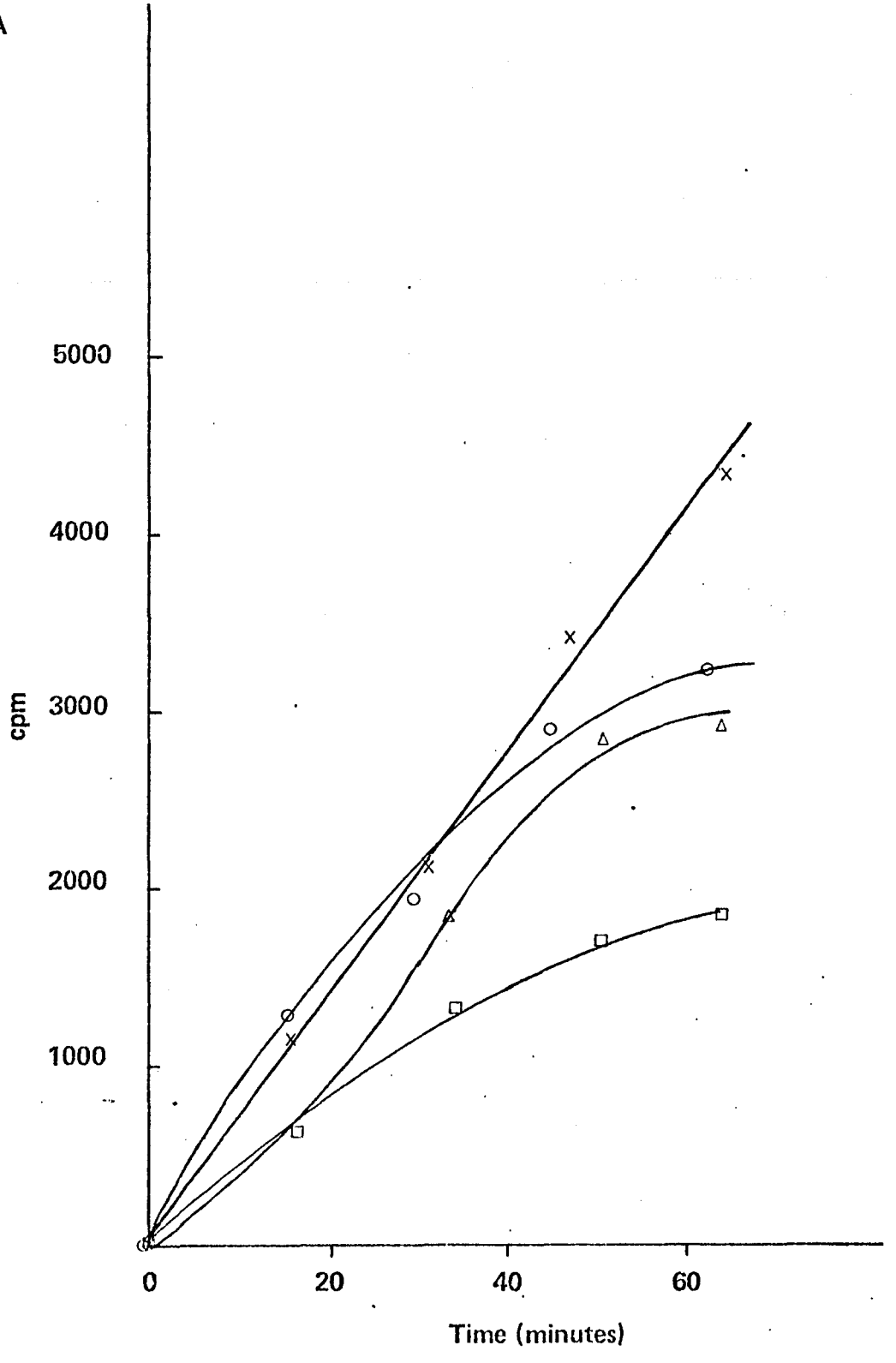
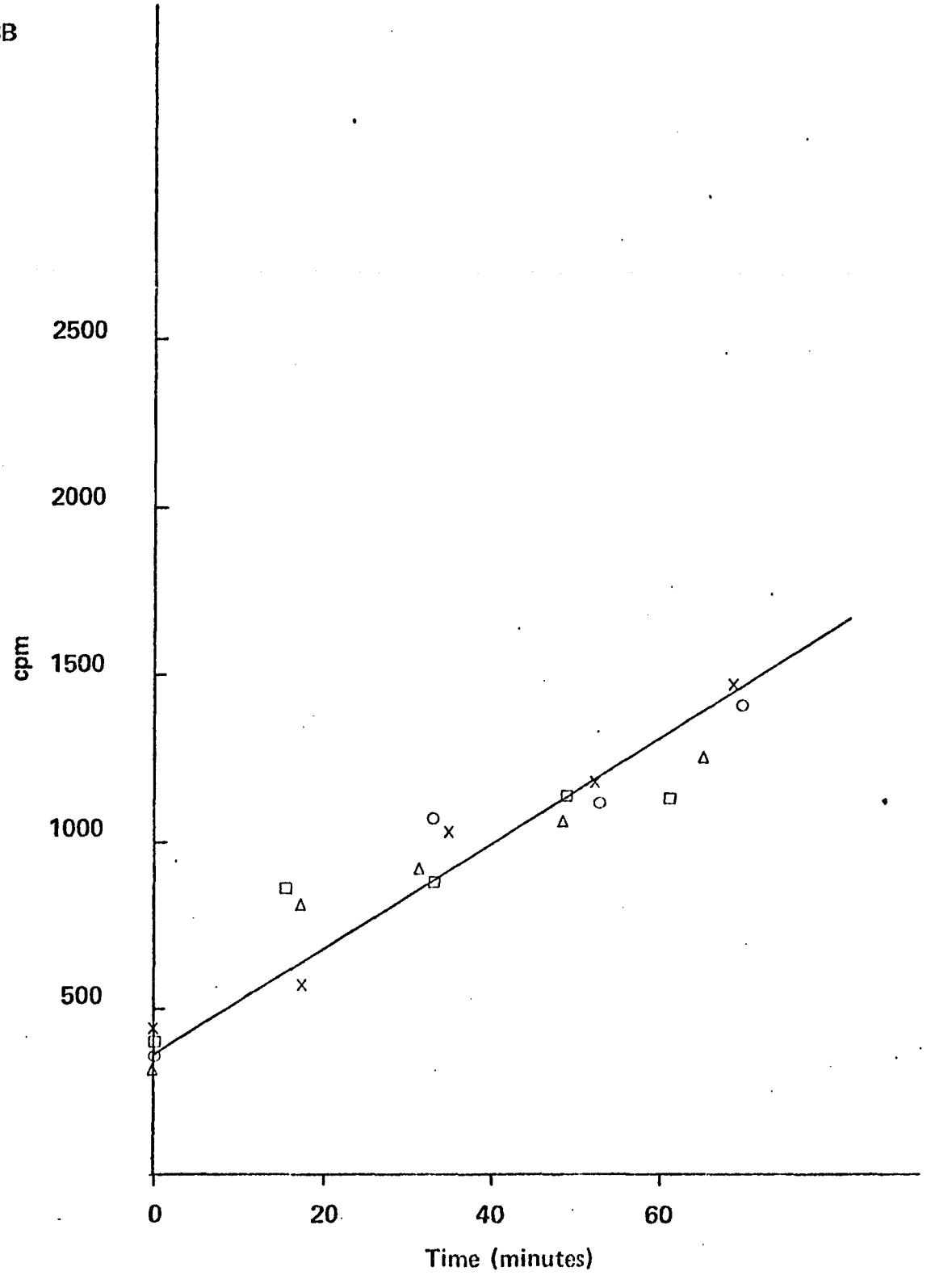


Fig. 3B



LEGEND FOR TABLE 1

Log phase cells of strains PA1 and PA2, cultured in supplemented medium, were transferred to media containing ^{14}C-2-acetate, and valine where indicated, and incubated as described in the text. After incubation, the total lipid extract was fractionated by thin layer chromatography and the distribution of ^{14}C was determined by zonal scanning, as described in the text. The plus or minus value appearing after each distribution is the 95% confidence interval.

TABLE 1

Relative Percentage Distribution of ^{14}C from ^{14}C-2-acetate in phospholipids.

Genotype	Valine	Origin	Phosphatidyl-glycerol	Phosphatidyl-ethanolamine	Cardio-lipin	Neutral lipids
Rapid-Rapid						
PA1	-	4 ± 1	10 ± 0.8	60 ± 2	15 ± 2	6 ± 2
PA2	-	5 ± 0.7	11 ± 0.7	59 ± 3	16 ± 1	6 ± 3
PA1	+	8 ± 1	13 ± 2	62 ± 8	12 ± 4	5 ± 2
PA2	+	5 ± 1	15 ± 1	56 ± 2	15 ± 1	6 ± 2
Rapid-Slow						
PA1	-	5 ± 1	14 ± 2	49 ± 4	16 ± 1	10 ± 4
PA2	-	4 ± 1	12 ± 1	51 ± 5	19 ± 3	8 ± 4
PA1	+	6 ± 2	16 ± 2	50 ± 3	13 ± 1	11 ± 3
PA2	+	7 ± 4	14 ± 1	46 ± 6	16 ± 4	13 ± 5

E. Summary

The synthesis of ribonucleic acid, protein and total lipids, and the relative distribution of ^{14}C-2-acetate among the major phospholipids were assayed in two strains of E. coli, isogenic except for the rel gene. The bacteria were cultured at 400 epm until they reached mid-log phase, and then were assayed for incorporation of radioactive substrates at shaking speeds of either 400 epm or 75 epm.

Ribonucleic acid and protein syntheses continue in both strains in the absence of valine. In the presence of valine, which inhibits the biosynthesis of isoleucine, protein synthesis is rapidly and drastically inhibited in both strains. RNA synthesis is similarly affected in stringent cells in the presence of valine, but relaxed cells continue to accumulate RNA in the presence of valine. These characteristic responses are elicited regardless of the speed of agitation during assay.

Total lipid synthesis is not as drastically or rapidly inhibited in either strain by the presence of valine during assay at 400 epm. During assay at 75 epm, valine has no observable effect upon total lipid synthesis in either strain.

The mild inhibition of lipid synthesis observed during assay at 400 epm does not appear to be a primary and direct effect of expression of the rel gene.

The relative distributions of ^{14}C-2-acetate among the major phospholipids obtained during 400 epm and 75 epm incubation were compared in the presence and absence of valine. The slower speed of agitation results, in both strains, in a decreased proportion of labeling in the phosphatidylethanolamine fraction.

F. Discussion and Conclusions

The research presented in this thesis was designed to elucidate metabolic consequences of the rel gene, a gene which exerts a profound effect upon bacterial metabolism by regulating the processes of transcription and translation.

At the time the research was initiated, several hypotheses had been advanced to explain how RNA synthesis was controlled in E. coli. One of the more interesting hypotheses suggested that transcription of RNA ceased because one or more of its four nucleotide substrates, ATP, GTP, CTP and UTP was present in limiting concentration. Several investigators had attempted to measure ribonucleoside triphosphate intracellular pool levels in stringent cells starved for amino acids, and had obtained conflicting results (93-95, 98). The hypothesis also predicted that transcription of all classes of RNA would be coordinately regulated. At that time, evidence supporting both a coordinate and a non-coordinate system of regulation of RNA synthesis had been obtained. CTP and ATP are required substrates for the synthesis of phospholipid in E. coli. ATP is also required for the synthesis of CTP itself. Thus, the continued synthesis of lipids in the absence of RNA synthesis in stringent cells starved for amino acids would indicate that regulation of RNA synthesis is not achieved through limited availability of nucleotide substrates ATP and CTP. Accordingly, lipid synthesis was investigated in relaxed and stringent cells in the presence or absence of a required amino acid, as a means of determining the availability of ATP and CTP.

The first chapter of this thesis describes published observations showing that lipid synthesis continues to a significant extent during

amino acid starvation. The conclusions drawn from these observations, that lipid synthesis is not significantly inhibited by amino acid starvation and that rel gene control of RNA synthesis is not exerted through limitation of nucleotide substrates (136) differed from the published conclusions of Sokawa, Nakao, and Kaziro (127) that starvation for an essential amino acid significantly inhibits the synthesis of lipids and that lipid synthesis is regulated by the rel gene.

The inhibition of lipid synthesis (as measured by the incorporation of labeled acetate into a chloroform-methanol soluble fraction) that Sokawa, Nakao, and Kaziro noted in stringent cells deprived of amino acids is not as complete nor as rapidly instituted (126, 136) as the inhibition of RNA synthesis observed in the classic stringent response. The amino acid starvation even caused a mild inhibition of lipid synthesis in relaxed cells (126, 136). When Sokawa, Nakao, and Kaziro measured incorporation of ^{14}C-glucose into the chloroform-methanol soluble fraction (127), both relaxed and stringent exhibited a larger, more rapidly instituted degree of stringency of lipid synthesis. The observation that phosphorylation of glucose is severely amino acid dependent in stringent cells (99) suggests that this increased stringency of lipid synthesis in both strains may be due to various metabolic effects resulting from a decreased availability of glucose-6-phosphate, rather than to a specific effect on lipid synthesis. Therefore, the mild inhibition of lipid synthesis during amino acid starvation of relaxed and stringent cells is probably a secondary and indirect, rather than a primary and direct consequence of rel gene expression.

The second chapter of this thesis presents results of investigations designed to resolve the conflicting results obtained by the two laboratories.

Addition of valine to stringent cells causes virtually complete cessation of net RNA and protein synthesis when assayed at either 400 or 75 epm, but net synthesis of total lipids still continues. Thus net lipid synthesis can be uncoupled from net RNA or protein synthesis under these conditions. Similar results of the effect of shaking were obtained when arginine deprivation instead of valine addition was the method of imposition of amino acid starvation. (L. Meade, unpublished results) Ballesta and Schaechter treated cells of E. coli K12 with antibiotics to inhibit protein, DNA or RNA synthesis, and also observed that lipid synthesis could be uncoupled from RNA and protein syntheses (138).

The data presented in chapter two show that the mild sensitivity of lipid synthesis to valine, noted at 400 epm during assay periods of 60-70 minutes, disappeared when the speed was decreased to 75 epm during assay. The incorporation of ^{14}C-2-acetate observed at the higher shaking speed is very similar to that obtained by Sokawa, Nakao and Kaziro (126).

The synthesis of RNA, however, is still regulated by the rel gene, regardless of the shaking speed during assay. The net incorporation of ^{3}H-6-uracil into RNA in relaxed cells in the presence or absence of valine, and into stringent cells minus valine was inhibited approximately 50% when the growth rate was halved by decreasing the shaking speed.

The effects of valine addition to relaxed and stringent cells on the relative distribution of ^{14}C-2-acetate in phospholipids were assayed at the two different speeds. Differences due to a shift-down in shaking speeds were considered, as were differences due to valine addition. No significant changes in the proportion of label were noted at either speed. The relative distributions of ^{14}C-2-acetate into phosphatidylglycerol and

phosphatidylethanolamine obtained during shift-down reported in the third chapter are, however, slightly lower than those reported in the second chapter. This effect may be due to the exaggerated difference in shaking speeds used in obtaining the second chapter's data.

The induction of shift-down in the growth rate by decreasing the shaking speed involves no change in the concentration or nature of the exogeneous carbon or nitrogen sources. The imposition of amino acid starvation by rapid addition of valine to the medium rather than by deprivation of arginine obviates the necessity for chilling, centrifuging and resuspending the cells, and eliminates possible effects of temperature shock on metabolic pools or other perturbations of the normal physiological state of the cell. However, similar results were obtained by both methods of amino acid starvation.

Although some of the conflicts in results obtained by measuring nucleotide pools (93-95, 98) were probably due to different solvents and conditions of extraction, it is also possible that some of the differences reflected alterations in ribonucleoside triphosphate pools due to varied rates of macromolecular synthesis at differing shaking speeds.

Powell has suggested (G. Powell, personal comm.) that the similar amounts of labeled acetate incorporated by both relaxed and stringent cells may be a result of a decreased specific activity of the total lipids synthesized in the absence of valine. This decreased specific activity is proposed to be a result of dilution of incorporated label due to the relatively larger size of the intracellular pools of acetate in the absence of valine. He is at present investigating the specific activity of the total lipids synthesized. Even if his results indicate the specific

activity of the total lipids is affected by the presence of valine, they still would not prove that lipid synthesis is also regulated by the rel gene.

The following conclusions are drawn from these studies:

1. Regulation of lipid synthesis is probably not a primary and direct consequence of the rel gene.

2. Rates of net lipid synthesis become insensitive to the mild regulation effected by amino acid starvation during shift-down imposed by a decrease in shaking speed.

3. Lipid synthesis is not dependent upon simultaneous net protein or RNA synthesis in rel^+ strains plus valine.

4. No significant alterations in phospholipid distributions are associated with expression of the rel gene.

When the research was begun, Escherichia coli was the only organism known to exhibit relaxed control of RNA synthesis. Since that time, relaxed mutants of Salmonella typhimurium (6, 46) and of Bacillus subtilis (139) have been characterized. The discovery of relaxed mutants in gram-positive, as well as gram-negative organisms, indicates that relaxed control is an aberration of a fundamental type of transcriptional control, and therefore understanding of its mechanism could be invaluable in investigations of cellular metabolism.

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