

CELL DIVISION IN A TEMPERATURE-SENSITIVE MUTANT
OF ESCHERICHIA COLI

by

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ABSTRACT

A temperature-sensitive division mutant, Escherichia coli BUG-6, has been investigated. This organism divides normally when grown at 30 C but fails to divide at 42 C. Growth continues at 42 C to produce very long, multinucleate filamentous cells. When returned to 30 C, or a high osmotic environment is added at 42 C, the filamentous cells divide rapidly to produce cells of normal size. The kinetics of cell division of the filaments at 30 C depends on the period at 42 C. During filament formation, DNA, RNA and protein synthesis continue as measured by radio-isotopic incorporation and chemical cell fractionation. DNA segregation occurs as shown by autoradiography.

The rapid division of filaments replaced at 30 C cannot be prevented by novobiocin or cycloserine but is prevented by vancomycin and penicillin suggesting that de novo synthesis of cell wall precursors is not required for division but that positioning and cross-linking of the precursors is required.

Filaments growing at 42 C were treated with nalidixic acid for different lengths of time. On returning these filaments to 30 C in nalidixic acid the number of divisions was proportional to the length of time at 42 C in the absence of nalidixic acid, i.e. proportional to the amount of DNA synthesized at 42 C.

Inhibition of protein synthesis, by chloramphenicol, does not prevent the division of filaments on replacing at 30 C provided that the

period of filamentation at 42 C was greater than 6 minutes and less than 110 minutes. The maximum amount of division in the absence of protein synthesis occurred after a longer lag and slower than in non-inhibited control cultures.

If protein synthesis was inhibited in filaments at 42 C the ability of such treated cells to divide at 30 C was rapidly lost. This loss of 'division potential' has a half-life of about 0.5 minutes, ie. 0.5 minutes of protein inhibition at 42 C reduces the subsequent division at 30 C by 50%. The normal presence of 'division potential', therefore requires the synthetic doubling rate to be in excess of 0.5 minutes. Very short periods at 42 C indicate that 10 minutes incubation at 42 C is required to produce this extremely fast synthetic rate. A model for the production and expression of 'division potential' is presented.

A biochemical analysis of the cell envelope of the filamentous cells and of normally dividing cells is presented. The major phospholipid compositions are the same. However, the fatty acid contents differ especially with regard to the cyclic fatty acids. When the filaments are allowed to divide by replacing at 30 C their fatty acid composition very rapidly reverts to that of normally dividing cells. The rates of individual phospholipid syntheses appear to change during the rapid cell division phase, however this may be an artifact resulting from an overall increase in the rate of phospholipid synthesis during this period.

An analysis of the proteins within the cell envelope by radio-active

double labelling techniques and followed by gel electrophoresis indicates that a protein(s) of molecular weight 80,000 - 90,000 exists in the envelope of filamentous cells which is not in the envelope of normal cells or is made at a much slower rate in normal cells. The protein is not incorporated into septa during the division of filaments at 30 C and little turnover occurs in the major proteins synthesized at 42 C when these cells are placed at 30 C. The possibility exists, however, that this protein is a product of filamentation and not the temperature sensitive gene product.

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INTRODUCTION

Studies of bacterial cell-division can be separated into investigations of normally growing and dividing bacteria and investigations involving inhibitors or mutants of the cell division process.

Observations of normally growing cells:

Studies of the first type are, of necessity, involved with the relationship of cell division to the whole cell cycle. Many microscopic investigations of normally dividing cells have been carried out and these have led to several conclusions regarding cell growth and division. Observations of bacteria growing on agar surfaces have shown that the growth rate determines the type of growth, ie. at slow growth rates the cells extend unidirectionally but at faster growth rates they extend bidirectionally (27). The rate of cell extension has been observed to be linear for most of the cell cycle and then double as cell division approached (2). Comparison of the growing cell to a fixed external marker in the agar (27) or examination of the distribution of young and old flagella (10), assuming that length is equivalent to age and flagella are only synthesized as the cell wall is synthesized, have led to the conclusion that cell extension is asymmetric. The region of cell wall growth has been investigated by direct microscopy. Antiserum, specific for the cell surface (19) or mucopeptide (45), was combined with a fluorescent dye and used to

determine the location of newly synthesized areas of cell surface or mucopeptide. The cells were first coated with non-fluorescent antibody so that following growth, only newly synthesized regions would be available for attachment of the fluorescent antibody and these regions would fluoresce when observed by ultra-violet microscopy. The results indicated that in gram-negative cells, growth occurs over most of the cell surface but gram-positive cells have specific localized regions of growth (19). The result for gram-negative bacteria was corroborated by an autoradiographic investigation of cell wall growth using radioactive diamino-pimellic acid as a specific label for bacterial cell walls (111). The actual mechanism of cell division differs as has been observed by electron microscopy, ie. gram-negative cells appear to divide by constriction whereas gram-positive cells first produce a membranous invagination and then the cell wall grows inward as an annulus, whereas the external cell diameter at the area of division shows little change (107).

Paulton (77) has observed, by using cell-wall specific staining, that Bacillus subtilis cells growing at different rates actually demonstrate a constant period between the initiation of a septum and cell division involving that septum. The division rate is determined by the rate of initiation of septa. Escherichia coli is not multiseptate but Adler et al. (2) observed microscopically that septum formation is apparent between half and three-quarters of the way through the cell cycle.

Further investigations of normally growing cells have used synchronised populations so that change in the cell cycle could be monitored sequentially. The growth rate has been found to change with cell age however, reports differ in how the rate changes, varying from several different rates per cycle (67) to a simple doubling in rate as a specific age per cell cycle (59). Changes have been found for enzyme activity (always equated to enzyme synthesis although not proven) such that some enzymes demonstrate distinct changes in their activity at points within the cell cycle, presumably due to a doubling in the gene controlling that enzyme production (33). The nucleoside triphosphate pools have been shown to fluctuate with cell age, the changes being consistent with changes in the known pattern of discontinuous DNA synthesis. A correlation has also been suggested between the level of one or more nucleotides and the control for cell division (46). This suggestion of linking a DNA precursor with cell division control is based on the knowledge, also obtained from synchronised cultures, that completion of a round of DNA replication is necessary for cell division (16, 42). Inhibition of DNA synthesis before completion blocks cell division, but inhibition of DNA synthesis after completion, ie. during the next cycle, does not block cell division (16, 42). The completion of a round of DNA replication changes the number of DNA synthetic regions and would be expected to alter the pool levels of precursors. This could be the required situation to initiate the division processes (46).

Adler et al. (2) and Paulton (77) observed microscopically that cell division was initiated considerably before cell separation. Clark (17) used sonication and bacteriophage to produce localized breaks in the cell surface and showed that in a synchronous population an increase in survivors occurred before cell division, indicating a period of physiological division before physical division. This was substantiated by Daniels (22), who, using the rate of incorporation of glycerol-2-³H as a measure of membrane synthesis, found a dramatic increase in the rate of synthesis of membrane slightly before cell division in a synchronised population presumably due to septum synthesis. Recently Groves and Clark (37) have demonstrated that the timing of synthesis of autolytic enzymes is also related to the cell cycle. Using chloramphenicol to block the synthesis of such enzymes at different cell ages, the subsequent cell lysis in the presence of ampicillin indicates a possibility of separating the autolytic enzymes temporarily within the cell cycle and also into those involved with definite physiological processes such as cell division or nuclear segregation.

The benefit of a synchronized culture, ie. enrichment of the cell division process, can be mimicked by using a nutritional shift-down as described by Ballestra and Schaechter (5). Using this technique they have described the changes in the ratios of the different phospholipids of Escherichia coli specifically occurring during cell division. The rate of synthesis of phosphatidyl-ethanolamine increased whereas that of phosphatidyl-glycerol decreased. A second type of shift, that

of temperature, has also been used on normal cells and it has been found that the cell division process appears to be particularly sensitive to high temperatures. Steed and Murray (107) were able to show by electron microscopy a cross membrane, visible prior to division, in gram-negative organisms which had been placed at 45 C, thus showing a change from the normal gram-negative division by constriction. Smith and Pardee (103) described a thermolabile protein synthesized early in the cell division cycle of Escherichia coli which was inactivated by a period of 15 minutes growth at 45 C. This protein was required for cell division to occur. This latter work, together with those of Adler (2), Groves et al. (37), Daniels (22), Paulton (77) and Clark (17) described earlier, suggest that the visible process of cell division is only the termination of a series of metabolic events which may have started one or more cell generations earlier.

Cell division inhibitors:

Many inhibitors are known to prevent cell division and allow filamentation eg. penicillin (30) which blocks cell wall synthesis (108); m-cresol (23) which interferes with membrane integrity; mitomycin C (99), ultra-violet light (6, 78) and nalidixic acid (34), all inhibitors of DNA synthesis; azaserine (14) an inhibitor of purine biosynthesis; 5-diazouracil (83) and D-serine (38), whose direct actions are not yet ascertained but probably affect RNA (82) and pantothenate (39) biosyntheses respectively; novobiocin (70) which inhibits many biochemical steps (104); magnesium (114) and thymine

(18, 32) deficiencies, and the application of extreme pressure (118). Filamentation is the result of inhibition of cell division without complete inhibition of cell growth. Because of the varied sites of inhibition of the above inhibitors, filament induction cannot be attributed to blockage of a single biochemical reaction, although the above inhibitors could conceivably all affect the same reaction indirectly. It has been proposed, however, that changes in cell shape, and induction of filamentation particularly, are due to a specific inhibition of cell wall synthesis (8, 76). Support is furnished by the finding that very low levels of penicillin specifically block cell division and higher concentrations preferentially cause cell lysis at division sites (97).

When protein synthesis is inhibited, e.g. by addition of chloramphenicol or amino acid starvation, filamentation does not occur. A period of residual cell division results, followed by inhibition of both cell division and growth (80, 94). Residual division also occurs following a block in DNA synthesis e.g. nalidixic acid addition, thymine starvation (16, 42), however filamentation then follows as inhibition of DNA synthesis does not inhibit cell growth.

Cell division mutations:

As an alternative to the addition of external inhibitors much use has been made of "internal inhibitors", ie. mutations blocked at a stage in cell division. Most mutations of this kind are conditional as their expression is lethal, however there are at least some cell

division processes in Escherichia coli for which mutations have been found which are not conditional. Examples are septum positioning, which is apparently incorrectly controlled in the "mini-cell" mutation (1), producing very small DNA-less cells; cell separation, which does not occur in a mutant described by Normack, Boman and Marsson (72), and therefore produces chains of cells; and cell size control which is lost in the mon mutation (4), giving rise to abnormally large cells.

Conditional mutations have added much to the knowledge of cell division, however many of the mutations are only indirectly related to cell division. Mutants are available which under the non-permissive conditions do not synthesize DNA (44, 110), or do not reinitiate DNA synthesis following completion of a round of DNA replication (58), or do not segregate completed genomes (44). From such mutants the direct linkage between DNA synthesis and cell division has been confirmed. Mutants in cell wall synthesis are also available (68, 109), and in one case, the cell wall content of choline and ethanolamine can be varied (109). This affects the substrate for autolytic enzymes which in turn has shown a requirement for the expression of autolytic enzymes in cell division.

There are, however, many mutants which appear to be involved directly with cell division and its control. Most conditional mutants are temperature sensitive. Mutants of this type have been described which have lost the coordination of DNA synthesis and cell division such that even when DNA synthesis is inhibited, cell division continues

giving rise to DNA-less cells.(43,47). Other mutants appear to lose the "trigger" for division at the non-permissive temperature and although all cellular syntheses continue, cell division does not occur and long coenocytic filaments are formed (44, 60, 71, 110). Some mutants still divide at the non-permissive temperature, but this division is irregular giving rise to bizarre cell shapes (13) or the excessive production of membranes (115). A mutant has been described which, if incubated at 45 C in liquid media and subsequently grown on solid media at either 37 C or 45 C; divides if at 45 C but not if at 37 C; ie. a period at 45 C conditions the cell division mechanism to function on solid media at 45 C but not at 37 C (102). A second group of conditional cell division mutants exist which only divide normally when the external environment is supplemented. Bacillus subtilis mutants have been described which grow as very misshapen cells unless a high osmotic environment is supplied (90). A mutant of Escherichia coli is known which forms filaments unless canavanine is added to the medium (61).

A much studied conditional mutant is that described as lon⁻ in Escherichia coli K12 (3) and as uvs in Escherichia coli B (25). The ability to divide is lost by this mutant following an exposure to a low level of ultra-violet irradiation, although growth is not inhibited and filaments are thus produced (3).

Comparison of dividing and non-dividing cells:

The inhibition of cell division allows the comparison of dividing cells with non-dividing cells. If the inhibition or conditional

mutation is reversible then the requirements for, and the events preceding and during the onset of division of the no longer inhibited cells, can be studied. The biochemistry of non-dividing cells, especially the membrane and cell wall composition, has been compared with that of dividing cells using both inhibitors and mutations to prevent division. Filaments produced by addition of penicillin (106) have been shown to produce more cardiolipin and less phosphatidyl glycerol than normally dividing cells and on addition of penicillinase, this situation is reversed until the control rates of phospholipid syntheses are obtained. The phospholipid and fatty acid composition of filaments produced by a DNA "initiator" mutant (unable to initiate new rounds of DNA replication at the non-permissive temperature) have been compared with the parent cell composition (79). The mutant contains much more anionic phospholipids (cardiolipin and phosphatidyl glycerol) than the parent. The fatty acid composition also differs in the two strains with a significant increase in the cyclic fatty acids in the filamentous cells. The proteins contained within the cell envelope of dividing and non-dividing cells, have also been compared (49, 51, 98, 100). Use has been made of a doubling labelling technique whereby the proteins produced by filaments are labelled with one type of radioactive precursor, eg. tritium labelled, and the proteins of dividing cells are labelled with the same precursor compound containing a different radioactive label, eg. carbon-14. Extraction of the proteins and comparison of the label of each kind in each protein by gel-electrophoresis allows the definition

of proteins made preferentially by dividing or non-dividing cells. All non-dividing cells, as reported by Inouye and co-workers (49, 51), preferentially produce a protein of molecular weight 39,000 and in some cases a second protein of molecular weight 80,000. If filamentation is produced by DNA inhibition, either by addition of inhibitors or use of conditional DNA synthesis mutants, then the filaments also lack proteins (5) which are presumed to be involved in DNA synthesis (49, 98, 100).

The cell wall structure has been analysed chemically in dividing and incorrectly dividing cells, and differences in the degree of mucopeptide cross-linking have been reported (88). The abnormal cells have a mucopeptide with less cross linking. Electron microscopy of cells grown at the non-permissive temperature has demonstrated that in one case (20) the abnormal morphology can be correlated directly with the absence of a cell wall layer, tentatively identified as the mucopeptide layer, combined with the random localization of septa.

Removal of cell division inhibition:

Removal of the inhibition of division has given the most direct information on the requirements for cell division. Removal of inhibition can sometimes be done by removal of the inhibitor or replacement in the permissive conditions, but in some cases, conditions which are not the reverse of those used to inhibit division, have been found to permit cell division. The production of a high osmotic environment allows a temperature-sensitive filament-former to divide at the non-

permissive temperature (86). A different temperature sensitive mutant, described by Kirby, Jacob and Goldthwaite (56), divides if more specific additions are made, ie. addition of guanine plus cytosine. Reversion to a normal morphology occurs by addition of glutamine to a rod⁻ mutant of Bacillus subtilis (89). A decrease in growth rate, ie. shifting from rich to minimal medium, has been found to induce division in filaments produced by ultra-violet irradiation (2). Addition of pantoyl-lactone also caused these filaments produced by irradiation, to divide (38), although this may be a result of a deleterious effect of this compound on growth rate (2), and not a specific effect on the cell division mechanism. A complex substance, which is lipase and temperature sensitive, has been extracted from Escherichia coli which on addition to lon filaments induces division without slowing the growth rate (29). A substance extracted from yeasts and similarly affecting cell division of yeasts, has also been described (112).

When a non-dividing cell is permitted to proceed to division, processes directly involved in division may be more easily demonstrated. The use of DNA synthesis inhibitors allowed the demonstration that only when DNA replication was completed was cell division permitted (16, 17 42). A direct correlation has been found between the ratio of protein:DNA in the division of filaments produced by thymine starvation, ie. on resumption of DNA synthesis there is no division until the protein:DNA ratio has returned to normal (26, 28). This is not true for division of lon⁻ filaments, which do not divide until considerably

after the protein:DNA ratio has returned to normal (62). Comparison of mutants blocked in cell division by arginine starvation with the same cells following addition of arginine, has demonstrated that the internal ratio of spermidine to putrescine is important for cell division control (50), ie. a high putrescine to spermidine ratio is needed for division to occur.

As yet no definite biochemical step has been demonstrated as a "division process" although several of the mutations have been mapped (1, 4, 44, 58). The involvement of proteins in the control of cell division has been demonstrated however by genetic means, as two specific genetic division releasing conditions have been found. An ochre suppressor mutation placed in a lon⁻ strain decreases filamentation indicating that the genetic lesion is expressed as a protein which can be suppressed into a functional form (65). In the second system, the presence of a recA⁻ mutation allows the division of a temperature sensitive mutant at the non-permissive temperature (48). This suggests that the recA⁺ product normally inhibits division by acting as a negative control factor but as yet no real knowledge of the actual proteins involved in either system is available.

In the present work, I describe a reversible temperature sensitive mutation which is apparently directly involved in cell division. By use of the permissive and non-permissive conditions, it is possible to produce the situations of division, non-division, filamentation and "recovery" division of filaments, ie. division following the removal

of division inhibition. I have investigated the control of division as related to macromolecular syntheses and also the biochemistry of the cell envelope in the three division situations.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

1. Organisms

Many temperature-sensitive cell division mutants were derived by Groves (37) from Escherichia coli AB-1157 Sm^r , gal^- , xyl^- , mtl^- which was obtained from E.A. Adelberg. All mutants behave normally at the permissive temperature (30 C) and exhibit abnormal division patterns at the non-permissive temperature (42 C). One of these division mutants, laboratory strain BUG-6, was selected for detailed analysis.

2. Media

Cultures were incubated in Erlenmeyer flasks containing nutrient broth (3 g beef extract per l, 5 g peptidase per l and 5 g NaCl per l), adjusted to pH 7.3 maintained at constant temperature in a shaking water bath. For experiments in which DNA, RNA protein or lipid synthesis was measured, a minimal salts medium (007) previously described (16) was supplemented with 1 g peptidase per l and 2 g glucose per l. For experiments in which membrane proteins were analyzed, the concentration of minimal salts was halved and the supplements were 80 mg peptidase per l and 2 g glucose per l. The cell numbers and cell size were measured with a modified Coulter Counter coupled to a pulse-height analyzer (16).

3. Basic experimental conditions

One of the experimental designs was used repeatedly. A culture

growing at the permissive temperature (30 C) was divided and part of the culture placed at the non-permissive temperature (42 C). After an undefined time at the non-permissive temperature, the culture was again subdivided and a portion returned to the permissive temperature (30 C). This incubation at the permissive temperature is referred to as the "recovery period".

4. Synchronised growth

Synchronised populations of cells were obtained by using the membrane technique devised by Helmstetter and Cummings (41). Because the cells were grown in a rich medium, it was necessary to use a Millipore membrane of pore size 0.8 μ .

5. Removal of antibiotics from cultures

Some experiments required the addition and removal of an antibiotic from the medium. Removal was accomplished by filtering the cells from the medium plus antibiotics using a Millipore membrane of pore size 0.45 μ , followed by washing and resuspending in preconditioned medium. (Preconditioned medium consists of the same medium as used in the experiment in which the same organism has grown to approximately the same cell density as that used in the experiment. The organism has been removed by filtration using a Millipore membrane of pore size 0.22 μ .)

II. Cell fractionation

1. Dry weights of cell suspensions

Duplicate or triplicate 1 ml samples of the concentrated cell

suspensions, in previously weighed aluminum foil containers, were heated at 100°C until the moisture had evaporated, and then were transferred to individual screw-cap jars, each containing a layer of activated silica gel dessicant (Davidson Chemical Company). In this manner, they were dried to constant weight, and the results obtained were corrected for the presence of buffer.

2. Protein, RNA and DNA extraction

A modification of the procedure of Roberts et al. (87) was employed in the chemical fractionation of the cells. Two cell samples were compared, one grown at 30°C and one at 30°C and then at 42°C for 60 mins. The cultures were rapidly chilled and each resuspended in 6 ml of distilled water.

Five ml of this suspension were added to 5 ml of cold 10% trichloroacetic acid (TCA) in a Pyrex centrifuge tube and the reaction mixture was held on ice for 30 min. Following centrifugation at 7,500 x g for 15 min, the supernatant fluid (cold TCA-soluble fraction) was removed and the pellet was resuspended in 5 ml of 75% ethanol (pH 2.5 by addition of dilute H_2SO_4), with the aid of a Vortex mixer. The extraction was carried out at 45°C for 15 min, after which the ethanol-soluble fraction was recovered by centrifugation. Five ml of 5% TCA were added to the pellet, and after thorough mixing, the tube was covered and was incubated at 90°C for 10 min. After cooling, the tube was centrifuged and the supernatant fluid (hot TCA-soluble fraction) was removed. The residual pellet (hot TCA-insoluble fraction) was

dissolved in 5 ml of 0.1 N NaOH by heating at 50 C for 5 min.

The acidic-alcohol soluble fraction and the hot TCA-insoluble fraction were assayed for protein (see section III-1). The hot TCA-soluble fraction was assayed for RNA and DNA (see sections II - 2 and 3).

3. Lipid extraction

The technique of Bligh and Dyer (11) was modified. Chloroform and methanol were added to the cell suspension in the ratio of 1 volume chloroform: 2 volumes methanol; 0.8 volumes cell suspension. This mixture was blended (Waring Blender Model 700S) for two minutes, a further 1 volume of chloroform added and the mixture blended again for 30 seconds. One volume of water was added followed by 30 seconds blending before placing the mixture in a separating funnel. The lower chloroform layer was collected as the major lipid extract. The upper alcoholic layer was re-extracted by addition of chloroform and two chloroform extracts combined, evaporated to dryness at 40 C to 50 C under vacuum and the resulting material dissolved in a small volume of chloroform: methanol: water in the ratios by volume 60:30:4.5.

This solution was placed on top of a column (diameter: height ratio 1:10) containing Sephadex G25. The Sephadex G25 was prepared by washing three times with a large volume of distilled water, six times with acetone, drying with suction followed by air drying and suspending in chloroform: methanol: water in the ratio by volume of 60:30:4.5 for ten minutes before placing in the column. The lipid extract was eluted from the column by addition of more of the same

mixture of chloroform:methanol:water followed by chloroform:methanol in the ratio by volume 2:1. For each gram of Sephadex G25 used, 15 ml of the chloroform + methanol + water mixture and 5 ml of the chloroform + methanol mixture can be run through the column before elution of non-lipid material begins.

The eluant was evaporated under vacuum and the resulting material redissolved as required for individual lipid separation or total lipid and phosphorus assays.

III. Assay Procedures

1. Protein

The method of Lowry et al. (63) was employed to determine protein concentration. Crystalline egg albumin in concentrations ranging from 8 - 42 $\mu\text{g/ml}$ in the reaction mixture were used in preparation of standard curves.

2. Deoxyribonucleic acid

The method described by Schneider (96) employed diphenylamine, which reacts with purine-bound deoxyribose, was used for the determination of DNA. Standard curves were prepared from purified calf thymus DNA (Nutritional Biochemicals Corp.) in the range of concentration of 18 - 100 $\mu\text{g/ml}$ in the reaction mixture.

3. Ribonucleic acid

Ribonucleic acid was determined with the use of a modification of the orcinol procedure of Schneider (96), which measures purine-bound pentose. The reagent was prepared by adding 1.0 gm of orcinol, in

10 ml of 95% ethanol, to 100 ml of concentrated HCl containing 0.2 gm of anhydrous FeCl_3 , immediately prior to use. Standard or test samples contained in 1.5 ml were added to 1.5 ml of the reagent; the tubes were covered and placed in a boiling water bath for 45 min, and after cooling, the OD at 660 m μ was measured on a Beckman model B spectrophotometer. Standard curves were prepared with yeast RNA (Nutritional Biochemicals Corp.) using concentrations between 3 - 18 $\mu\text{g/ml}$ in the reaction mixture.

Because DNA reacts significantly with the orcinol reagent, standard curves using DNA were made with each assay. Thus, with prior knowledge of the DNA concentration in a sample, a correction could be made for the contribution made by DNA to the OD in the orcinol reaction.

4. Phosphorus in phospholipid

A modification of the technique of King (55) was used. The samples were placed in 5 ml graduated tubes, which had been prewashed (24 hours in chromic acid, 5 water rinses, 1 6N HCl rinse and 5 distilled water rinses), and the samples evaporated to dryness on a steam bath. 0.5 ml of 70% w/v perchloric acid was added and the samples digested for two hours on a sand-bath at 220 C. Following cooling 1 ml of water, 3 ml of 0.4% w/v ammonium molybdate and 0.2 ml of an aminonaphthol sulphonic acid solution (30 g NaHSO_4 or 28.5 g $\text{Na}_2\text{S}_2\text{O}_5$ + 6g Na_2SO_3 + 0.5 g aminonaphthol sulphonic acid, made up to 250 ml with water) were added to each tube. The tubes were placed at 100 C for 10 minutes and the final volume in each tube brought to 5 ml by addition

of water. The OD at 820 mμ was read on an Hitachi-Perkin-Elmer spectrophotometer model 124.

The standard curve was prepared from dried KH_2PO_4 (1.5 g KH_2PO_4 per 100 ml of water is 0.3414 g phosphorus per 100 ml of water) and was in the range 0.5 - 3 μgP per tube.

IV. Separation and isolation of individual phospholipids

1. Thin layer chromatography

Separation and qualitative identification of individual phospholipids was accomplished by unidirectional thin layer chromatography. 27 g of Silica gel G was suspended in 60 ml of water, spread on glass plates at 0.5 mm thickness. These were air dried for 20 to 30 minutes, then oven dried (110 °C) for two hours and stored in a desiccator. The solvent used for phospholipid separation was chloroform:methanol:water in the ratio by volume 95:36:6. For demonstration of the presence and number of neutral lipids the plates were prepared in the same way and the same solvents used however their ratio by volume was changed to 99:10:1. (No further work was done on neutral lipids).

2. Column chromatography

Preparative separation of individual phospholipids was accomplished by column chromatography. The column material was Silic AR-CC7 (Mallinckrodt- Montreal). Five g Silic AR-CC7 were used for each milligram of phosphorus (in phospholipids) to be run through the column (see section III-4 for phosphorus assay). The Silic AR-CC7 was suspended

in a small volume of chloroform for 10 minutes before placing in the column. Several volumes of chloroform were run through the column until even packing was obtained. The sample was placed on the top of the column in a small volume of chloroform.

Elution was accomplished by sequential addition of increasingly more polar solvents. The volume of each successive solvent added was determined by checking the resulting eluant fractions for the presence of lipid by the char test (see section VI - 1) ie, the solvent was not changed until at least 10 successive fractions contained no lipid material. A small sample from each fraction was also applied to a thin layer plate (see section IV-1) and the phospholipid(s) present identified (see section VI-1). The following elution sequence was found to be successful for separation of the major Escherichia coli phospholipids;-

- i) 100 ml of chloroform - collected in bulk; contains neutral lipids and free fatty acids.
- ii) 150 ml of acetone - collected in 5 ml fractions; contains 3 unidentified compounds which could not be further resolved on Silic AR-CC7.
- iii) 200 ml of acetone:methanol (9:1) - collected in 5 ml fractions; fractions 1 to 10 contains 1 unidentified compound. Fractions 15 to 30 contain phosphatidyl glycerol.
- iv) 100 ml of chloroform:methanol (9:1)- collected in 5 ml fractions; fractions 1 to 15 contain only cardiolipin but fractions 15 to 20 also contain traces of phosphatidyl ethanolamine.

- v) 200 ml of chloroform:methanol (5:1) - collected in 5 ml fractions; contains phosphatidyl ethanolamine only.
- vi) 100 ml methanol - collected in bulk contains only 1 compound tentatively identified as lysophosphatidyl ethanolamine.

V. Separation of fatty acids from phospholipids

The fatty acids were removed from the phospholipids and methylated by treatment with the Sweeley reagent (31) consisting of 8.6 ml of concentrated HCl and 9.4 ml of water made up to 100 ml with methanol. The phospholipids were placed in a tube, evaporated to dryness, the Sweeley reagent added (1 ml per milligram of sample), the tube sealed and placed in an oven at 70 C for 18 hours. The resulting material was washed into a separating funnel with 10 volumes of petroleum ether. A small volume of water was added. After vigorous shaking the top ether phase was collected and washed several times with water until the aqueous phase exhibited a neutral pH. The washed ether phase was evaporated to dryness under vacuum. The resulting methylated fatty acids were dissolved in a small (<100 μ l) volume of redistilled hexane and a portion of this material was injected into a Hewlett-Packard gas-liquid chromatography system for qualitative separation of the fatty acids. The gas-liquid chromatography system was equipped with a 6 ft. glass column of 0.25" diameter containing diethylene glycolsuccinate on an inert carrier. The operating temperature was 160 C.

VI. Identification of compounds separated by chromatography

1. Phospholipids

Following separation on a thin layer chromatography plate as described in section (IV-1), the sequential treatment of the same plate with different reagents allows the visualization of NH_2 -containing compounds, P- containing compounds, carbohydrate containing compounds, and finally all carbon containing compounds. On all plates, standard markers were run in parallel with the unknown for identification purposes. All standards and reagents were kindly supplied by Dr. D.J. Hanahan.

The NH_2 -containing compounds, eg. phosphatidyl ethanolamine were visualized by spraying the plate with ninhydrin. The phosphorus containing compounds (all phospholipids) were visualized by spraying with a molybdenum reagent consisting of 25 ml of solution A + 25 ml of solution B + 90 ml of water, (Solution A was 40.11g MoO_3 added to a litre of 25N H_2SO_4 boiled until total dissolution; solution B was 1.78 g of powdered molybdenum added to 500 ml of solution A, boiled for 15 minutes, cooled and decanted from any residue). Carbohydrate containing compounds were identified by a pink colour reaction to naphthoresorcinol after 5 minutes at 100 C. (Naphthoresorcinol reagent consists of equal parts of 20% H_2SO_4 and a solution of 0.1 g naphthoresorcinol in 50 ml of ethanol.) Finally the plates were sprayed with concentrated H_2SO_4 and placed in a very hot sealed oven for 5 minutes. All carbon containing compounds char and appear as black spots. The

plates were photographed after each spray treatment so that a permanent record was obtained.

2. Fatty acids

Immediately following the gas-liquid chromatography of the unknown methylated fatty acids standard compounds were applied to the column for comparison of R_f values. A standard curve, used for estimation of carbon-chain lengths of unidentified compounds, was produced by plotting the logarithm of the distance of peaks from the solvent front against the length of the carbon-chain of the compound producing each peak.

To ensure correct identification of compounds, a sample of the unknown was hydrogenated and then compared by gas-liquid chromatography with the unknown before hydrogenation. A peak tentatively assigned to a compound containing an unsaturated bond should disappear and the size of the peak identified as that containing the same carbon-chain length compound but no unsaturated bonds, should increase. Hydrogenation was accomplished by dissolving the material in a small volume of hexane under 50 lb. of hydrogen pressure per square inch in the presence of a platinum oxide catalyst with continuous shaking for 2 hours at room temperature. The catalyst was removed by filtration before the material was rechromatographed.

VII. Determination of rates of macromolecular syntheses

1. Deoxyribonucleic acid

DNA synthesis was measured by incorporation of C^3H_3 -thymidine over a period of 5 minutes. A 1 ml cell sample was added to 0.1 ml of labelled thymidine consisting of 2.5 μ l C^3H_3 -thymidine (specific activity 20.6 Ci/mM; 1 mCi/ml; Schwarz Bioresearch Inc., Orangetown, N.Y.), diluted with unlabelled thymidine to a final concentration of 0.5 μ g/ml thymidine. Synthesis was stopped by adding 2 ml of cold 7.5% trichloroacetic acid (TCA) containing 200 μ g/ml of thymidine. Each sample was filtered through a 0.45 μ Millipore membrane and washed with 5 volumes of cold TCA and 3 volumes of 90 C water. The membranes were dried and placed in scintillation vials and toluene based scintillation fluid added. The vials were counted in a Nuclear Chicago scintillation counter.

2. Ribonucleic acid

The same technique as used for DNA measurements was employed for RNA labelling with the following modifications. 0.1 ml of a 1/10 dilution of uracil-2- ^{14}C (specific activity 46.7 mCi/mM; 0.1 mCi/ml; Schwarz Bioresearch Inc., Orangeburg, N.Y.) was added to the 1 ml cell samples and the trichloroacetic acid contained 200 μ g/ml of unlabelled uracil.

3. Protein

The same technique as used for DNA measurements was employed for protein labelling with the following modifications. 0.1 ml of a

1/10 dilution of a mixture of 15 different nonspecifically, tritium labelled amino acids (specific activity of 16.1 mCi/mg; New England Nuclear Corp., Boston, Mass.) was added to 1 ml of cell sample. The trichloroacetic acid contained 200 µg of pepticase/ml.

4. Lipid

i) The same technique as used for DNA measurements was employed for total lipid labelling with the following modifications. A 1 ml cell sample was added to 0.1 ml of labelled glycerol consisting of 1 µl glycerol-2-³H (specific activity 420 mCi/mM; 1 mCi/ml; Amersham/Searle, Des Plaines, Ill.), diluted with unlabelled glycerol to a final concentration of 0.5 µg glycerol/ml. The incorporation period was reduced from 5 minutes to 3 minutes and trichloroacetic acid containing 200 µg of glycerol/ml.

ii) When the rate of synthesis of individual phospholipids was investigated, the following modifications were necessary. Each sample consisted of 5 ml of cells and the glycerol-2-³H, although the same concentration as in i) above had double the specific activity. The Millipore membrane plus washed cells were not placed in scintillation vials but were extracted with chloroform:methanol:water by the procedure of Bligh and Deyar (11) described in section (II-3). The Sephadex-G25 column purification step was omitted.

The extracted phospholipids were evaporated to dryness by bubbling a stream of dry nitrogen through the chloroform solution. The phospholipids were redissolved in a known volume of chloroform

and 95% of each sample was chromatographed in a parallel with the other samples on thin layer plates as described in section (IV-1). The remaining 5%'s of each sample, was spotted on a portion of the plate which would not be approached by the solvent system. Following chromatography, the spots were localized under ultra-violet light, each spot was scraped from the plate, placed in a scintillation vial and counted. The 5% of each sample not affected by the solvent was also scraped from the plate and counted so that an estimate of the total radioactivity in each sample could be obtained. By comparison of the counts from each identified compound with the total number of counts applied, the relative contribution of each phospholipid, and any change in the amount of material applied to the plate which could not be identified could be determined.

VIII. Analysis of cell envelope protein

1. Isolation procedures

Two isolation procedures were used so that results from total cell envelope preparations could be compared to results from purified cytoplasmic membrane preparations. In both cases, a double labelling technique was employed whereby cells grown under one condition were labelled with ^{14}C -amino acids and cells grown under the second conditions were labelled with ^3H -amino acids. Comparison of the percentage of each label in a particular protein indicated the relative amounts of that protein synthesized under the two conditions.

i) Total cell envelope preparations were obtained by a modification of the technique of Inouye and Guthrie (49). The cells were grown in the medium as described in section (1 - 2), the length of time and the temperature of labelling differed depending on the particular experiment. In no case did the cell density exceed 1×10^8 cells/ml. For each litre of medium, either 20 μ Ci or non-specifically labelled 14 C-amino acids (specific activity 0.1 mCi/ml; 0.067 mg/ml; New England Nuclear, 575 Albany St., Boston, Mass.) or 100 μ Ci of non-specifically labelled 3 H-amino-acids (Specific activity 0.5 mCi/ml; 0.0342 mg/ml, New England Nuclear, 575 Albany St., Boston, Mass.) were added. On completion of the labelling period the cultures were rapidly chilled by placing the containing flask in a bath of acetone containing solid carbon dioxide. If removal of the radioactive label was required, then filtration and resuspension in preconditioned non-radioactive medium was used as described in section (1 - 5).

The chilled cell suspensions, labelled with 14 C-amino acids under one condition and 3 H-amino acids under the second condition were mixed and centrifuged at 4 C at 5000 rpm for 15 minutes in a Servall SS-3 refrigerated centrifuge. The cell pellet was washed 3 times with 0.01 M phosphate buffer, pH 7.1 and then resuspended in 10 ml of the same buffer at 4 C. The suspension was sonicated for four 1 minute periods using a Biosonik model Bronwill sonicator, at setting '60'. The suspension was then centrifuged for 15 minutes at 5000 rpm in a Servall SS-1 at 4 C. The supernatant was collected. The

pellet was resuspended, sonicated again using the same protocol and following centrifugation this supernatant was mixed with the original supernatant. The combined supernatants were centrifuged at 15000 rpm in a Servall SS-1 at 4 C for 90 minutes. The resulting supernatant was removed, freeze dried and stored for later investigation by polyacrylamide disc-gel electrophoresis as the "non-particulate fraction". The pellet was washed twice using the same buffer and centrifuged settings. The final pellet constituted the cell envelope fraction.

ii) Purified cell membranes were obtained exactly as described by Shapiro et al. (98). The basic difference from the technique described in section (VIII-1i) being the use of ethylene diamine-tetraacetic acid + lysozyme to digest away mucopeptide material and the use of sucrose gradients to separate the membrane from contaminating material.

2. Disaggregation of proteins

i) Cell envelopes as prepared by technique (i) above, were disaggregated in 0.01 M sodium phosphate buffer, pH 7.1 + 1% w/v mercaptoethanol + 1% w/v sodium dodecyl sulphate + 10% w/v glycerol at 70 C for 20 minutes. (Other temperatures and times were used but little variation in final results occurred and the described conditions appeared optimum for disaggregation.)

ii) Cell membrane preparations, and the "non-particulate fraction" were disaggregated as described by Shapiro et al. (98)

using 40 minutes at 40 C.

3. Electrophoresis

The 7.5% polyacrylamide gel solution was prepared by mixing 6 ml of buffer (0.1 M sodium phosphate, pH 7.1 + 0.1% w/v sodium dodecyl sulphate) + 5.4 ml of acrylamide solution (3.9 gm acrylamide + 0.15 gm bis-acrylamide in 25 ml of the same buffer) + 0.6 ml of ammonium persulphate solution (15 mg/ml of water, made freshly on each occasion) + 5% of N,N,N',N-tetramethylethylenediamine (Canal Industrial Co., Rockville, Md.). The solution was pipetted into glass tubes and a small volume of water carefully layered on the surface of the polymerising material so that when the gel solidified (approximately 20 minutes after mixing the reagents) it would have a flat upper surface.

The water was removed when the solution had gelled. The gels were placed in an electrophoresis apparatus, the sample plus tracking dye carefully placed on the top of the gel, both reservoirs filled with 0.1 M phosphate buffer + 0.1% sodium dodecyl sulphate and the electrophoresis was run for 7 hours at 5 milliamps/gel. Following electrophoresis, the gels were fixed in 20% w/v sulphosalicylic acid for 12 - 18 hours.

4. Gel slicing and counting

The fixed gels were washed with water and then frozen. The frozen gels were sliced into 1 mm thick discs using a mechanical gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, England).

The discs were placed in scintillation vials and 0.5 ml of NCS solubilizer solution (Amersham/Searle Co., Des Plaines, Ill.) was added to each vial. The NCS was diluted 9 volumes NCS to 1 volume water before adding to the vials. The vials were incubated at 50 °C for 6 - 8 hours before 10 ml of scintillation fluid was added. (4 gm of 2,5-diphenyloxazole + 0.05 gm of 1,4-(2-(5-phenyl oxazolyl))-benzene per litre of toluene). The samples were counted in a Nuclear Chicago scintillation counter model Unilux II adjusted so that less than 0.1% of tritium counts registered in the carbon-14 channel. The final counts were corrected for efficiency of counting in each channel and for percentage of carbon-14 counts registered in the tritium channel. Calculations were based on results obtained using known standards prepared by the same technique. The radioactivity in a single disc is expressed as a percentage of the total radioactivity of that isotope which entered the gel.

5. Standard curve for molecular weight estimation

A standard curve of R_f values was produced from cytochrome C (molecular weight 12,400), myoglobin (molecular weight 17,800), chymotrypsinogen (molecular weight 25,000), ovalbumin (molecular weight 45,000) and albumin (molecular weight 67,000).

The standards were treated with the SDS-disaggregation solution before being run in parallel with the experiment gels. The standard proteins were localized by chemical staining.

IX. Autoradiography

Cells were grown in minimal medium with 1 gm peptidase per l, 2 gm dextrose per l, 250 mg deoxyadenosine per l to ensure incorporation of thymidine (12), 5 mg thymidine per l and 5 ml C^3H_3 -thymidine per l (specific activity 12.2 Ci/mM; 1 mCi/2 ml; Schwarz BioResearch Inc., Orangeburg, N.Y.) at 30 C for a total of 20 generations, and diluted to give a culture containing 5×10^7 cells/ml. The cells were filtered from the medium and washed three times with fresh medium minus the C^3H_3 -thymidine and resuspended in this medium at 42 C. Samples were taken after various periods at 42 C and autoradiography carried out as described by Caro (15). Ilford L4 Nuclear Emulsion was used. After exposure and development, the preparations were observed by phase contrast using a Zeiss microscope equipped for photography.

X. Microscopy

1. Light microscopy

Cells were grown in nutrient broth at 42 C until long filaments were produced. These were inoculated by means of a pasteur pipette onto nutrient agar covered microscope slides and placed in a humid atmosphere at 30 C. After periods of time at 30 C, slides were removed and the fragmenting filaments and developing microcolonies observed and photographed by phase contrast microscopy.

2. Electron microscopy

During filamentation at 42 C and subsequent recovery at 30 C,

9 ml cell samples were added to 1 ml of 5% v/v glutaraldehyde buffered to pH 7.2 in 0.2 M sodium cacodylate (92). The samples were immediately centrifuged and resuspended in 1 ml of buffered 0.05% glutaraldehyde for 10 hours.

Secondary fixation at room temperature in acetate-veronal-buffered 1% osmium tetroxide, and subsequent washing, agar embedding and treatment with uranyl acetate were done according to Ryter and Kellenberger (91). Dehydration and Epon embedding were done according to Luft (64).

Sections were cut with a glass knife on an LKB Type 4801A ultratome and placed on carbon-formvar-coated grids. All sections were stained for 3 minutes with alkaline lead citrate (85). Examination and photography were done with a Phillips EM 300 electron microscope.

RESULTS

I. Division kinetics of *Escherichia coli* BUG-6

1. Determination of non-permissive temperature

During the initial isolation of the cell division mutants (37), 42 C was always used as the non-permissive temperature, however it was possible that the known inability of *Escherichia coli* BUG-6 to divide at 42 C would extend to temperatures below 42 C, nearer the optimum temperature for growth.

Escherichia coli BUG-6 was grown for several generations at 30 C and the culture then subdivided. The subcultures were placed at 36 C, 38 C, 40 C and 42 C. The 42 C culture stopped dividing almost immediately and the cell number remained constant for the remainder of the experiment. The culture at 40 C, stopped dividing after approximately 10 minutes and then resumed dividing at a rapid rate, 35 minutes after the 30 C → 40 C shift. During the period of non-division, cell size increased. The culture shifted from 30 C to 38 C or 36 C, did not stop dividing but divided at a slower rate and eventually assumed the rate characteristic of *Escherichia coli* BUG-6 grown at 38 C and 36 C, Figure 1. From this result, 42 C was subsequently used as the non-permissive temperature and 30 C as the permissive temperature.

2. The effect of different time periods at 42 C on recovery at 30 C .

Escherichia coli BUG-6 was grown for several generations at

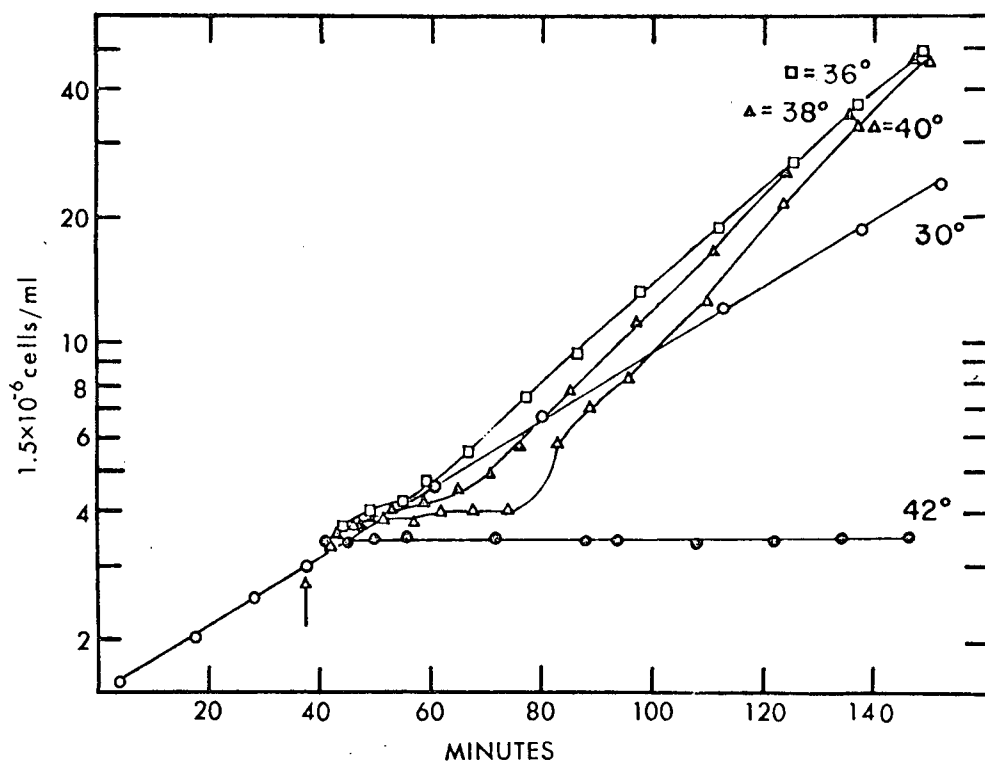


Figure 1. The effect on cell division of shifting Escherichia coli BUG-6 from 30° C to higher temperatures. E. coli BUG-6 was grown in broth for several generations at 30° C (○) and then subdivided at the time indicated by the arrow and subcultures placed at 36° C (□), 38° C (Δ), 40° C (◄) and 42° C (●).

30 C and then shifted to 42 C for different lengths of time. In Figure 2 zero time is the time when the 42 C subcultures were replaced at 30 C. The open circles represent growth of the control culture at 30 C. One minute incubation at 42 C (Figure 2a), has little effect on the division of the cells, but a two minute pulse (Figure 2b), at 42 C, causes a lag before division resumes and 34 minutes is required at 30 C before the control value is attained. These experiments are summarized in Figure 3. The time required for the cell number of the culture incubated at 42 C to reach the 30 C control value is plotted against the length of the period at 42 C. This time is 34 minutes for pulses of 2, 3, and 4 minutes (Figures 2b, 2c, 2d). The period decreases rapidly to a lag of 22 to 24 minutes for temperature pulses exceeding 10 minutes (Figure 2i, 2j). For temperature pulses between 4 and 10 minutes intermediate length lags are observed (Figures 2e - 2h).

This recovery period remains constant until the length of the 42 C pulse exceeds 35 minutes when the recovery period required to reach the 30 C control cell number increases again. In Figure 4, the recovery patterns at 30 C following periods of 45, 60, 75, 90 and 105 minutes at 42 C are shown. In each case, 15 minutes is required before cell division starts and then one rapid doubling of cell number occurs followed by a slower rate of division until the control rate of cell division at 30 C is obtained.

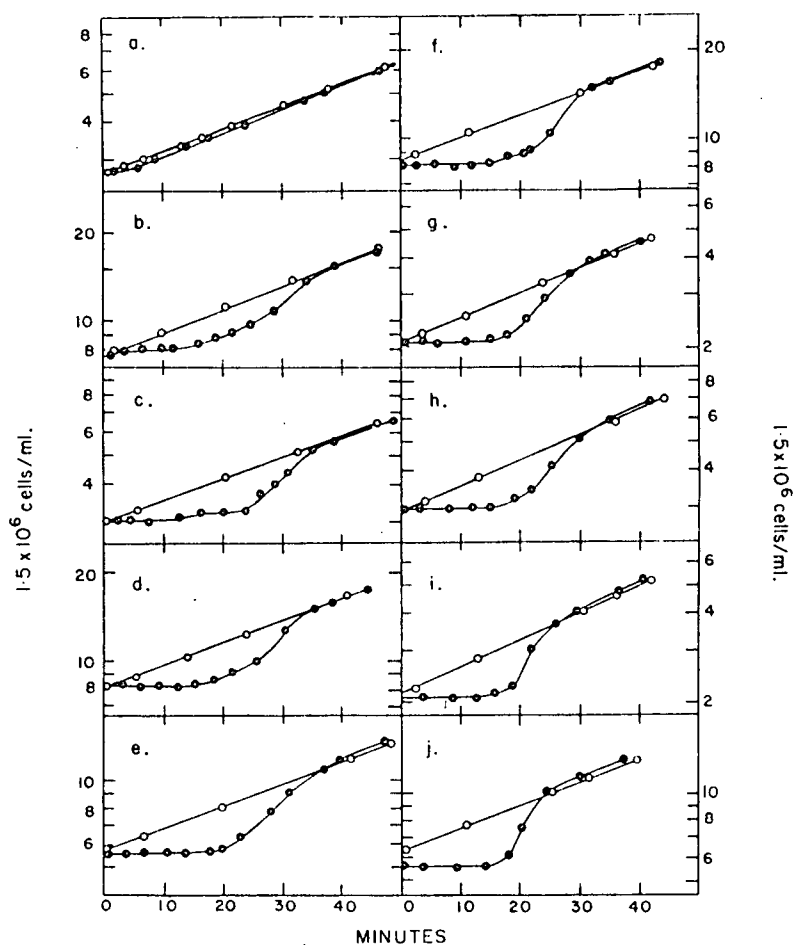


Figure 2. The effect on cell division of shifting *Escherichia coli* BUG-6 from 30°C to 42°C for different time-intervals. *E. coli* BUG-6 was grown in broth for several generations at 30°C (0) and then shifted to 42°C (0) for, a) 1 min; b) 2 min; c) 3 min; d) 4 min; e) 5 min; f) 6 min; g) 7 min; h) 8 min; i) 10 min; j) 12 min before replacing at 30°C. Time 0 min represents the time at which the 42°C cultures were replaced at 30°C.

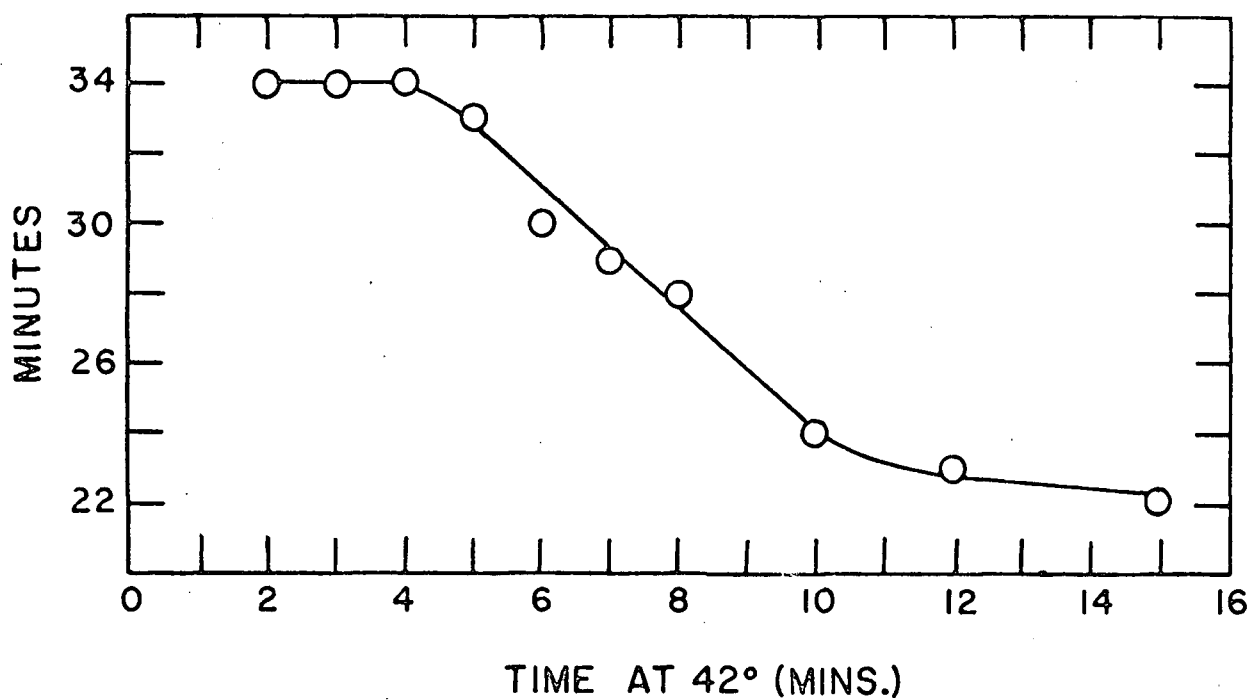


Figure 3.

The variation in recovery time for cultures of *Escherichia coli* BUG-6 placed at 42 C for different time intervals. *E. coli* BUG-6 was grown for several generations at 30 C and subcultures placed at 42 C for different time intervals before replacing at 30 C. The time required for the cell number of a subculture to attain the 30 C control value after being replaced at 30 C is plotted against the time that subculture was kept at 42 C.

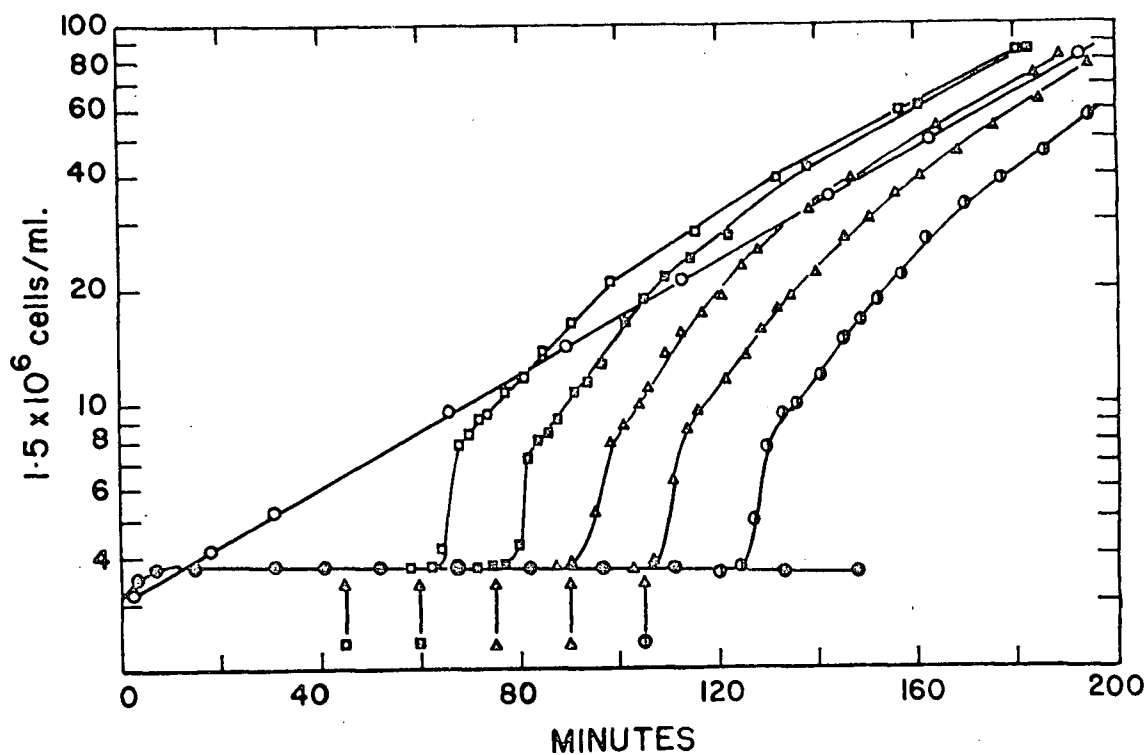


Figure 4.

The effect on cell division of shifting *Escherichia coli* BUG-6 from 30°C to 42°C for different time intervals. *E. coli* BUG-6 was grown in broth for several generations at 30°C (○) and at 0 min part of the culture was shifted to 42°C (●). Subcultures from 42°C were replaced at 30°C after 45 min (□), 60 min (■), 75 min (Δ), 90 min (▲) and 105 min (○) at 42°C.

II. Microscopy

1. Phase contrast light microscopy

The appearance of the filaments after 3 generations of growth at the non-permissive temperature is shown in Figure 5. Photographs were taken at 10, 45, 90, 150 and 210 minutes after shifting the cells back to 30 C (Figure 5). The time before filament fragmentation occurs on the solid medium is much longer than in liquid medium c.f. Figure 4. The resulting cells are smaller than normal Escherichia coli BUG-6 cells growing at 30 C (Figure 5) but eventually return to the normal size (Figure 5e, c.f. Figure 8b).

2. Electron microscopy

No cross-septa were found in filamenting cells (Figure 6a). Filaments were produced at 42 C and then replaced at 30 C. During the recovery period samples were removed 6, 8, 10 and 12 minutes after the 42 C → 30 C shift. The typical state of the septum at each time is shown in Figures 6, b,c,d, and e respectively. Twelve minutes after the 42 C → 30 C shift, the filament is divided but is not yet separated.

3. Autoradiography

Segregation of the nuclei was followed by labelling the DNA at 30 C with C^3H_3 -thymidine for 20 generations and then allowing filaments to form at 42 C in the absence of radioactive label. Autoradiograms of the filaments indicate normal segregation, as seen in Figure 7. i.e. the DNA is segregated throughout the length of the filament.

Figure 5. Fragmentation of BUG-6 filaments during recovery at 30 C. After three generations at 42 C, filaments were inoculated on slides precoated with nutrient agar and incubated at 30 C. Slides were removed at 10 (a), 45 (b), 90 (c), 150 (d), and 210 (e) minutes and examined by using a Zeiss phase-contrast microscope. Magnification of these photographs is approximately 5,000 X.

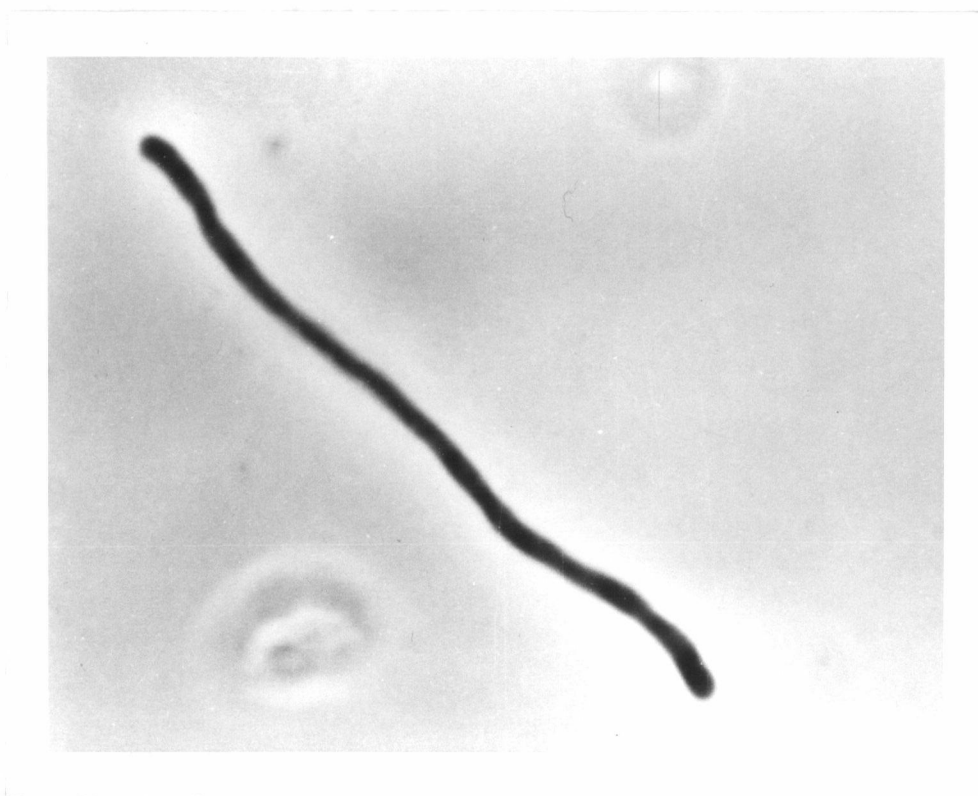


Figure 5 (a)

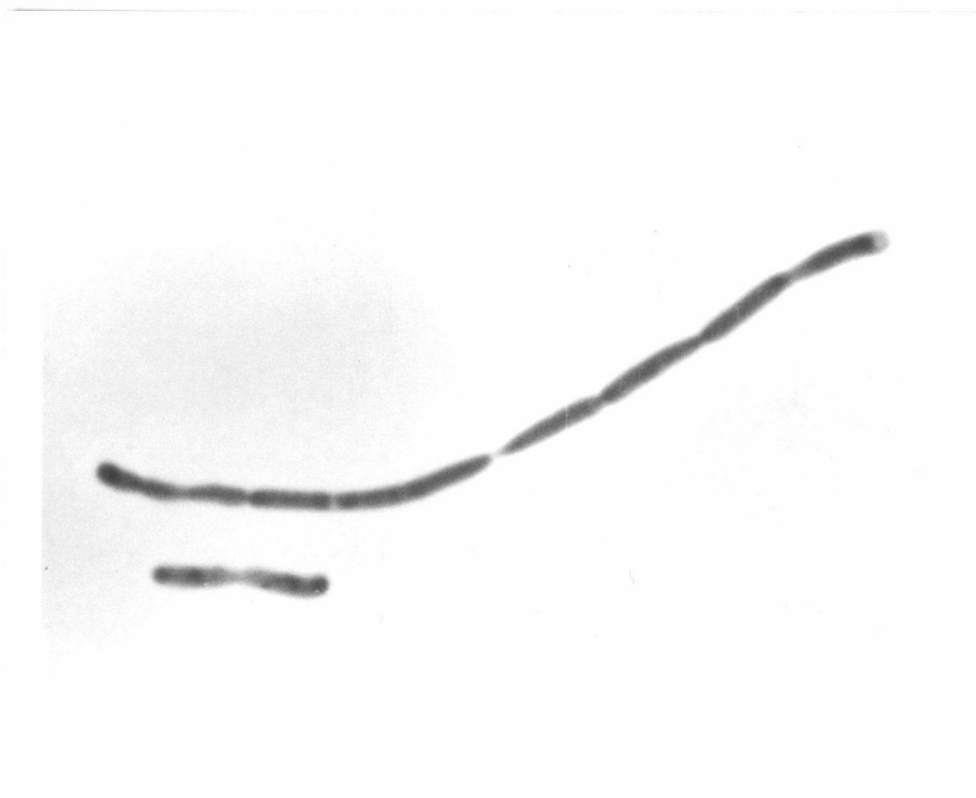


Figure 5 (b)



Figure 5 (c)

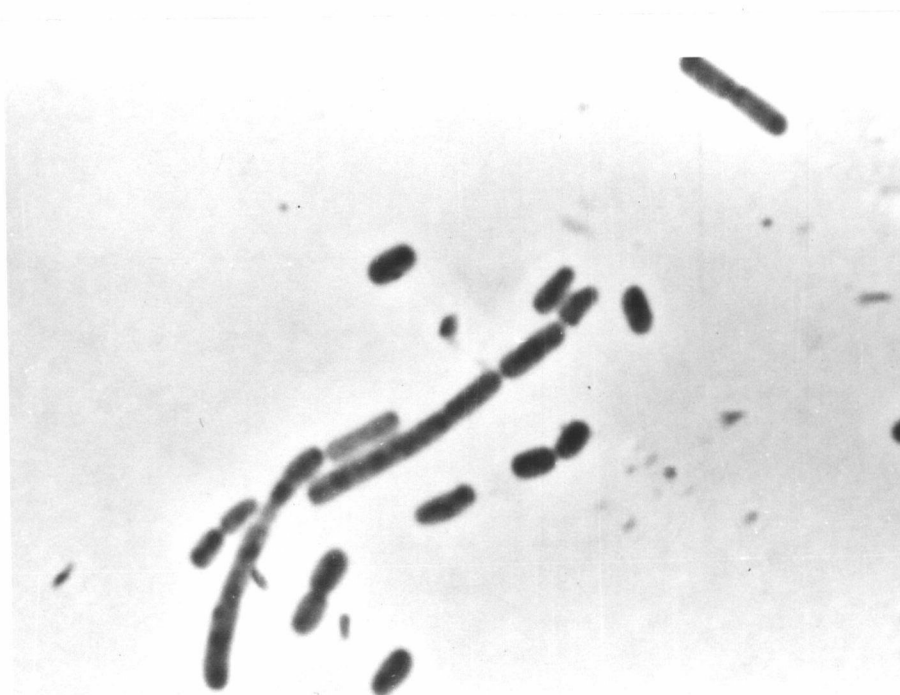


Figure 5 (d)

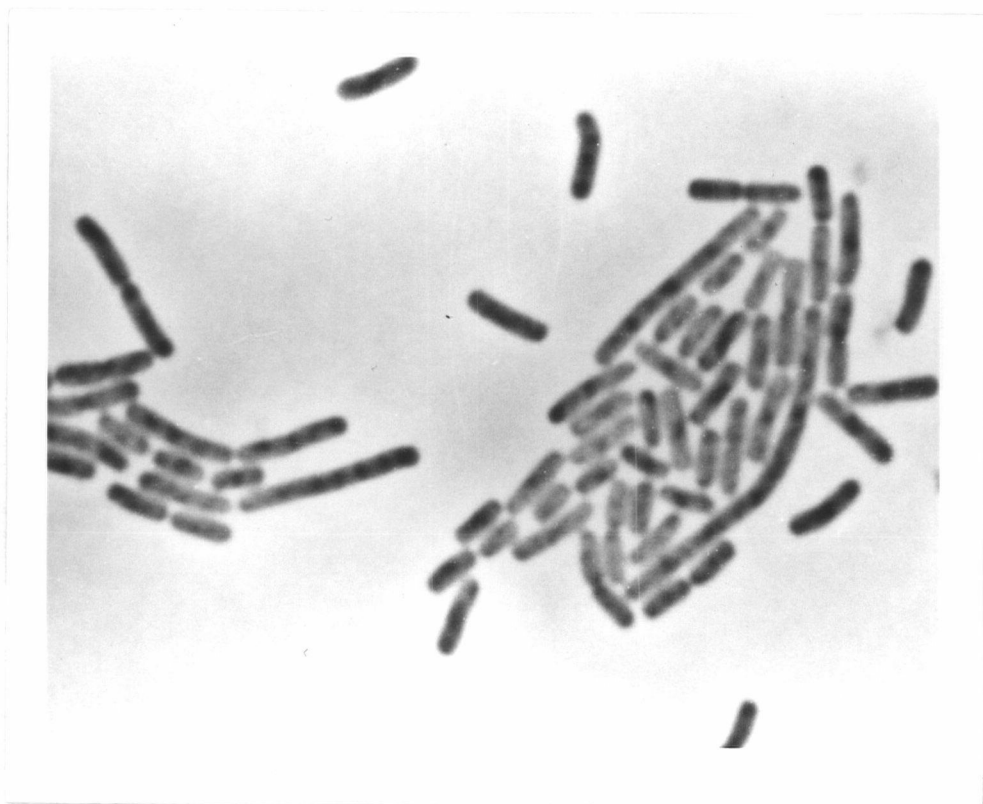
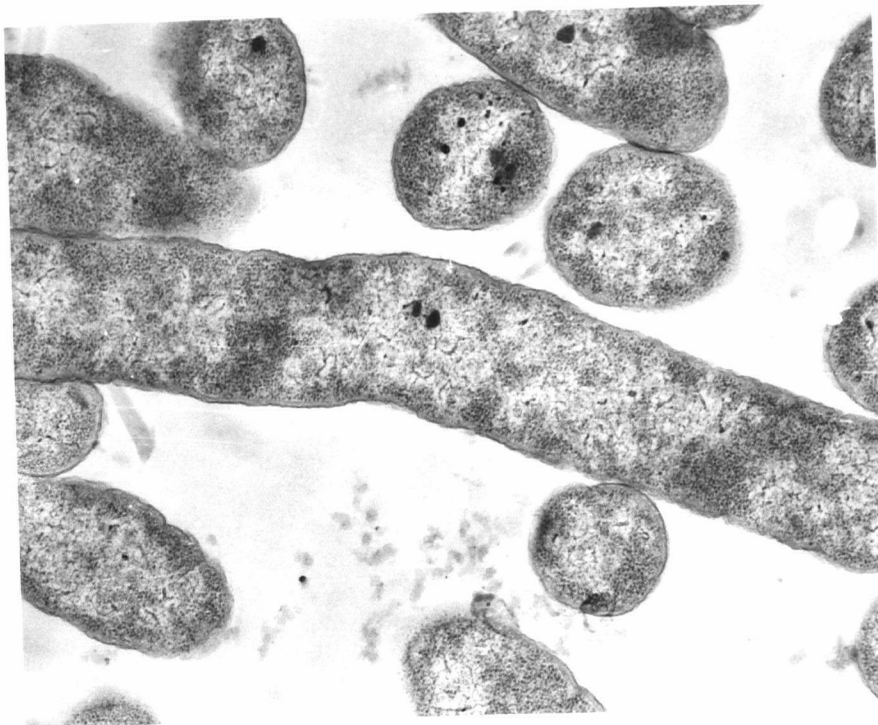


Figure 5 (e)

Figure 6.

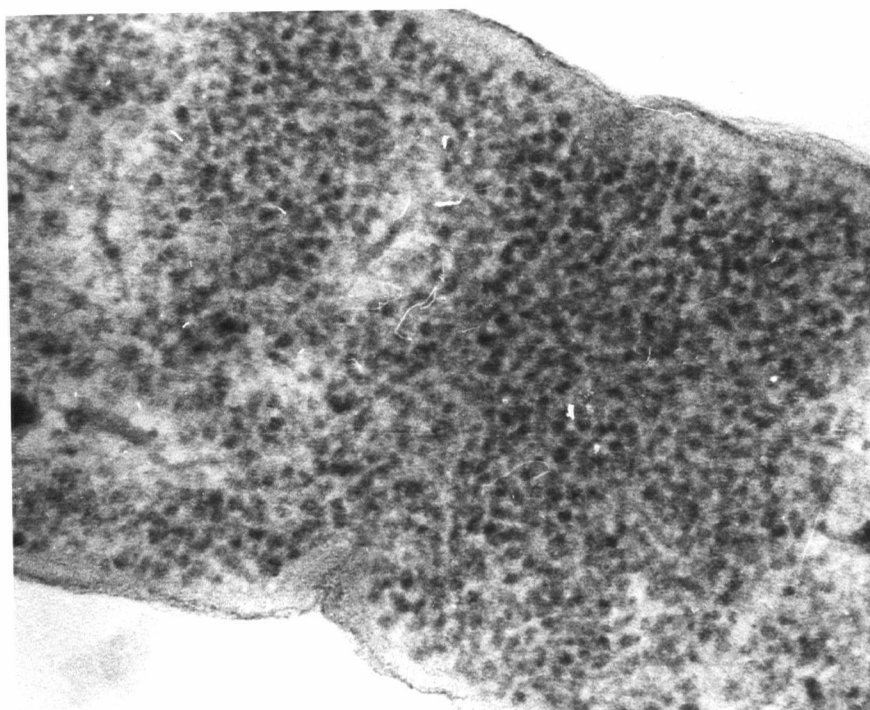
Electron microscopy of Escherichia coli BUG-6 grown at 42 C and during recovery at 30 C. A culture of E. coli BUG-6 was grown for 60 min at 42 C in broth and then replaced at 30 C. Samples were removed at a) 0 min; b) 4 min; c) 6 min; d) 8 min; e) 10 min, and f) 12 minutes after the 42 C to 30 C shift and prepared for and observed by electron microscopy as described in Methods, section (X-2). Magnifications are approximately 6a - 30,000; 6b-g - 120,000 X.

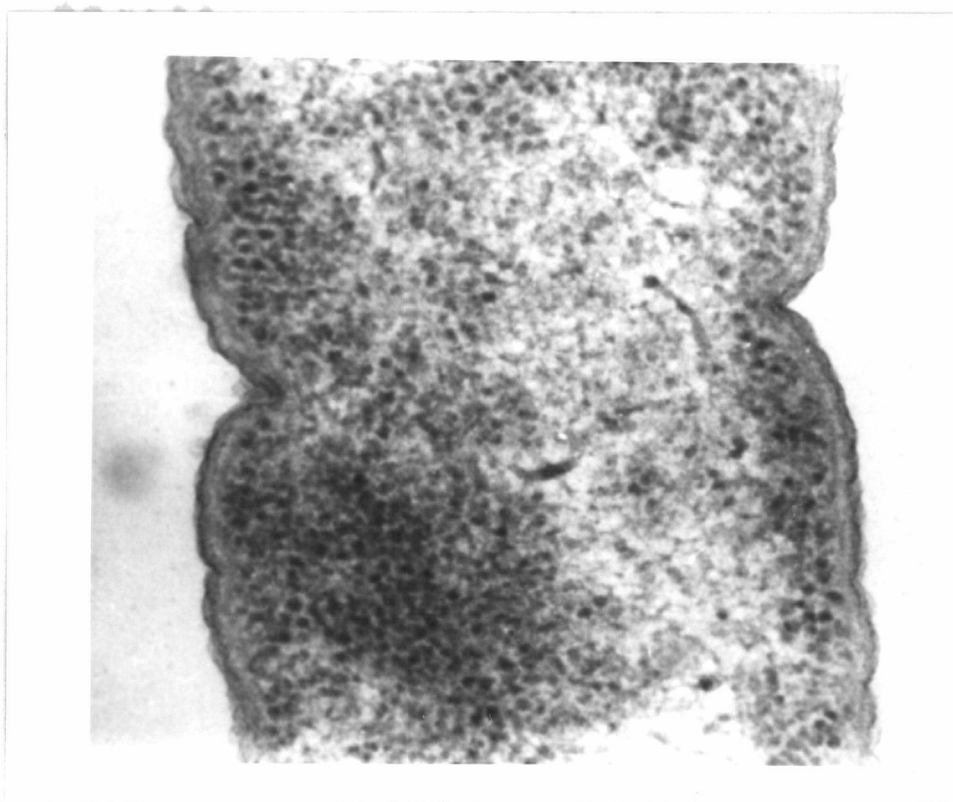


(a)

Figure 6

(b)

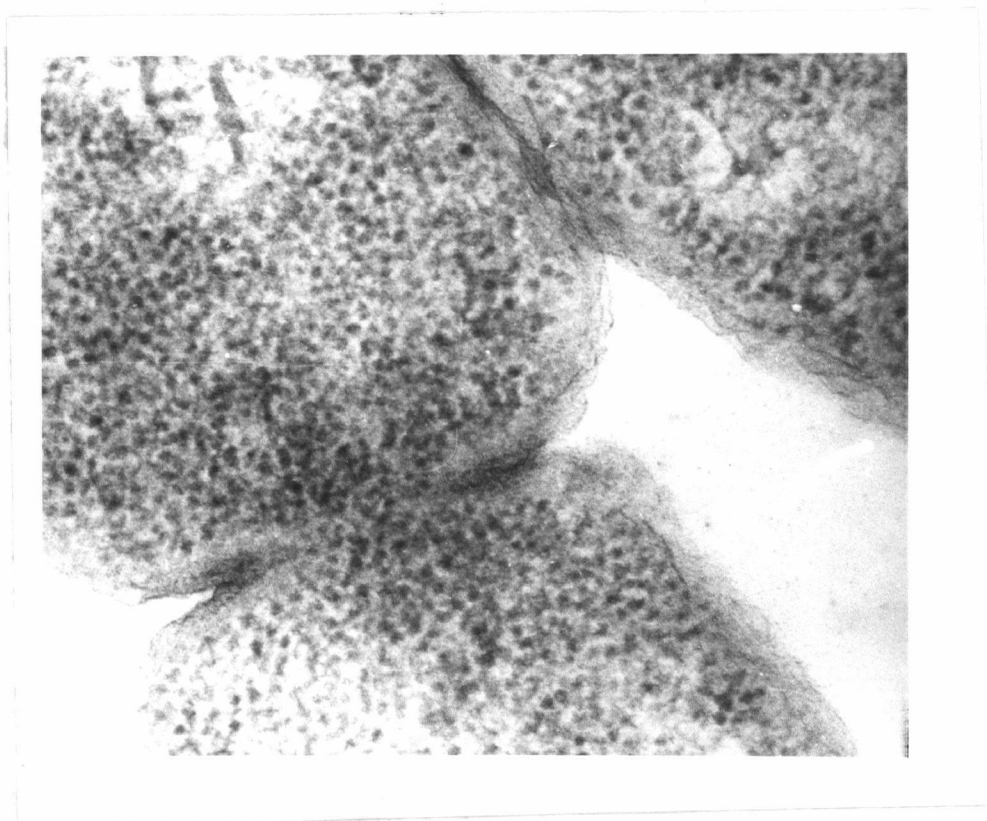


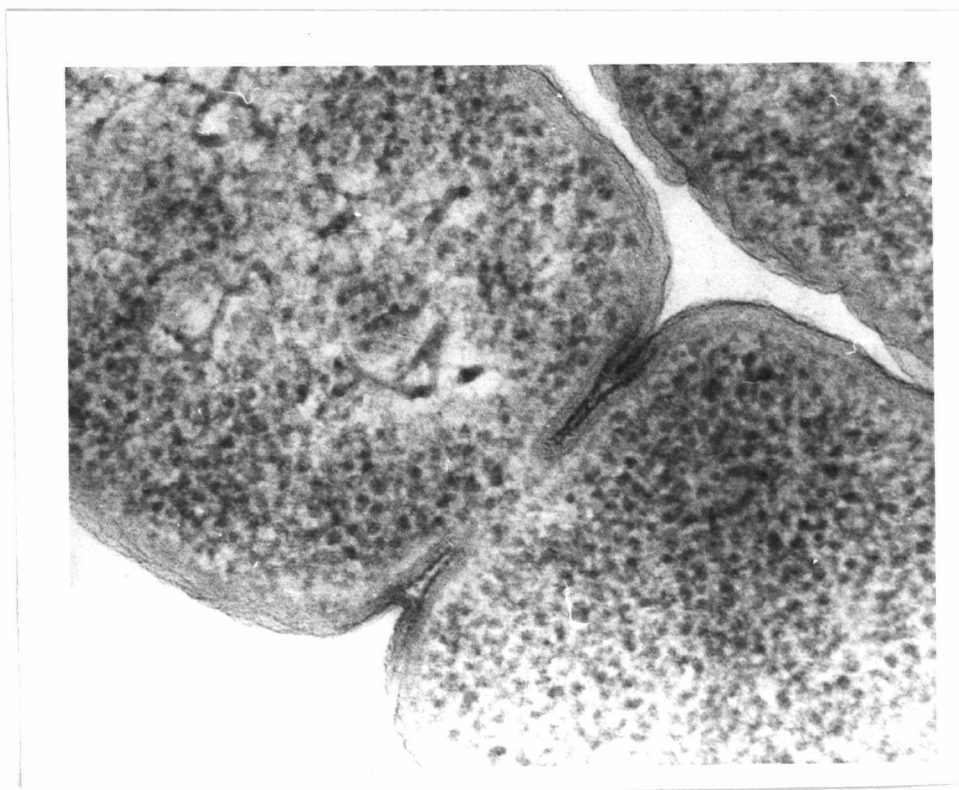


(c)

Figure 6

(d)

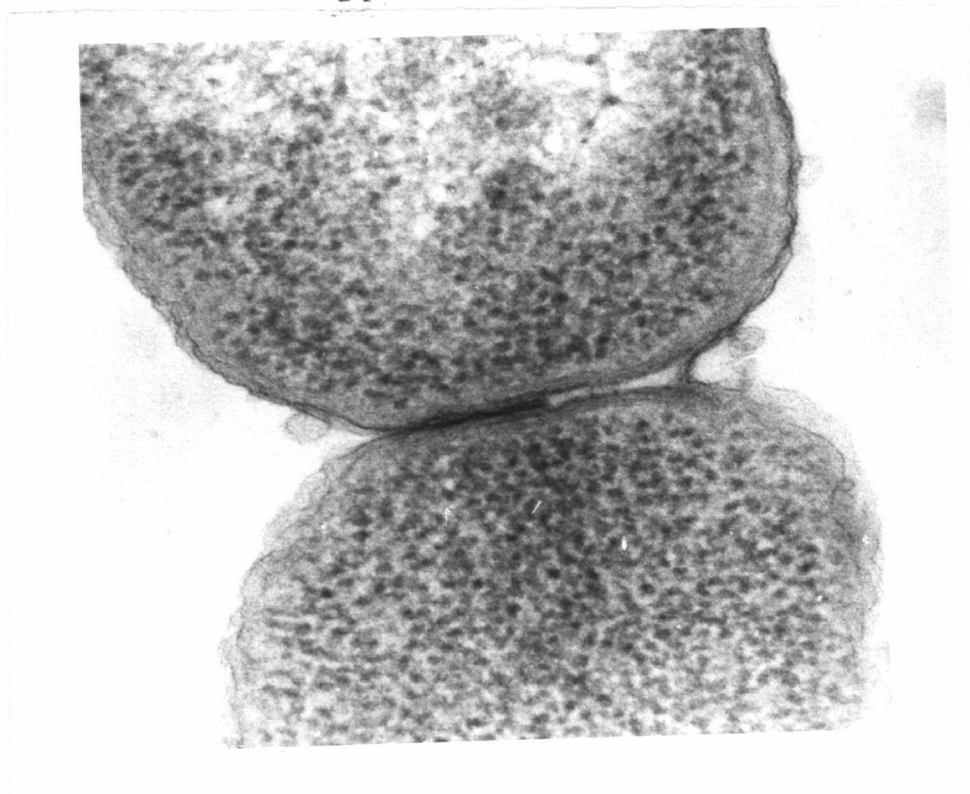




(e)

Figure 6

(f)



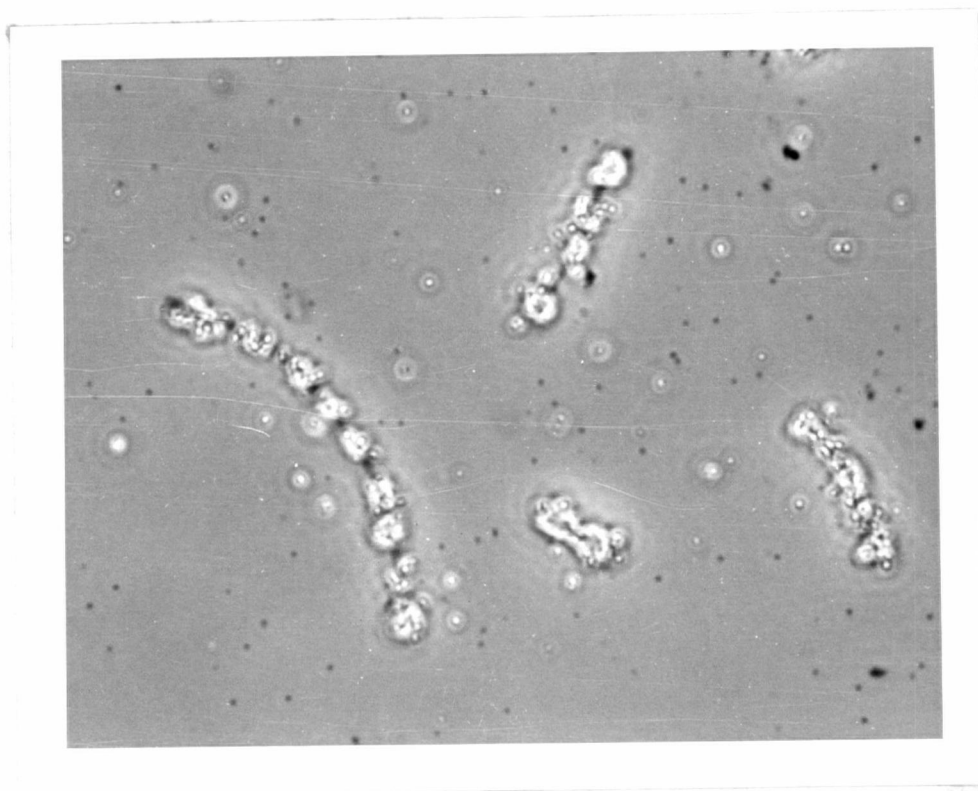


Figure 7. Nuclear segregation in BUG-6 at the non-permissive temperature (42 C) as determined by autoradiography. Cells were grown at 30 C in 5 μ g C^3H_3 -thymidine per ml at a specific activity of 1 μ C/ μ g plus 250 μ g deoxyadenosine per ml for at least 20 generations. The cells were washed, resuspended in the same medium plus unlabelled thymidine and grown at 42 C. Samples removed at intervals were fixed on slides and coated with Ilford L4 emulsion. The slides were developed after an appropriate period of exposure and examined microscopically. Magnification is 3000 X.

III. Cell division and macromolecular synthesis at 30 C and 42 C.

Exponential cells which had been grown and measured for at least 6 generations prior to zero time were measured carefully for increase in cell number for 25 min. At this time, a sample was removed from the control flask at 30 C and shifted to 42 C. As seen in Figure 8a, there is an abrupt cessation in increase in cell numbers and the cell number remains constant for the duration at 42 C. On the other hand, the mass continues to increase as indicated by an increase in the peak channel position in the Coulter Counter (Figure 8b). After 45 min at 42 C, a sample was returned to 30 C and the recovery of the cells at the permissive temperature was observed. An abrupt increase in cell number occurs after a lag of about 10 - 15 min during the recovery period (Figure 8b). The average cell size returns to slightly smaller than that of a cell during balanced growth at 30 C (Figure 8b).

Analysis of the rate of total protein, RNA and DNA synthesis during the course of the experiment is given in Figure 9. The rate of protein synthesis per cell is constant during growth at 30 C and increases exponentially during incubation at 42 C. As the cells are shifted to 30 C during the recovery period, the rate quickly assumes the control rate coincident with the abrupt increase in cell number at about 90 min. The rate of RNA synthesis as measured by the incorporation of ^{14}C -uracil during short pulses, was almost identical to the pattern observed for protein synthesis (Figure 9).

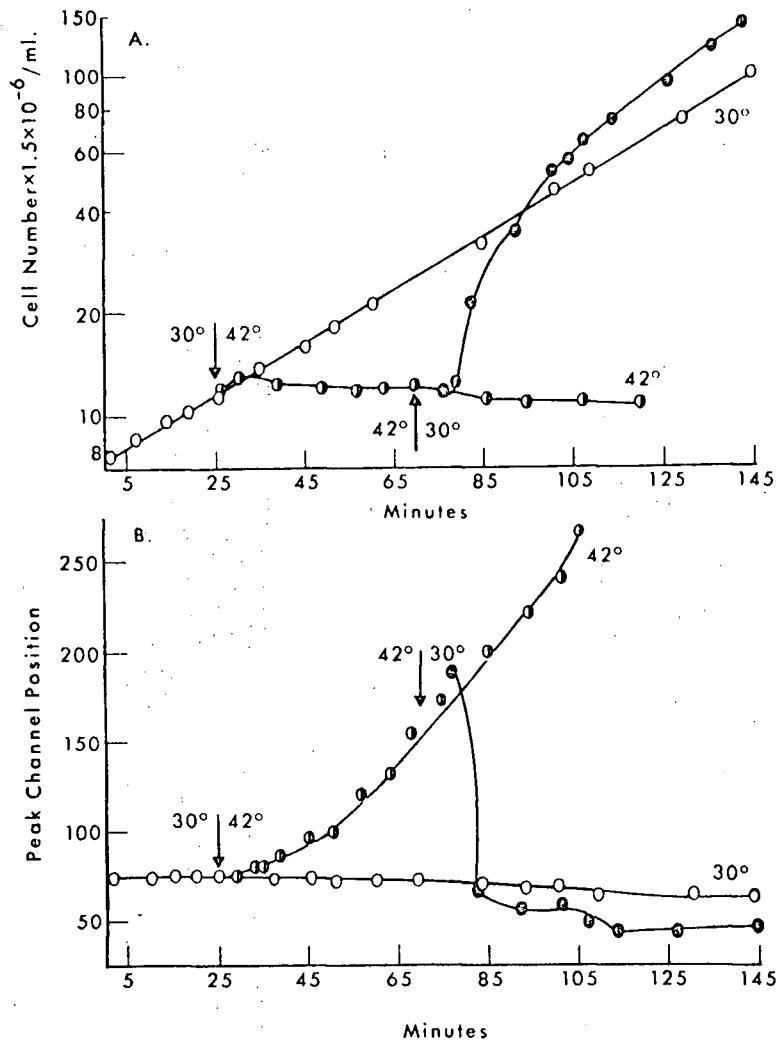


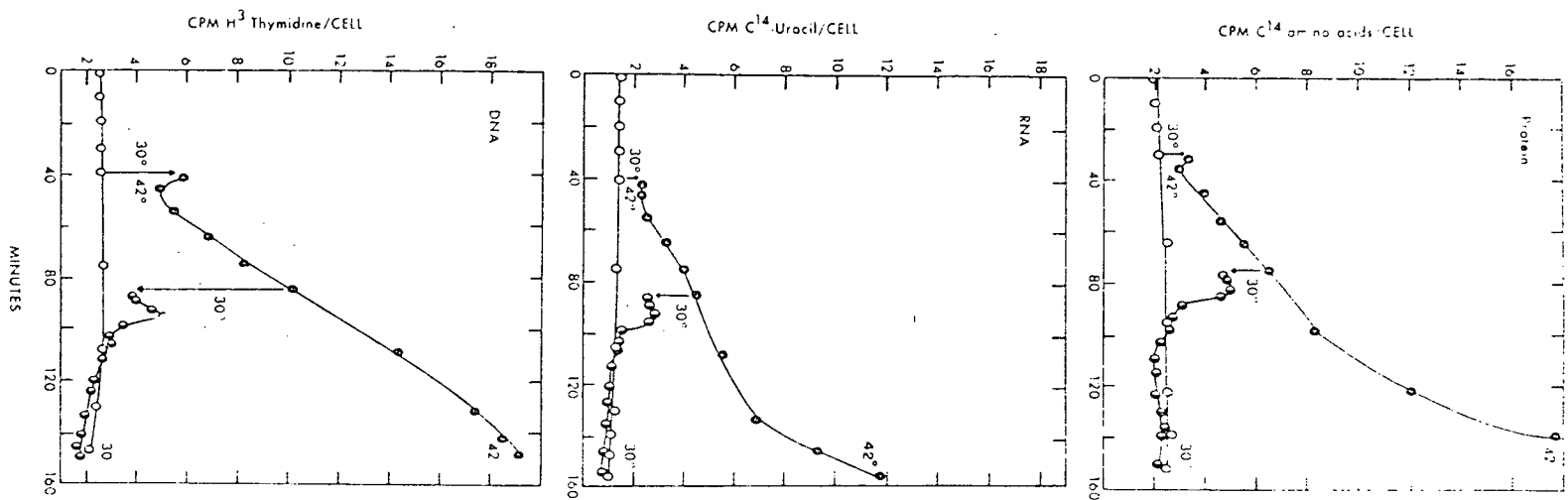
Figure 8.

Cell number (A) and cell size (B) of BUG-6 as a function of time at the permissive (30°C) and at the non-permissive (42°C) temperature. Part of a culture grown for several generations at 30°C (○) was shifted to 42°C at 25 min (○). After 45 min at 42°C, part of this culture was returned to 30°C (●). Cell size changes are monitored by plotting the position of the peak of the size distribution obtained from a pulse height analyser attached to a Coulter counter. This machine is capable of separating a population of cells into 512 relative sizes and depicting the number of cells of each size.

Figure 9.

The rate of protein, RNA and DNA synthesis per cell number in BUG-6 as a function of time at the permissive (30 C) and non-permissive (42 C) temperature. A culture in the steady state at 30 C (0) was divided, and part of the culture was placed at 42 C (●). After a period of growth at 42 C that culture was again divided, and part of it was returned to 30 C (○).

(i) Protein, (ii) RNA, and (iii) DNA synthesis were measured by 5-min exposures of 1 ml samples to (i) 1 μ Ci per ml of 3 H-amino acid mixture at a specific activity of 16.1 mCi/mg to the 007 medium containing 1 mg of peptidase per ml, (ii) 5 μ g of uracil-2- 14 C at a specific activity of 46.7 μ Ci/ μ mole, and (iii) 0.5 μ g of thymidine- 3 CH₃ at a specific activity of 3.87 Ci/mmole. Incorporation was terminated by addition of two volumes of 7.5% cold trichloroacetic acid containing 200 μ g of the appropriate unlabelled compound/ml.



The rate of DNA synthesis per cell during growth at 30 C, 42 C and during the recovery period at 30 C is indicated in Figure 9. The control has a relatively constant count of 2.5 cpm per cell. At the time of the shift to 42 C there is a sudden increase in the rate of thymidine incorporation to about 6 cpm per cell. The rate drops slightly and then increases during incubation at 42 C to about 20 cpm per cell at about 160 minutes. Cells which are shifted back to 30 C after incubation at 42 C show an immediate decrease in the rate of thymidine incorporation, which corresponds closely to the control level (3.5 cpm per cell count), which increases at about 90 minutes and then, following division of the filaments, returns to the control level at about 160 minutes.

The rate of total lipid synthesis per cell during growth at 30 C, 42 C and during the recovery period at 30 C, measured by glycerol-2-³H incorporation, is shown in Figure 10. The rate of lipid synthesis per cell is constant during growth at 30 C and increases exponentially at 42 C. The changes in rate of lipid synthesis during the recovery period are different from the changes in rate observed for protein, RNA and DNA synthesis. During the period between the 42 C → 30 C shift and filament division, a high rate of label incorporation is maintained and even after the rapid division phase, at 90 minutes, a rate of incorporation double that of the 30 C control is observed. The rate then gradually decreases to that of the control by 120 minutes.

These results indicate that the filaments produced at 42 C

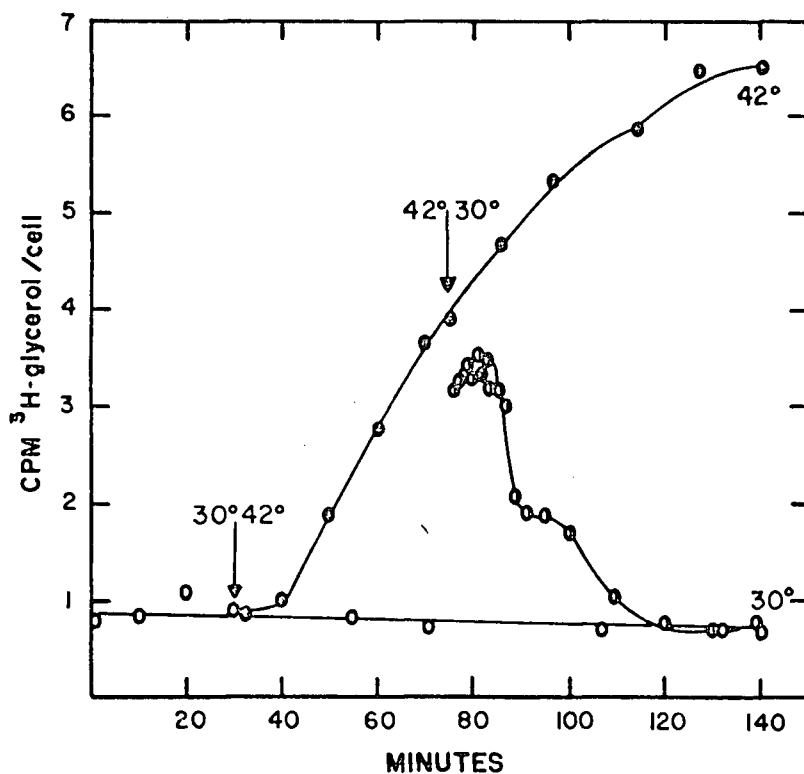


Figure 10.

The rate of lipid synthesis per cell number in *Escherichia coli* BUG-6 as a function of time at the permissive (30°C) and non-permissive (42°C) temperature. An exponential culture at 30°C (○) was divided and part placed at 42°C (●). After 45 minutes at 42°C that culture was again divided and part of it returned to 30°C (○). Lipid synthesis was measured by 3 minute exposures of 1 ml samples to 0.5 µg of glycerol containing 1 µCi of glycerol-2-³H. Incorporation was terminated by the addition of 2 volumes of 7.5% w/v cold trichloroacetic acid containing 200 µg glycerol/ml.

continue to synthesize protein, RNA, DNA and lipid and that on filament fragmentation cells which synthesize these macromolecules at the normal 30 C rate are eventually produced. Because of the abrupt change in the rate of C^3H_3 -thymidine incorporation following the 30 C \rightarrow 42 C shift, it was considered that perhaps this was an inaccurate measure of DNA synthesis and in order to check this possibility, chemical fractionation of cells grown at 30 C and 30 C plus 60 minutes at 42 C, was carried out. RNA, DNA and protein contents, as a percentage of dry weight, were compared (Table 1). Cells grown at 30 C and at 42 C, appear to have very similar DNA: protein ratios supporting the suggestion that the use of C^3H_3 -thymidine as an indicator of DNA synthesis gives inaccurate results immediately following a temperature shift (see above and (103)). The chemical fractionation also indicates a decrease in the percentage of RNA in cells grown at 42 C. This is supported by the uracil- ^{14}C incorporation studies (Figure 9), which indicates a slower rate of RNA synthesis than protein synthesis at 42 C, (Figure 9).

IV. Individual phospholipid synthesis at 30 C and 42 C

The high rate of incorporation of glycerol 2- 3H during the recovery period (Figure 10) suggested that the rate of lipid synthesis was elevated during this period. This would be predicted if rapid synthesis of septa were occurring. Ballestra et al. (5) have shown specific changes in phospholipid composition of cells undergoing

division. Starka et al. (106) described changes in the ratios of phospholipids when Escherichia coli cells were converted into filaments by penicillin treatment.

Table 1. The percentages of the total dry weight of cells, grown at 30 C and 30 C + 60 minutes at 42 C, as constituted by protein, RNA and DNA.

Percentage of dry weight of cells constituted by:-			
Growth temperature	Protein	RNA	DNA
30 C	51	36	3.1
30 C + 60 min at 42 C	56	27	3.2

The rate of synthesis of individual phospholipids was therefore investigated to determine which phospholipids were being labelled and whether this changed during filamentation or during the rapid division phase.

A large culture of Escherichia coli BUG-6 was grown at 30 C for several generations and then shifted to 42 C for 45 minutes before

being returned to 30 C. Five ml samples were taken at intervals and pulsed for 3 minutes with glycerol-2-³H. The phospholipids were extracted, separated and their individual radioactivity measured. The total radioactivity extracted from each sample, as phospholipids, was also measured. In Figure 11, the rate of synthesis of phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) are depicted. The radioactivity incorporated into each individual phospholipid is expressed as a percentage of the radioactivity incorporated into the material extracted by the Bligh and Dyer (11) lipid extraction technique.

The rates of incorporation of radioactivity into PE and PG remain constant at 30 C and 42 C until the recovery period, ie. filamentation does not alter the rate of synthesis of these phospholipids. During the recovery period, there is at first an apparent drop in the rate of synthesis of PE and increase in the rate of synthesis of PG. However, when the filaments divide, at about 90 minutes, the reverse appears to occur and by 120 minutes, the same rates as before the 42 C → 30 C shift are recovered.

Escherichia coli BUG-6 contains other phospholipids, namely cardiolipin (CL) and lysophosphatidyl ethanolamine. However, the amount of radioactivity incorporated into these fractions during 3 minutes is very small and at no time does it exceed 5% of the total extracted radioactivity. The fluctuation in these minor components is very small and probably within experimental error. For this reason

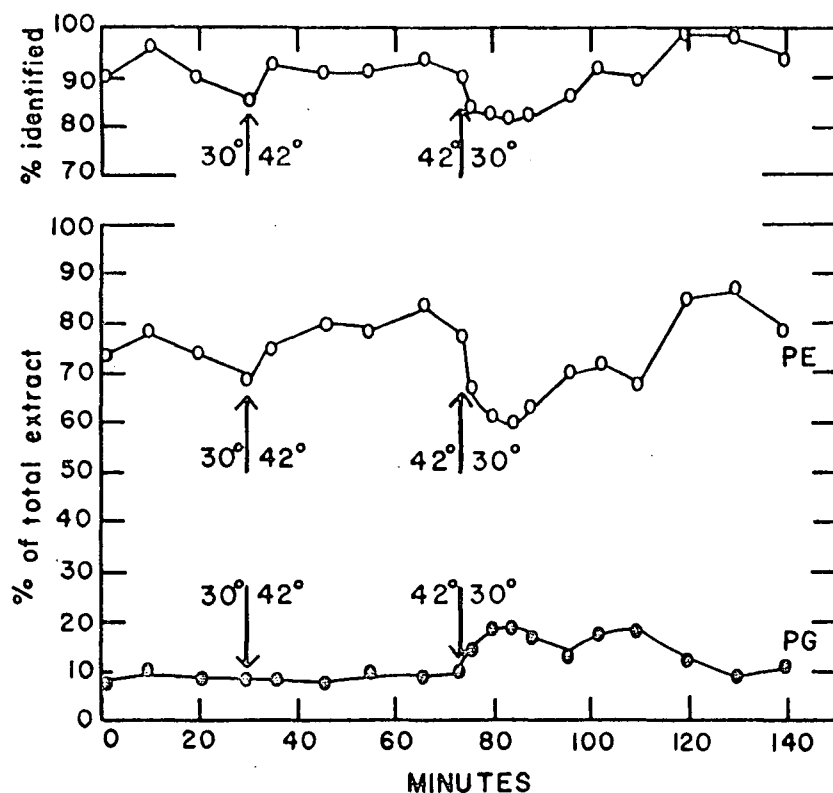


Figure 11. The rate of synthesis of phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) in *Escherichia coli* BUG-6 as a function of growth at the permissive (30 C) and non-permissive (42 C) temperatures. Five ml samples were exposed for 3 minutes to 0.5 μ g glycerol/ml containing 2 μ Ci of glycerol-2- 3 H/ml. The phospholipids were extracted and separated, as described in "Materials and Methods" and the radioactivity in each individual phospholipid was measured. At the top of the graph the "% identified" is time percentage of extracted radioactivity identified in known phospholipids for each sample.

these components were omitted from Figure 11 except for calculations of the "% identified". This is the sum of the radioactivity in each identified phospholipid expressed as a percentage of the total extracted radioactivity. The "% identified" remains above 90% throughout the growth at 30 C and filamentation at 42 C but drops to about 80% during the early part of the recovery period, coincident with the apparent drop in the rate of synthesis of PE. During the rapid division phase the "% identified" returns to above 90%.

The drop in the "% identified" indicates that labelled material is being extracted but it is not one of the normally extracted phospholipids and does not form a spot on the thin-layer chromatography system employed. Possible explanations will be discussed later.

V. Fatty acid synthesis at 30 C and 42 C

Marr and Ingraham (66) demonstrated that an increase in temperature of incubation increased the degree of saturation found in the fatty acids of Escherichia coli. The composition of the fatty acids of Escherichia coli BUG-6, extracted from the total phospholipid, was determined for cells grown at 30 C, 30 C + 60 minutes at 42 C and 8 minutes after the 42 C → 30 C shift to ascertain whether the production of filaments was caused by an inability to regulate the degree of saturation found in the cells' fatty acids. The time of 8 minutes after the 42 C → 30 C shift was chosen as this coincided with the high

rate of phospholipid synthesis as shown in Results (section IV).

Because the fatty acid content of different phospholipids has been shown to differ markedly (24), the fatty acids were also extracted from the major individual phospholipids isolated from cells grown at 30 C and at 30 C + 60 minutes at 42 C. This would prevent the results from total phospholipid fatty acids masking a lesion in the control of fatty acid saturation in an individual phospholipid.

The results are shown in Table II, each fatty acid is expressed as a percentage of the total fatty acids applied to the gas-liquid chromatograph. The cyclic compounds were identified by their retention times and comparison with the published fatty acid compositions of Escherichia coli (24, 79). The compound labelled a, was not identified but its retention time suggested a carbon chain length longer than C-19.

The results indicate that Escherichia coli BUG-6 does not regulate the degree of saturation of its fatty acids and in fact, indicates that this can be accomplished very rapidly, as 8 minutes after the 42 C \rightarrow 30 C the fatty acid composition is already intermediate between the 30 C and 42 C compositions. The regulation also occurs correctly for the fatty acids contained by all the major individual phospholipid. During growth at 42 C, the two cyclic fatty acids apparently increase markedly. For comparison, the fatty acids of the parent strain, Escherichia coli AB1157 were isolated from cells grown at the same temperature (Table III). The actual percentages of

	Total Phospholipid			PE		Lyso-PE		PG	
Temp. °C	30	42	30 42 30	30	42	30	42	30	42
C ^{14:0}	2.7	4.6	3.1	1.9	2.1	2.1	4.3	1.4	2.0
C ^{16:0}	47.4	52.5	49.4	44.3	47.7	39.6	44.0	40.7	45.0
C ^{16:1}	17.5	13.6	14.7	23.1	14.7	19.5	14.3	12.8	11.2
C ^{18:0}	2.1	2.6	1.9	1.1	1.7	2.7	3.4	1.7	1.5
C ^{18:1}	29.9	20.6	26.2	29.6	26.4	33.5	23.0	43.4	30.8
C ^{17:0}	<1	1.3	2.3	<1	5.1	<1	7.2	<1	5.1
C ^{19:0}	<1	3.9	2.6	<1	3.0	1.9	3.7	<1	<1
C ^P	<1	<1	<1	<1	1.3	<1	<1	<1	4.3

Table II. The fatty acid composition of Escherichia coli BUG-6 grown at 30 C, 30 C + 60 minutes at 42 C, and 30 C + 60 minutes at 42 C, + 8 minutes at 30 C. Each fatty acid is expressed as a percentage of the total fatty acid composition of phosphatidyl ethanolamine (PE), lysophosphatidyl ethanolamine (lyso-PE) and phosphatidyl glycerol (PG) extracted from cells grown at 30 C and 30 C + 60 minutes at 42 C is also presented.

Table III. The fatty acid composition of Escherichia coli AB1157 grown at 30 C, 30 C + 60 minutes at 42 C and 30 C + 60 minutes at 42 C + 8 minutes at 30 C. Each fatty acid is expressed as a percentage of the total fatty acids applied to the gas-liquid chromatography system.

Temperature °C	Total phospholipid		
	30 C	42 C	30 - 42 - 30 C
C ^{14.0}	4.5	4.8	4.1
C ^{16.0}	47.5	54.2	54.4
C ^{16.1}	22.3	19.6	18.2
C ^{18.0}	3.4	4.3	3.7
C ^{18.1}	22.9	17.1	21.6
C ^{17v}	<1	<1	<1
C ^{19v}	<1	<1	<1
C [?]	<1	<1	<1

the different fatty acids differ slightly from those found for Escherichia coli BUG-6, however the saturation of the fatty acids is controlled in the same manner. The most noticeable differences between wild type and mutant is the increase in cyclic fatty acids which occurs in the mutant at 42 C but apparently does not occur in the wild type. (DeSiervo (24) recently reported that acidic hydrolysis of phospholipids tends to degrade cyclic fatty acids so that the results obtained must be regarded as minimum values). A similar variation in cyclic C19-fatty acid can be found in the results of Peypoux et al. (79) in their analysis of a temperature-sensitive DNA mutant (Escherichia coli CR34T46) and its parent (Escherichia coli CR34). This mutant also forms filaments at the non-permissive temperature.

VI. The effect of high salt concentration on cell division at the non-permissive temperature

Escherichia coli BUG-6 resembles a temperature-sensitive division mutant of Escherichia coli strain PAT 84 (86), which is able to divide at the non-permissive temperature if supplemented with high concentrations of NaCl or sucrose. A similar effect is observed with Escherichia coli BUG-6, as illustrated in Figure 12. Escherichia coli BUG-6 was grown for several generations at 30 C and part was shifted to 42 C. At 74 minutes after the zero time, samples were removed and brought to a final concentration of 5, 7, 9 and 11 g of

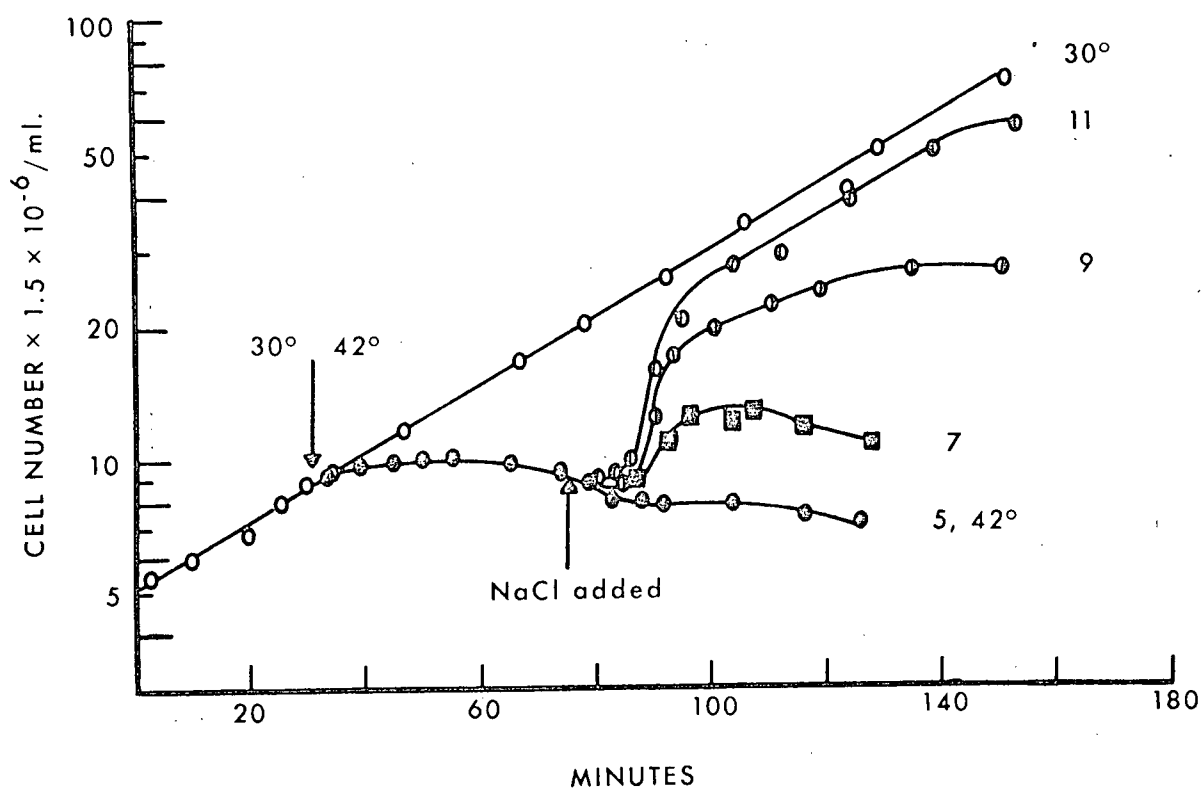


Figure 12. The effect of NaCl on BUG-6 filaments at 42 C. BUG-6 was grown at 30 C (○) in nutrient broth containing 5 μg NaCl/l. Part of the culture was shifted to 42 C (○) at 30 minutes. After 45 min at the non-permissive temperature, the culture was subdivided and NaCl was added to each culture giving a final concentration of 7 g/l (■); 9 g/l (○) and 11 g/l (○).

NaCl per liter. Cell division occurred approximately 15 minutes after the addition of salt (Figure 12). As the concentration of NaCl is increased to 11 g/l, there is a proportional increase in the amount of residual division. In the experiment illustrated by Figure 13, the concentration of NaCl was brought to 12 g/l at the time indicated by the arrow at 30 C, and part of the culture placed at 42 C. Division stopped at both 30 C and 42 C for 20 minutes and then both cultures began to divide rapidly until normal division rates were obtained. In this case, division continued at 42 C.

VII. The effect of pantoyl lactone on cell division at 42 C

It has been reported that the addition of pantoyl lactone to filamentous cells causes them to divide (3, 38). The effect of addition of pantoyl lactone on Escherichia coli BUG-6 at 42 C was investigated. A culture was grown at 30 C for several generations and placed at 42 C for 45 minutes. At this time, various concentrations of pantoyl lactone were added to subcultures. In Figure 14 the effect of the pantoyl lactone on the increase in cell size of the filaments is depicted. In no case does cell size decrease, indicating that cell division did not occur. The rate of increase in cell size decreases, as the concentration of pantoyl lactone added increases. This confirms previous reports that pantoyl lactone has a deleterious effect on growth rate (3). An increase in optical density occurred in the presence of pantoyl lactone but Coulter counter measurements gave no increase in cell division.

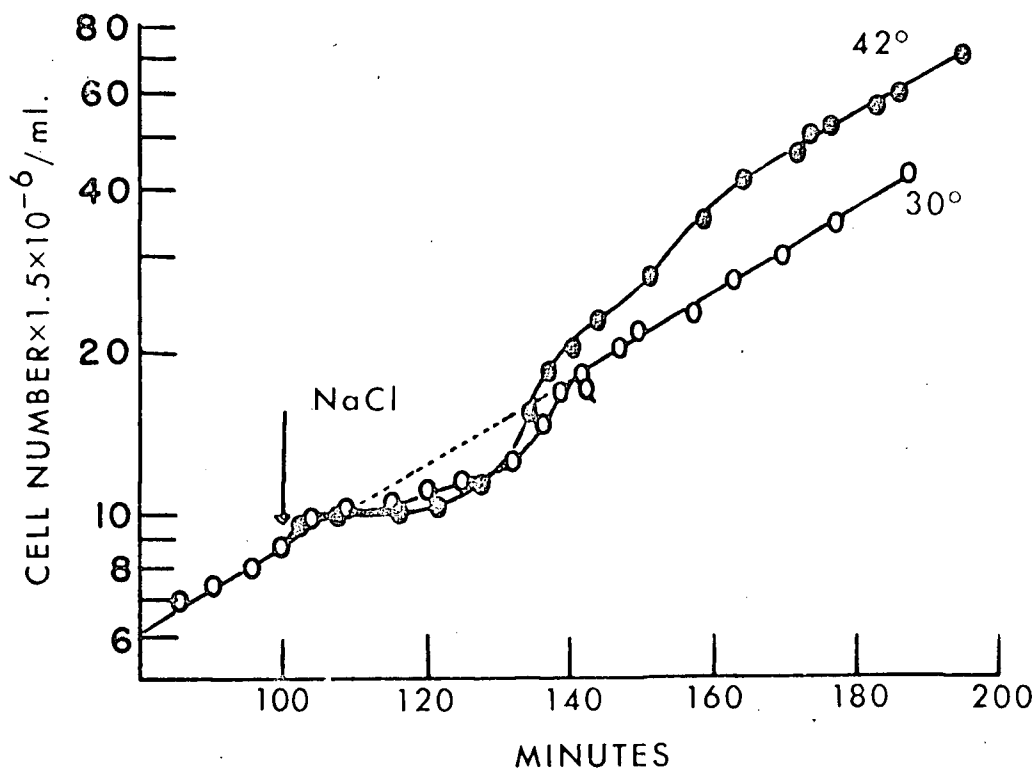


Figure 13. The effect of NaCl on *Escherichia coli* BUG-6 at 30 C and 42 C. *Escherichia coli* BUG-6 was grown in broth containing 5 g NaCl/l for several generations (O). At the time indicated by the arrow, NaCl was added giving a final concentration of 12 g/l. Also, at this time, part of the culture was shifted to 42 C (●).

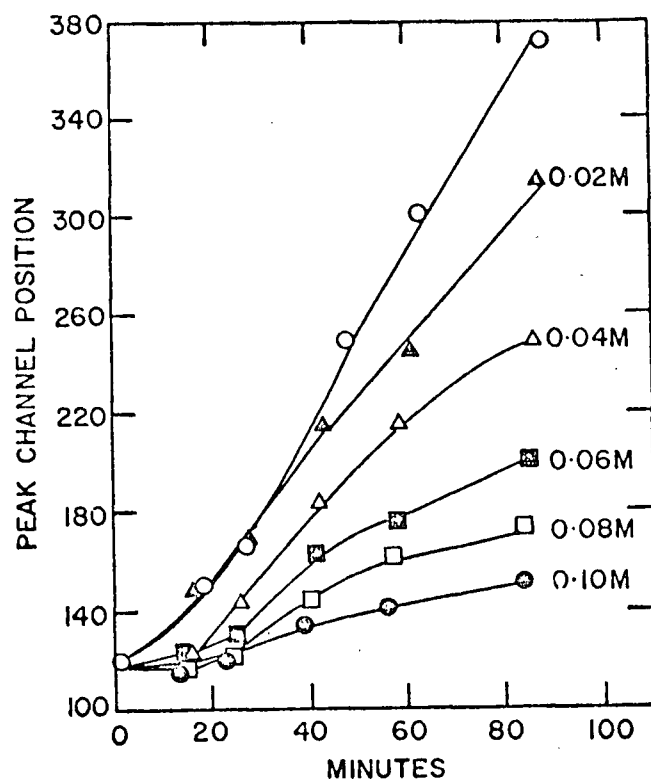


Figure 14.

The effect of pantoyl lactone on the rate of size increase of *Escherichia coli* BUG-6 at 42 C. *Escherichia coli* BUG-6 was grown for several generations at 30 C and then placed at 42 C for 45 minutes. At this time, 0 minutes in the graph, the culture was subdivided and pantoyl lactone added to each culture giving a final concentration of 0.01 M (○), 0.08 M (□), 0.06 M (■), 0.04 M (Δ) and 0.02 M (Δ). A control culture was maintained at 42 C without the addition of pantoyl lactone (○).

VIII. The effect of inhibitors of cell wall synthesis on cell division during the recovery period.

In an attempt to determine the nature of the temperature-sensitive lesion, inhibitors of cell wall synthesis were added to cultures during the recovery period and cell number recorded as a function of time. Cells grown at 30 C were shifted to 42 C to form filaments and then returned to 30 C at 80 minutes coincident with the addition of one of each of the following antibiotics: cycloserine, novobiocin, vancomycin and penicillin. The concentration of each antibiotic corresponded to an amount which blocked cell division in Escherichia coli BUG-6 at 30 C. The effect of each antibiotic on cell division during the recovery period is shown in Figure 15. The addition of cycloserine and novobiocin have little effect on cell division but do affect continued growth of the cells. On the other hand, penicillin completely inhibits cell division during this period. Vancomycin allows some residual division but inhibits cell division markedly compared either to the control or to those inhibited with cycloserine or novobiocin.

IX. The effect of inhibitors of macromolecular synthesis on cell division during the recovery period.

The requirement for protein synthesis during the recovery period was examined using chloramphenicol (CAM) and puromycin as inhibitors. Figure 16 shows the results of an experiment in which CAM was added

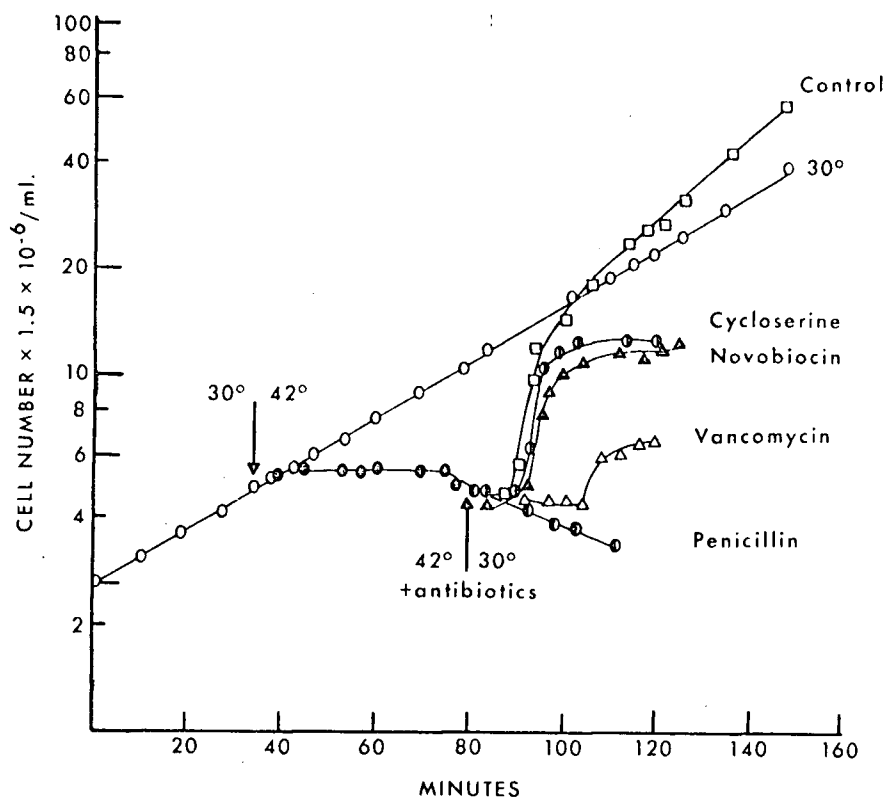


Figure 15. The effect of cell wall inhibitors on cell division of filaments during recovery at 30°C. BUG-6 was grown at 30°C (○) in nutrient broth for several generations. Part of the culture was shifted to 42°C (●). After 45 min at 42°C, the culture was divided and the subcultures placed at 30°C coincident with the addition of: 20 units penicillin/ml (○); 400 µg vancomycin/ml (Δ); 100 µg novobiocin/ml (Δ) or 30 µg cycloserine/ml (○). A control was returned to 30°C with no addition (□).

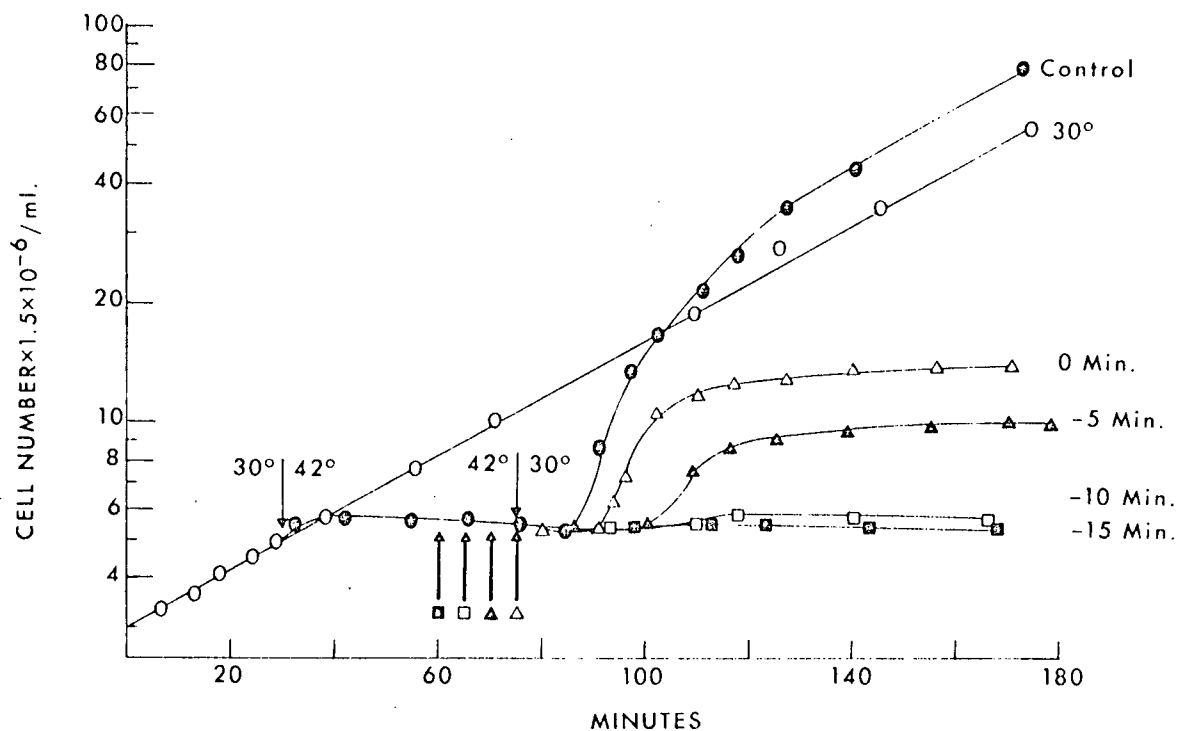


Figure 16. The effect of chloramphenicol at 42 C on the cell division of filaments of BUG-6 during the recovery at 30 C. BUG-6 was grown in nutrient broth at 30 C (0). Part of the culture was shifted to 42 C at 30 min and returned to 30 C at 75 min (0). Chloramphenicol (150 $\mu\text{g/ml}$) was added to subsamples of the 42 C culture at: 0 min (Δ); 5 min (\blacktriangle); 10 min (\square) and 15 min (\blacksquare) prior to the return to 30 C at 75 min.

to individual cultures at the time of the shift-back to the permissive temperature or at 5, 10 and 15 minutes prior to the shift-back to the permissive temperature.

The control in which CAM was omitted from the culture shifted back to 30 C shows an abrupt increase in cell number to a level which is slightly higher than the control which remained at 30 C. If CAM is added to the culture at the time the culture is shifted from 42 C to 30 C, there is a two-fold increase in cell number although the final plateau is considerably below that of the control. The addition of CAM, 5 min before the shift to the permissive temperature, decreases the amount of residual cell division. If added 10, 15 min prior to the shift-back to 30 C, there is little or no division. The results obtained with puromycin are similar to those obtained with CAM.

Clearly, "division potential" is present when the cells are incubated at the non-permissive temperature since a shift-back to 30 C results in cell division in the absence of protein synthesis (Figure 16). On the other hand, this potential is destroyed at 42 C since a 10 min incubation at 42 C in the absence of continued protein synthesis removes the potential for cell division at the permissive condition. In an effort to describe with more precision the stability of the division potential at 42 C, a culture was treated similarly to that described in Figure 16, except that CAM was added at 1 min intervals to separate samples before and after

the shift from 42 C to 30 C. The final cell number at 30 C is plotted as a function of the time at which CAM is added. As seen in Figure 17A, the addition of CAM at 7 - 8 min prior to the shift-back to 30 C results in no increase in cell number over the amount of cells present in the 42 C control. An abrupt increase in division potential is observed during the period of 6 min to 2 min prior to the shift-back to 30 C. After the shift-back, there is a slower increase in the number of cells obtained at the plateau increasing with a doubling time of about 20 minutes. A plot of the log percent residual increase of the cells at 30 C as a function of the time of the addition of CAM, gives an estimate of the stability of the division potential at 42 C. Such a plot is shown in Figure 17B.

The increase in "division potential" at 30 C does not appear to be as rapid as at 42 C. An experiment designed to determine the extent of de novo synthesis of "division potential" was done using a CAM pulse of 10 min at the non-permissive temperature to remove all division potential, and observing subsequent division of the filaments at 30 C. Cells grown in broth for several generations were shifted to 42 C at 20 min. After 45 min at 42 C, the cells were shifted back to 30 C. Sub-cultures were treated with a 10 min pulse of CAM at 10, 20 and 30 min prior to the shift-back to 30 C. The results are shown in Figure 18. A culture which is not treated with CAM divides rapidly about 12 min after the shift-back to 30 C. On the other hand, a culture which has been exposed to CAM

EFFECT OF CAM ON RESIDUAL DIVISION DURING RECOVERY

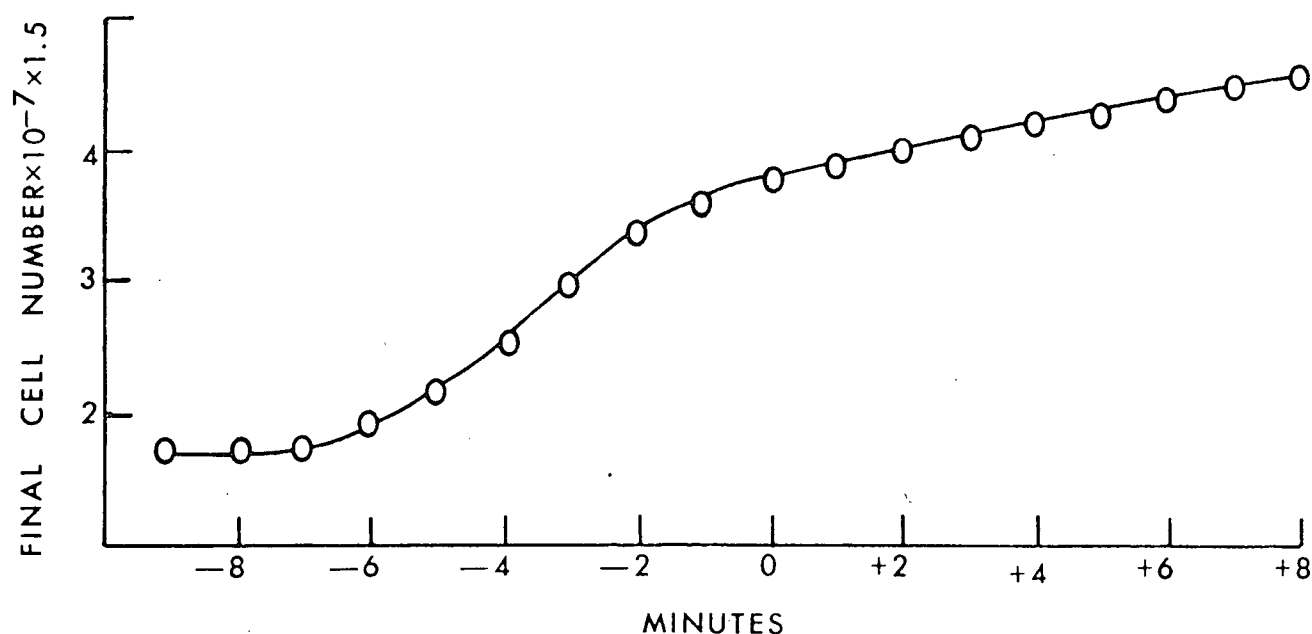


Figure 17A. The effect of chloramphenicol on residual division of filaments during the recovery at 30 C. BUG-6 was grown in broth at 30 C, shifted to 42 C for 45 min and then returned to 30 C. Aliquots were removed at 1 min intervals for 10 min before and 10 min after the shift-back and chloramphenicol (150 $\mu\text{g}/\text{ml}$) was added to each sample. The final cell number attained in each sample is plotted against the time of chloramphenicol addition to that sample. Zero is the time of the return to 30 C.

DECAY OF DIVISION POTENTIAL AT 42°

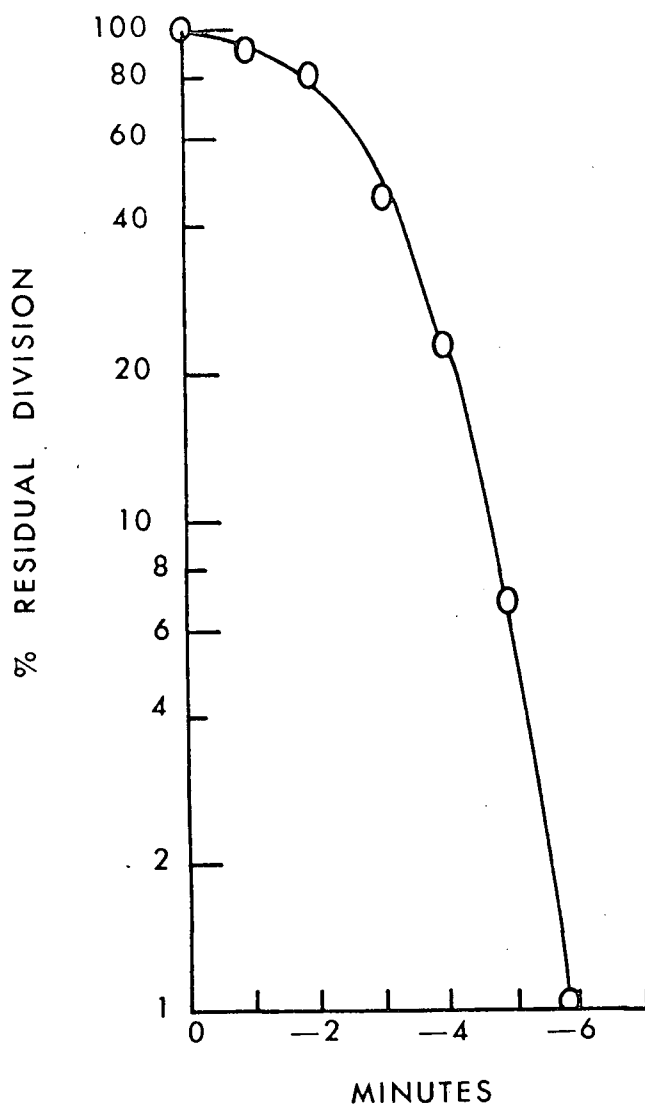


Figure 17B. Decay of division potential at 42 C. The data from Figure 17A prior to 0 time are used to construct this figure. The increase in cell number of chloramphenicol treated samples is plotted as a percentage of the increase in cell number to which chloramphenicol was added at the time of the return to 30 C. The increase in cell number of the culture with chloramphenicol added at the time of the shift from 42 C to 30 C is plotted as 100% and the increase in cell number of other samples is plotted as a percentage of this value.

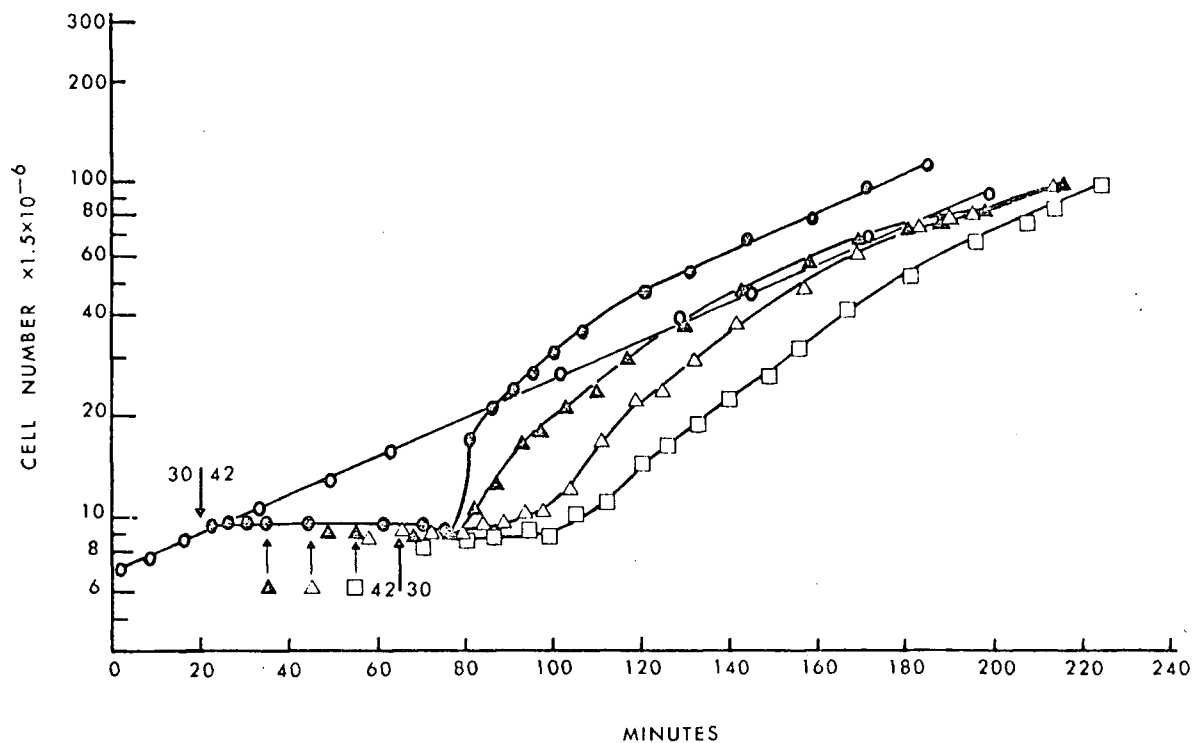


Figure 18. The effect of a 10 min treatment with chloramphenicol at 42 C on division of filaments during recovery at 30 C. BUG-6 was grown at 30 C (○) for several generations in nutrient broth. Part of the culture was shifted to 42 C at 20 min and returned to 30 C at 65 min (●). A 10 min pulse of chloramphenicol (150 μ g/ml) was given to subcultures of the 42 C culture at 10 min (□); 20 min (Δ) and 30 min (▲) prior to the return to 30 C at 65 min.

for 10 min, prior to the shift-back and then had the CAM removed, does not start to divide until nearly 35 min after the shift-back to 30 C, and the rate of division is much reduced. A 10 min pulse of CAM 20 min prior to a shift-back to 30 C allows for a 10 min synthesis period at 42 C. Cell division starts at about 40 min after removal of CAM. A 10 min pulse at 30 min prior to a shift-back to 30 C allows for a 20 min synthesis period at 42 C. Cell division starts at about 35 min after removal of CAM. With increasingly longer periods of protein synthesis at 42 C after the removal of CAM, the recovery division rates increase, supporting the suggestion that "division potential" is accumulated faster at 42 C than at 30 C.

Since high concentrations of salt were observed to reverse the block in division (Figure 12), the requirement for protein synthesis in the presence of high concentrations of NaCl was examined. Figure 19 shows the effect of CAM on cell division in the presence of a high concentration of salt compared with the effect of CAM on cell division during the recovery period at 30 C. A culture growing exponentially under balanced growth conditions was shifted to 42 C at 30 min. After growth at 42 C for one generation, samples were removed and treated (a) with high concentration of salt and CAM and left at the non-permissive temperature, or (b) shifted to 30 C in the presence of CAM. As indicated in Figure 19, the cells divide at the permissive temperature in the presence of CAM as had

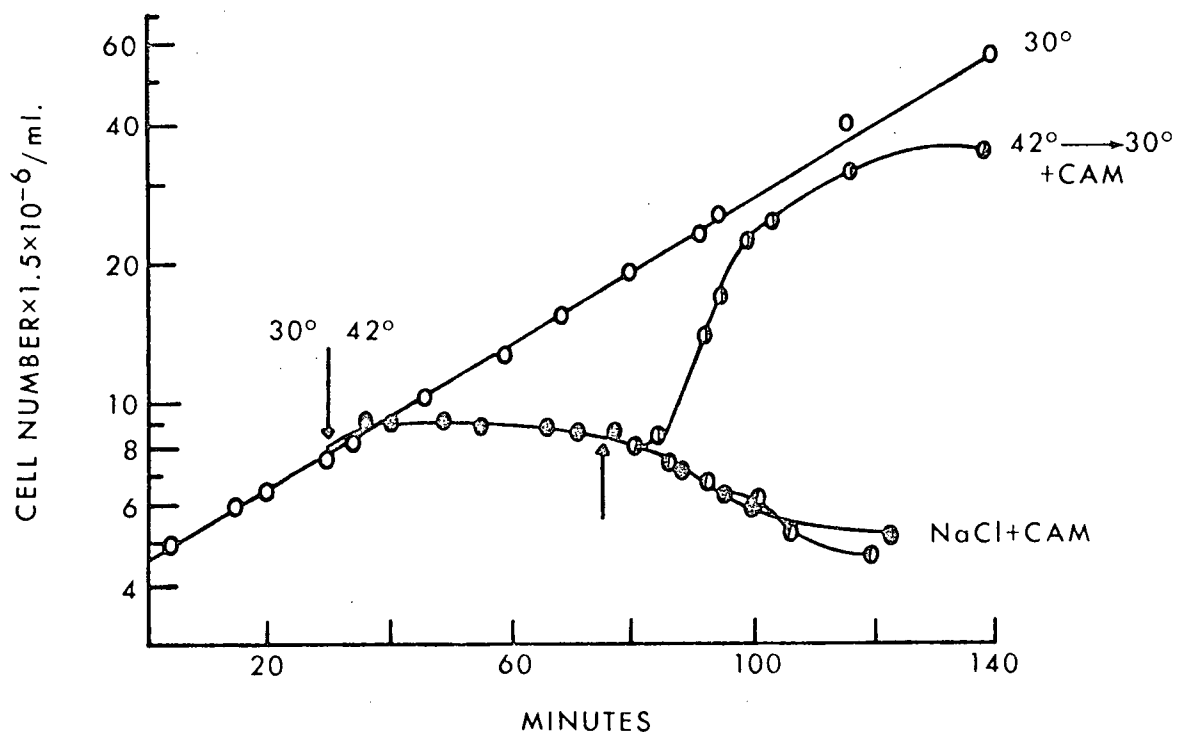


Figure 19. The differential effect of chloramphenicol on division of filaments following the addition of NaCl versus a shift from 42 C to 30 C. BUG-6 was grown in a nutrient broth for several generations at 30 C (O). Part of the culture was shifted to 42 C at 30 min (O). At 75 min, chloramphenicol (150 μ g/ml) was added to subsamples of the culture at 42 C, coincident with the return to 30 C (O) or coincident with the addition of NaCl (O) to a final concentration of 11 g/l.

been seen previously in Figure 16. On the other hand, cells which normally divide in the presence of high concentrations of salt (see Figure 12), do not divide in the presence of high salt if CAM is added.

It is well known that specific inhibitors of DNA synthesis also block cell division (6, 34, 78). A dependence of division upon DNA replication has been established (16, 42), although it is possible to obtain mutants in which cell division becomes uncoupled from DNA segregation (43, 47). In the normal division cycle, termination of the round of replication appears to be essential for division (16, 42). An examination of the coupling between cell division and DNA replication was examined in Escherichia coli BUG-6 by inhibition of DNA synthesis at intervals during filament growth at the non-permissive temperature. Figure 20 illustrates the effect of inhibition of DNA synthesis during filament formation at 42 C on subsequent cell division during the recovery period at 30 C. A culture of Escherichia coli BUG-6 grown for several generations at 30 C was measured carefully for increase in cell numbers and constancy of cell size to establish balanced growth of the culture. Twenty-five minutes after zero time, a portion of the culture was removed and shifted to 42 C. At subsequent times of 30, 40, 50 and 60 min, samples from the culture at 42 C were each transferred to a flask at the same temperature (42 C) containing 10 µg of nalidixic acid (NAL) per ml final concentration. At 70 min, each of the cultures treated with NAL was returned to the permissive temperature. A control culture

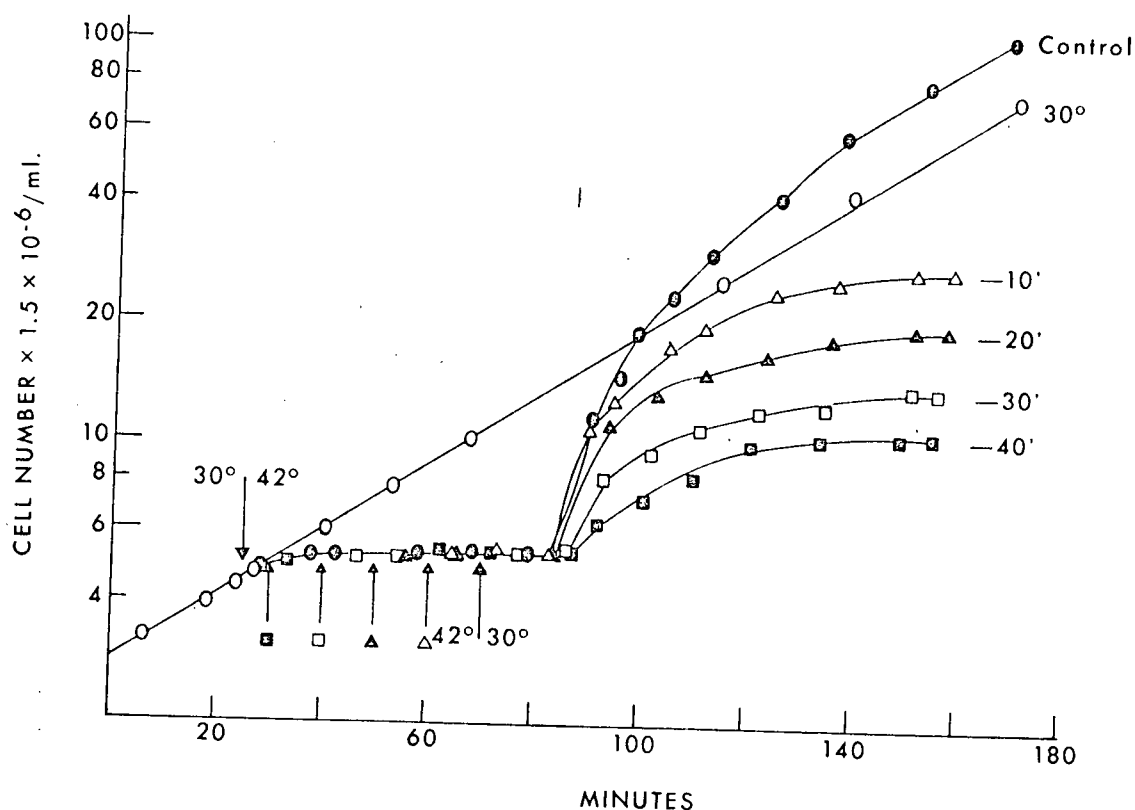


Figure 20. The effect of nalidixic acid on cell division of the filaments during recovery at 30 C. BUG-6 was grown at 30 C in nutrient broth for several generations. Part of the culture was placed at 42 C (●) at 25 min and returned to 30 C at 70 min. Nalidixic acid (10 μ g/ml) was added to subsamples at 42 C, 10 min (Δ); 20 min (\blacktriangle); 30 min (\square) and 40 min (\blacksquare) prior to the return to 30 C at 70 min.

which had been incubated at 42 C in the absence of NAL was returned to 30 C to record the increase in cell numbers following the shift-back to 30 C. As indicated in Figure 20 there is a proportional relationship between the amount of residual division during the recovery period and the time allowed for DNA synthesis at the non-permissive temperature. Thus, blocking DNA synthesis 5 min after shifting to the non-permissive temperature allows about a 50% increase in cell number. If DNA synthesis is allowed to continue for 45 min, there is nearly a 200% increase in the cell number during the recovery period.

Inhibition of DNA synthesis in an exponential culture does not stop cell division immediately. Apparently, DNA synthesis is required until the end of a round of replication before a cell can divide. After completion of the round, there is no further requirement for DNA synthesis, although division does not actually occur until a considerable time after the end of a round (80). Since there is a time lag between the end of a round of replication and actual separation of the cells, a large number of the cells in a random population continue to divide although DNA synthesis is blocked. This is illustrated in Figure 21. Cells growing exponentially are treated with NAL at 30 min and residual cell division is measured. If a portion of the culture is treated with NAL and simultaneously placed at 42 C, there is no division as a result of the effect of temperature. Returning this culture to 30 C after 45 min at the non-permissive temperature allows expression of the division potential which was

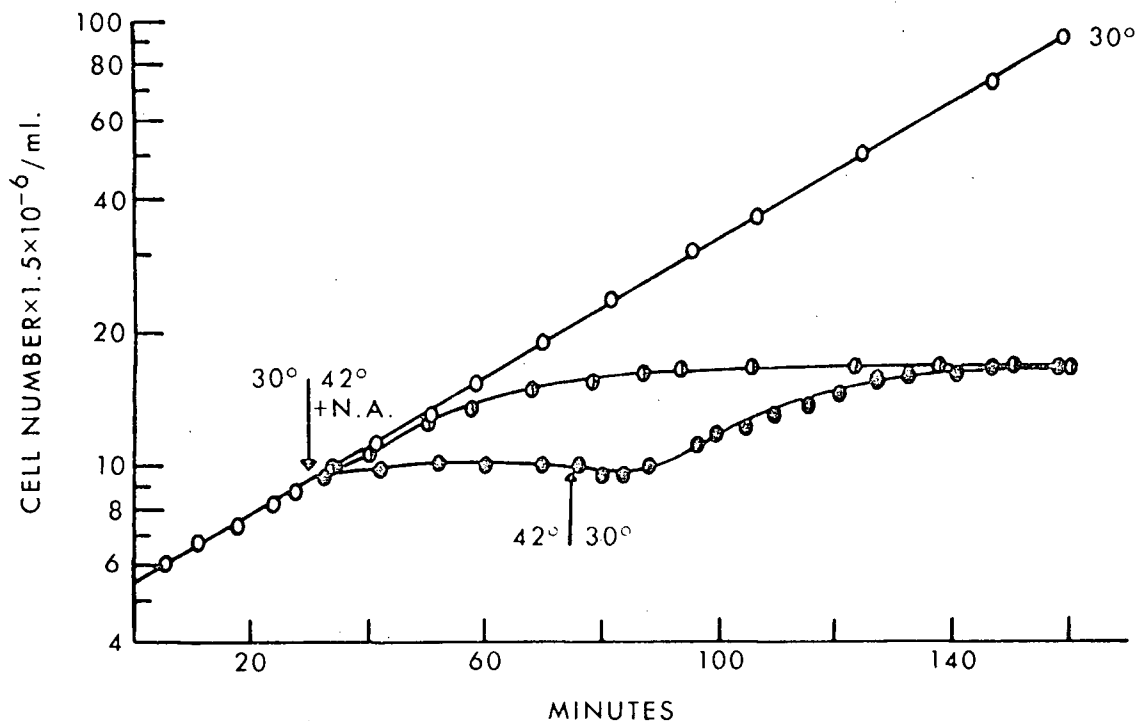


Figure 21. Residual division of BUG-6 following the addition of nalidixic acid. BUG-6 was grown in nutrient broth for several generations, at 30 C (0). Nalidixic acid (10 $\mu\text{g/ml}$) was added to part of the culture at 30 min. The culture treated with nalidixic acid was divided and one-half was left at 30 C (0). The other half was placed at 42 C for 45 min and then returned to 30 C (0).

present at 25 min. The manner in which the filaments divide after they have been exposed to NAL at the non-permissive temperature and then returned to 30 C is seen in Figure 22a. For comparison, filaments treated with CAM and then returned to 30 C are shown in Figure 22b. The latter divide regularly into "unit cells" whereas the former divide into cells of different lengths. The irregular division positions is consistent with random segregation of a subnormal amount of DNA assuming that all the cells, so formed, contain DNA.

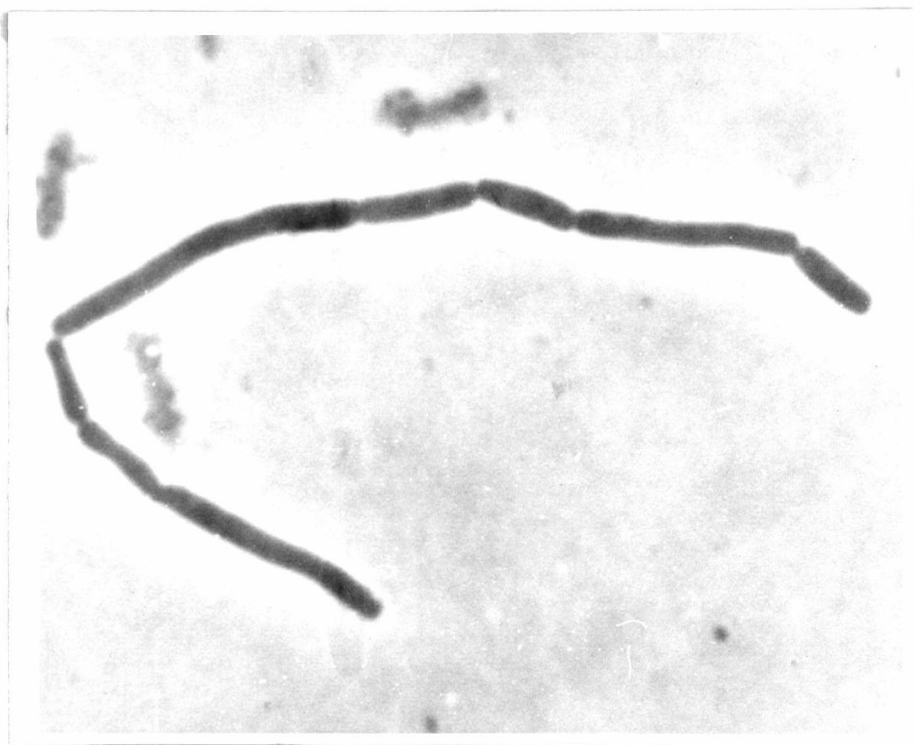
X. The effect of inhibitor macromolecular synthesis on cell division during the recovery period following different periods at 42 C.

In section (I-2) of the results, it was shown that the recovery division kinetics differ with the different periods at the non-permissive temperature. In section (VII) of the results, a detailed analysis of effect of macromolecular inhibitors on the recovery cell division following 45 minutes at 42 C was presented however it was thought necessary to determine the effect of these inhibitors after different periods at 42 C.

A culture of Escherichia coli BUG-6 was grown for several generations in broth and then a portion shifted to 42 C at 0 minutes as defined in Figure 23. After 35, 50, 65, 80 and 95 minutes, sub-cultures were returned to 30 C in the presence of CAM (150 µg/ml final concentration). Figure 4 is a similar experiment with no CAM addition. The results in Figure 23 show a longer lag before recovery

Figure 22A. Fragmentation of nalidixic acid filaments during recovery at 30 C. BUG-6 was grown for 3 generations at 42 C with nalidixic acid (10 µg/ml) for the last generation. The filaments were inoculated onto slides precoated with nutrient agar containing nalidixic acid (10 µg/ml), and incubated at 30 C for 2 hrs.

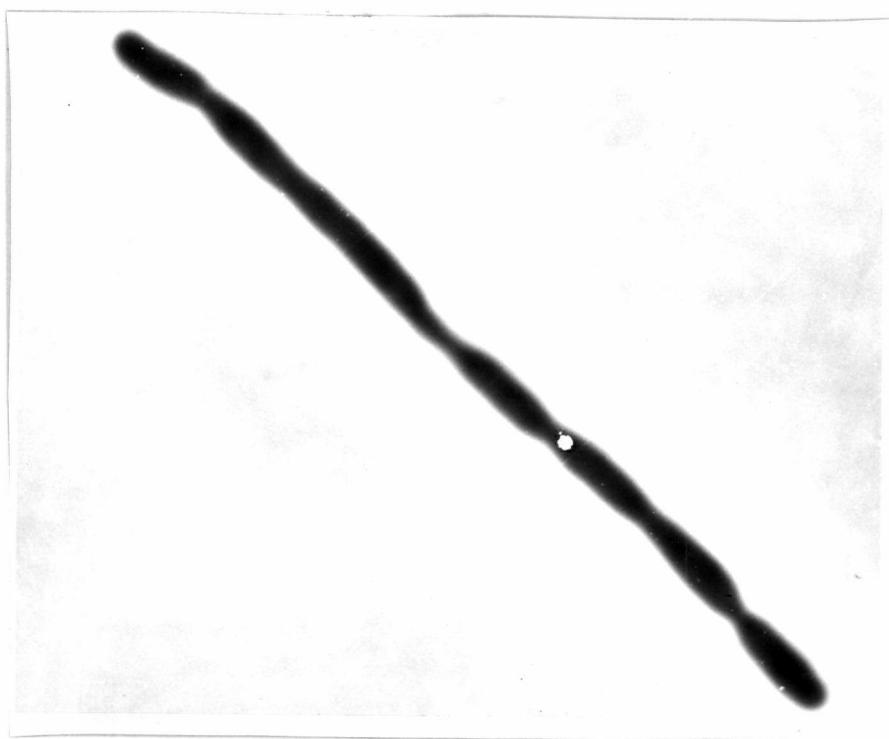
Figure 22B. Fragmentation of filaments of BUG-6 at 30 C in the presence of chloramphenicol. BUG-6 was grown for 3 generations at 42 C. Three minutes prior to returning the filaments to 30 C, chloramphenicol (150 µg/ml) was added and the filaments were then inoculated onto slides precoated with nutrient agar containing 150 µg CAM/ml, and incubated at 30 C for 2 hr.



(a)

Figure 22

(b)



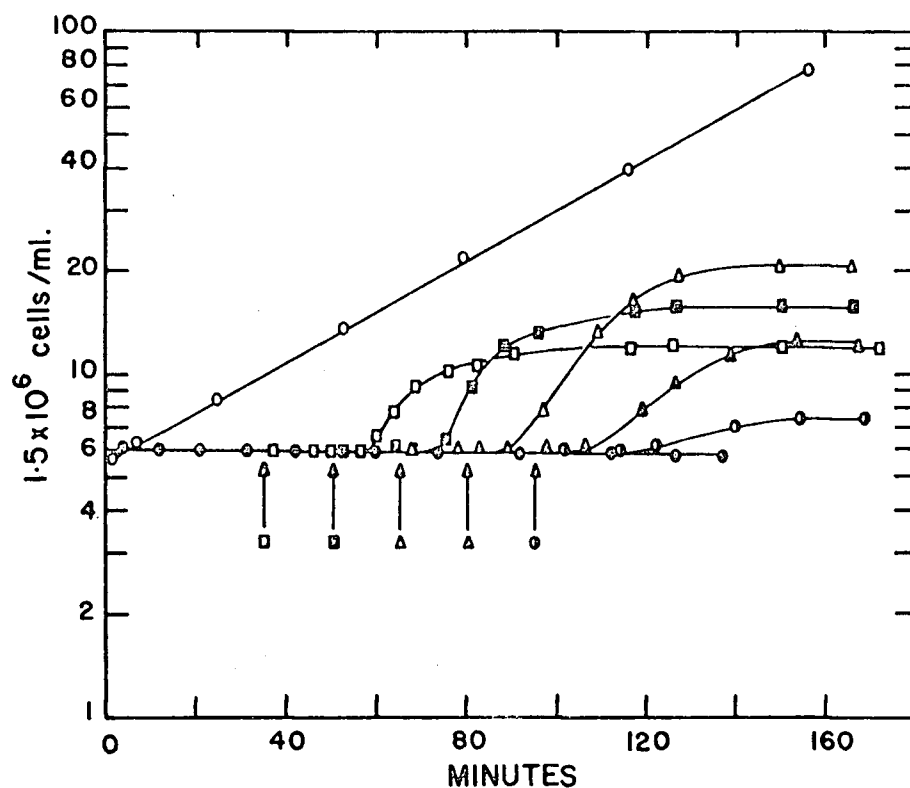


Figure 23. The effect on cell division of shifting *Escherichia coli* BUG-6 from 30 C to 42 C for different time intervals and then replacing at 30 C in the presence of chloramphenicol. *E. coli* BUG-6 was grown in broth for several generations at 30 C (○) and at 0 min part of the culture was shifted to 42 C (◐). Subcultures from 42 C were replaced at 30 C and chloramphenicol added (150 µg/ml; final concentration), after 35 min (◻), 50 min (◼), 65 min (Δ), 80 min (▲) and 95 min (◊) at 42 C.

division starts than shown in Figure 4, and the number of divisions accomplished by the filament decreases when the period at 42 C exceeds 50 minutes and CAM is present during the recovery. The results from Figure 4 indicate that division potential is always present regardless of the length of time at 42 C as division always occurs rapidly during the recovery period. However, the results from Figure 23 show that in the presence of CAM the expression of this potential is dependent on the length of time at 42 C.

A more detailed analysis of the expression of division potential in CAM is presented in Figure 24. A growing culture of Escherichia coli BUG-6 was placed at 42 C and at frequent intervals samples were removed and placed at 30 C in CAM. The final cell number attained is plotted against the length of time at 42 C. A maximum occurs at about 46 minutes and plateau values occur between 20 - 22 minutes and 50 - 75 minutes incubation at 42 C. No cell division occurs if the cells are kept at 42 C for more than 110 minutes or less than 6 minutes.

Samples were also removed from the cultures described in Figure 24 and at 42 C and placed at 30 C in 10 µg NAL/ml (final concentration). Figure 25a is the $OD_{420\text{ m}\mu}$ of the culture at 42 C, Figure 25b is the final number attained by the subcultures placed at 30 C in NAL, and Figure 25c is Figure 24 repeated for ease of comparison. The $OD_{420\text{ m}\mu}$ doubling rate is 35 minutes for 70 minutes and then begins to decrease slowly, but the initial doubling time for the final cell number reached

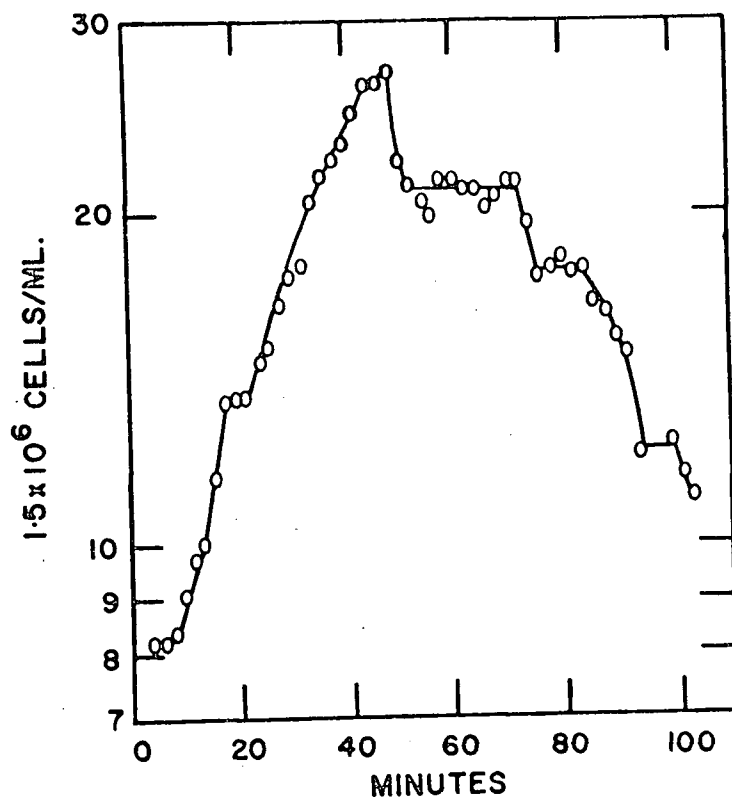


Figure 24.

The effect of chloramphenicol on residual division of filaments during the recovery at 30 C after different time intervals at 42 C. *Escherichia coli* BUG-6 was grown for several generations in broth at 30 C and then placed at 42 C. Subcultures were removed and replaced at 30 C in the presence of chloramphenicol (150 µg/ml; final concentration). The final cell number attained by each subculture is plotted against the time it was at 42 C.

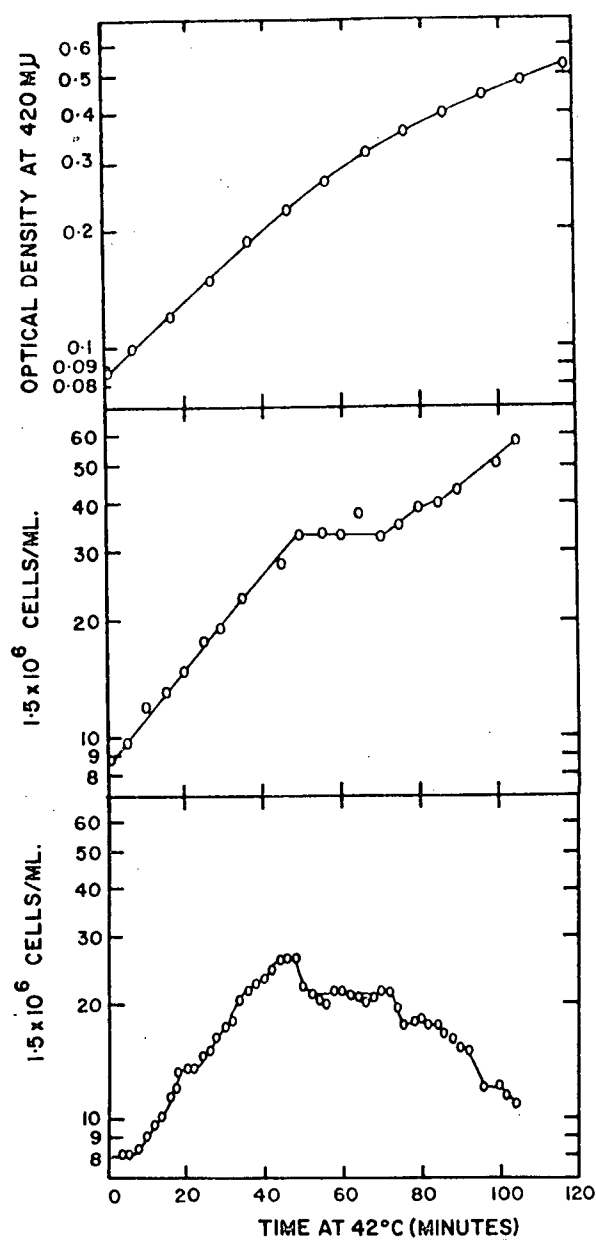


Figure 25. The effect of nalidixic acid or chloramphenicol on residual division of filaments during the recovery at 30 C after different time intervals at 42 C. *Escherichia coli* BUG-6 was grown for several generations in broth at 30 C and then placed at 42 C. Figure 25a is the change in OD_{420 mμ} at 42 C. Subcultures were removed and returned to 30 C in the presence of nalidixic acid (Figure 25b) (final concentration 10 μg/ml) or chloramphenicol (Figure 25c) (final concentration 150 μg/ml). The final cell number attained by each subculture is plotted against the time it was at 42 C.

by cells placed in NAL is 22 minutes. Filaments incubated at 42 C for 45 - 75 minutes divide, in NAL, to give the same final cell number. Filaments incubated for longer than 75 minutes divide in NAL to give increasingly more cells. If the final cell number doubling rate is summed over the first 75 minutes, its value is about 35 minutes, ie. the same as the $OD_{420\text{ m}\mu}$. The major plateau of the CAM treated cells and NAL treated cells occurs over approximately the same incubation period at 42 C.

It was considered that a constant level of division potential might be present in filaments which were continually increasing in length and so producing more "division sites" for use of this fixed amount of potential. If production of "division sites" was dependent on completed DNA replication (16, 42), then adding NAL at 42 C and allowing filamentation but no production of division sites should produce increasingly longer filaments which, with a fixed amount of "division potential" and a fixed number of "division sites" would all divide to the same extent. An experiment was done to test this hypothesis. Escherichia coli BUG-6 was grown at 30 C for several generations and a portion of the culture placed at 42 C. After 50 minutes at 42 C, NAL was added to a subculture maintained at 42 C and 30 minutes later CAM was added to the untreated culture and the NAL treated culture. Both cultures were replaced at 30 C at this time (Figure 26). This produced filaments grown for 80 minutes at 42 C which in the presence of CAM would give submaximum recovery

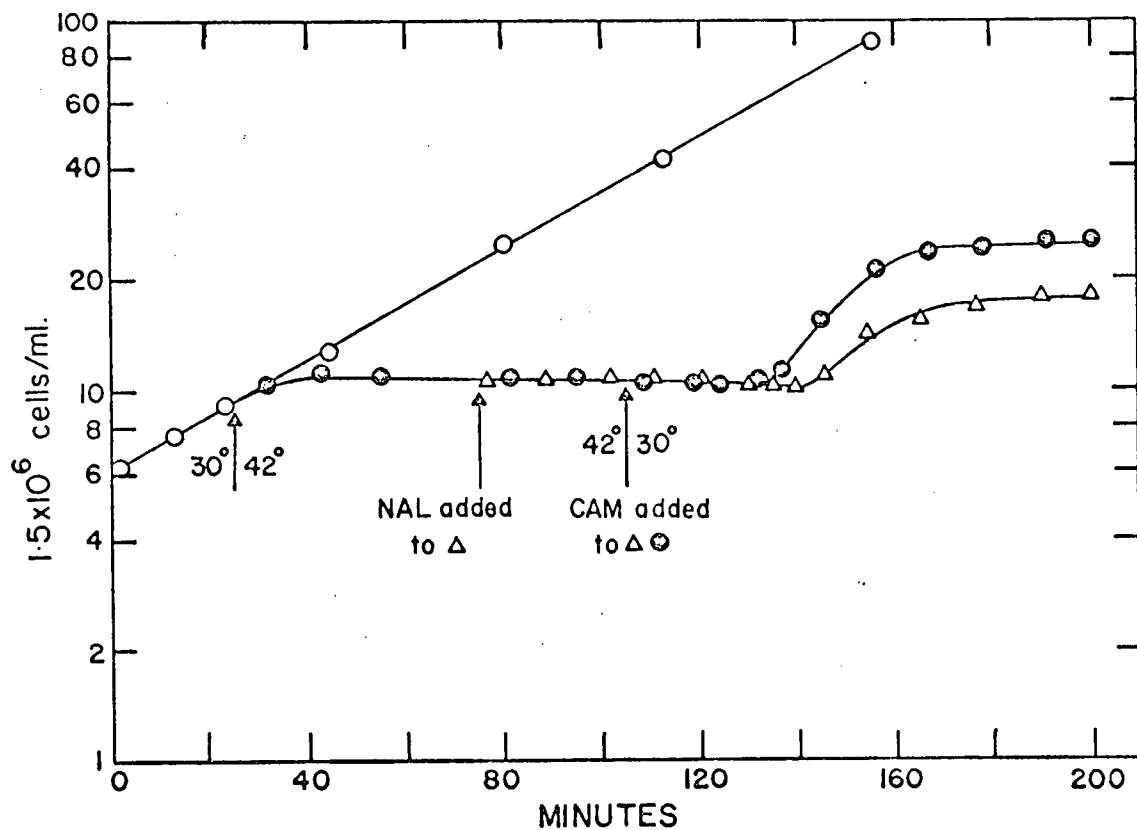


Figure 26.

The effect of a period of inhibition of DNA synthesis at 42°C on the residual division of filaments during the recovery at 30°C in the presence of chloramphenicol. *Escherichia coli* BUG-6 was grown for several generations at 30°C (○) before part of the culture was placed at 42°C (●). The culture at 42°C was halved after 50 minutes at 42°C and nalidixic acid (10 µg/ml final concentration) was added to one half (Δ). After a total of 80 minutes at 42°C, both cultures were returned to 30°C in the presence of chloramphenicol (150 µg/ml final concentration).

division (Figure 24). However, one of the cultures treated with CAM was blocked for DNA synthesis after 50 minutes filamentation, ie. at the time when, if CAM had been added and the cells placed at 30 C maximum recovery division would have occurred. If blocking DNA synthesis prevents "division site" production and the number of divisions possible with a fixed amount of "division potential" in the presence of CAM depends on the number of division sites, the culture treated with NAL at 50 minutes and so having a limited number of "division sites" should divide more at 30 C than the culture which was not so treated. The result was actually the opposite, ie. the combination of NAL + CAM gave less division during the recovery period than the addition of CAM alone.

XI. The effect of 30 C pulses on filaments produced at 42 C

It has been shown in previous sections that Escherichia coli BUG-6 filaments contain a high "division potential" which can be expressed at 30 C. A period of 15 minutes always occurs between the 42 C → 30 C shift and cell division when a nutrient broth medium is employed, (this period can be altered by use of different media giving different growth rates). It was of interest to ascertain at which point, if any, during the 15 minute period the filaments would become committed to divide even if replaced at 42 C.

A culture of Escherichia coli BUG-6 was grown for several generations at 30 C and then part of the culture placed at 42 C for 45 minutes.

Subcultures from the 42 C culture were placed at 30 C for 6, 8, 10 and 12 minutes before replacing at 42 C. A control culture was shifted to 30 C but not replaced at 42 C. In Figure 27, the subsequent division patterns are depicted. A 6 minute pulse at 30 C allowed virtually no subsequent division, 8 minute and 10 minute pulses allowed increasing amounts of division and a 12 minute pulse allowed an amount equivalent to one doubling of cell number. In Figure 4 it was shown that one doubling is the maximum amount of rapid division. Figure 6 indicated that division was complete after 12 minutes at 30 C, but cell separation was still required. In all cases, division started 15 minutes after the 42 C to 30 C shift.

The results in Figure 27 suggest that the "division potential" in the filaments is not expressed at one particular time point during the 15 minutes, but is required for at least the first 12 minutes of this period if maximum expression is to be obtained. The amount of "division potential" within a filament can be controlled by CAM addition and removal at 42 C, ie. a three minute pulse of CAM immediately before the 42 C \rightarrow 30 C shift reduces the potential by approximately 50% (Figure 17B). By such a procedure, filaments were produced with 50% of the normal amount of "division potential" and the experiment described in Figure 27 repeated. The 76 minute pulse of 30 C was omitted. The results are shown in Figure 28. There is no division following an 8 minute pulse at 30 C and the division following 10 and 12 minute pulses is very much reduced as compared to Figure 27. Thus

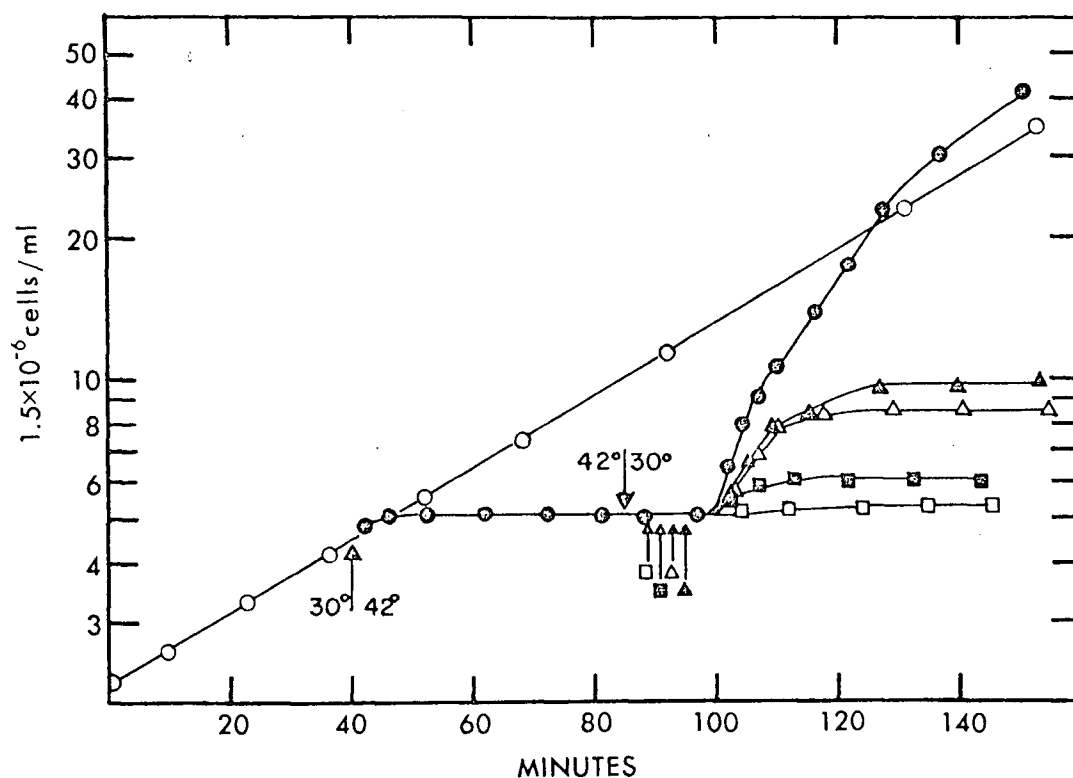


Figure 27. The effect on cell division of *Escherichia coli* BUG-6 of different length pulses of 30°C during 42°C growth. *E. coli* BUG-6 was grown for several generations in broth at 30°C (○) and at 40 min part of the culture was shifted to 42°C (●) for 45 min before replacing at 30°C. 6 min (□), 8 min (■), 10 min (Δ) and 12 min (▲) after the 42°C → 30°C shifts, subcultures were again shifted to 42°C.

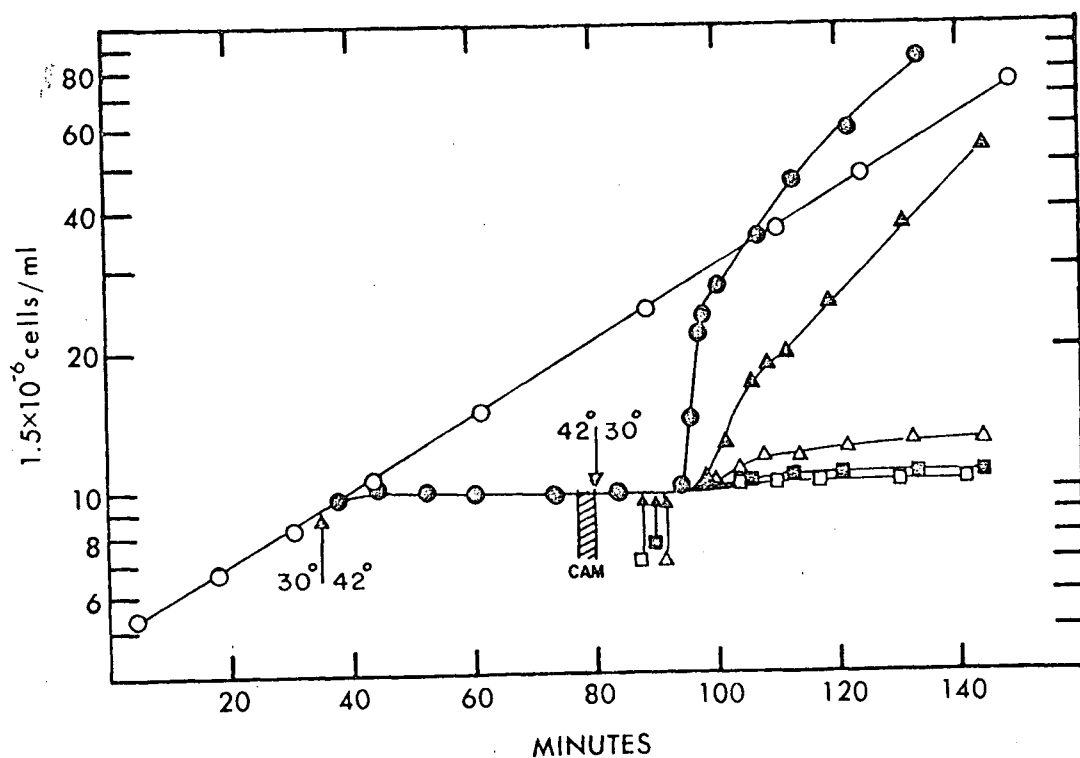


Figure 28. The effect on cell division of *Escherichia coli* BUG-6 of different length pulses at 30 C during 42 C growth when the temperature pulse is preceded by a 3 minute chloramphenicol pulse. *E. coli* BUG-6 was grown for several generations in broth at 30 C (○) and at 35 min part of the culture was shifted to 42 C (●) for 45 min before replacing at 30 C. Three minutes before replacing at 30 C, a portion of the culture was subjected to 150 μg/CAM/ml which was removed on replacing at 30 C (▲). 8 min (□), 10 min (■), and 12 min (△) after the 42 C → 30 C shift, subcultures of the CAM-pulsed culture were again shifted to 42 C

the amount of division following a pulse of 30 C is dependent on the amount of division potential within the cells produced at 42 C as well as the length of the pulse at 30 C. Figure 28 also shows a culture pulsed with CAM for the last three minutes before the 42 C → 30 C shift and then kept at 30 C. Comparison with the control culture not treated with CAM shows that the CAM treatment decreased the amount of rapid division, supporting the idea that the rapid division phase is an expression of potential accumulated at 42 C.

XII. The effect of a short period at 42 C on cells of different ages

A synchronized culture of Escherichia coli BUG-6 was produced by the Helmstetter and Cummings (41) membrane technique. For technical reasons the permissive temperature was 37 C and not 30 C as usually employed; however the mutant divides normally at this temperature. Samples were removed from the synchronous culture and placed at 42 C for 4 minutes before replacing at 37 C. (Four minutes is adequate time at 42 C to stop cell division in an exponential population (Figure 2)). The samples were removed from the culture at 4, 10, 15 and 20 minutes after the cells had been collected from the Millipore membrane. The parent culture began to divide 22 minutes after its collection from the membrane and the half-step ($T_{1/2}$) occurred 5 minutes later. In Figure 29 the division patterns of the subcultures, pulsed at 42 C for 4 mins is compared with the parent culture. Table IV is a summary of Figure 29.

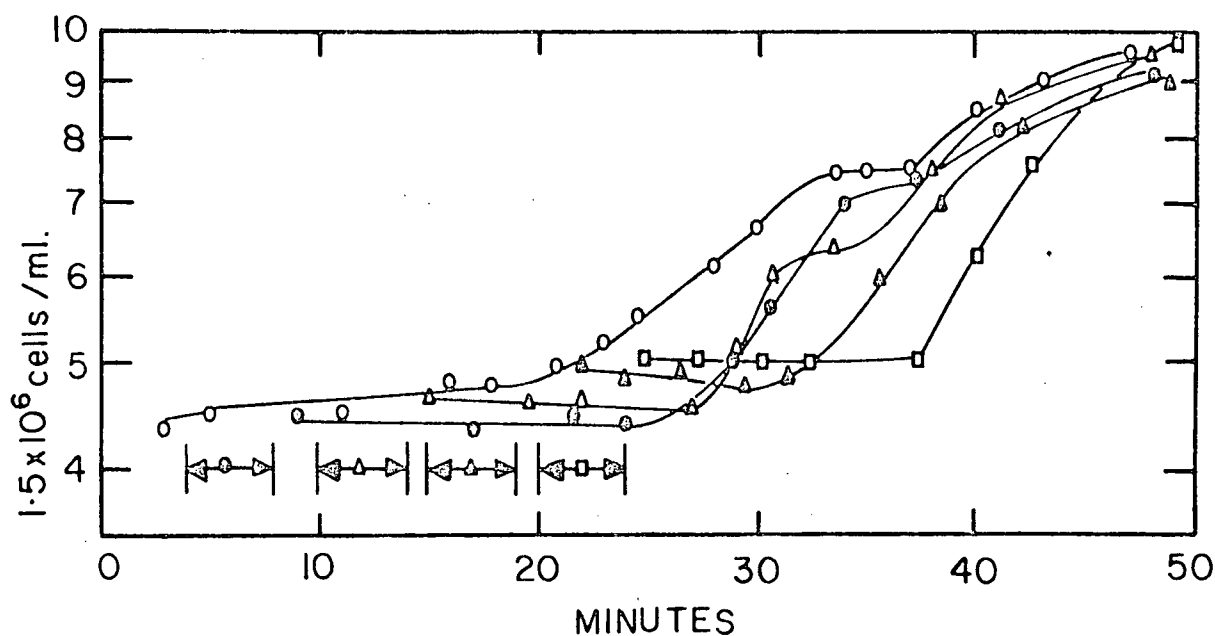


Figure 29. The effect of a 4 minute pulse at 42 C on the division of cells of different ages. A synchronized culture of *Escherichia coli* BUG-6 growing in broth at 37 C was produced by the technique of Helmstetter and Cummings (41). Subcultures were removed at 4 (○), 10 (Δ), 15 (▲) and 20 (◻) minutes after collecting the cells from the membrane and placed at 42 C for 4 minutes before returning to 37 C. The arrows indicate the period over which each subculture was maintained at 42 C.

Table IV. A comparison of the effect on cell division of a 4 minute pulse at 42 C on cells of different ages.

Time of 42 C pulse after collection of cells	Division began at:-	Time between 42 C → 37 C and division start	Time between 42 C → 37 C and $T_{\frac{1}{2}}$
No pulse, ie. control	22 minutes	-	-
4 minutes	25 minutes	17 minutes	23 minutes
10 minutes	28 minutes	14 minutes	17 minutes
15 minutes	33 minutes	14 minutes	17 minutes
20 minutes	38 minutes	14 minutes	16 minutes

It can be seen from Figure 29 and Table IV that division is delayed by the 42 C pulse by a constant period of 14 minutes for cells whose age is 10 minutes or more. Younger cells do not show this constant timing. This suggests that a 42 C pulse inactivates some material, required for division, which takes at least 14 minutes to replace or which is rapidly replaced but is used 14 minutes before cell division occurs. That the division of younger cells is only delayed by the length of the pulse supports this analysis and confirms that functional material cannot be made during a short 42 C pulse.

The results with synchronized populations therefore support the previous results with exponential populations that under a particular set of conditions, a constant period is always found between the time of the shift from non-permissive to permissive temperature and the onset of cell division.

XIII. Comparison of the proteins produced at 42 C with those produced at 30 C.

1. Cell envelope proteins

The analysis of division kinetics and use of macromolecular inhibitors suggests that filaments at 42 C contain protein(s) at a high concentration which are used during the rapid cell division when the filaments are placed at 30 C. Cells normally growing at 30 C would be assumed not to have this high concentration as they divide at a normal regulated rate. It was hoped that the high concentration of this protein(s) at 42 C would be achieved by an increased rate of synthesis as compared to 30 C and that this would enable the detection and identification of the protein(s). Cells were grown at 30 C in tritium labelled amino acids. A second culture was grown at 30 C, then placed at 42 C for 90 minutes, with carbon-14 amino acids present during the final 60 minutes at 42 C. The two cultures were rapidly chilled and mixed. Protein profiles of the cell envelopes and non-particulate fractions were produced by disc-gel electrophoresis. If a protein were synthesized more rapidly in one culture as compared to

the other culture then it should be enriched for the type of radioactive label added under the conditions of more rapid synthesis, when compared to the total amount of label incorporated.

In Figure 30, the membrane profiles are compared, and each type of radioactivity in a gel slice is expressed as a percentage of the total radioactivity of its particular isotope that entered the gel. At the top of the figure, the ΔP is plotted for each gel slice. This is the percentage of radioactivity of the isotope incorporated at 42 C in a gel slice minus the percentage of radioactivity of the isotope incorporated at 30 C in the same gel slice. A positive value indicates that material in that gel slice was made faster at 42 C than at 30 C and a negative value indicates the opposite. Figure 30 indicates that several regions of the profile contain proteins preferentially synthesized under one or other condition. However, before meaningful information can be obtained, the same experiment must be repeated with the radioactive labels reversed, ie. carbon-14 at 30 C and tritium at 42 C. Also, the same experiments must be done with the parent strain. These two controls remove the possibility of artifacts due to, i) the cells using the different radioactive labels differently, and ii) changes in protein profiles merely due to the temperature changes and not involved with filamentation. Figures 31 and 32 are these controls, with the labels reversed and with the wild type strain, respectively.

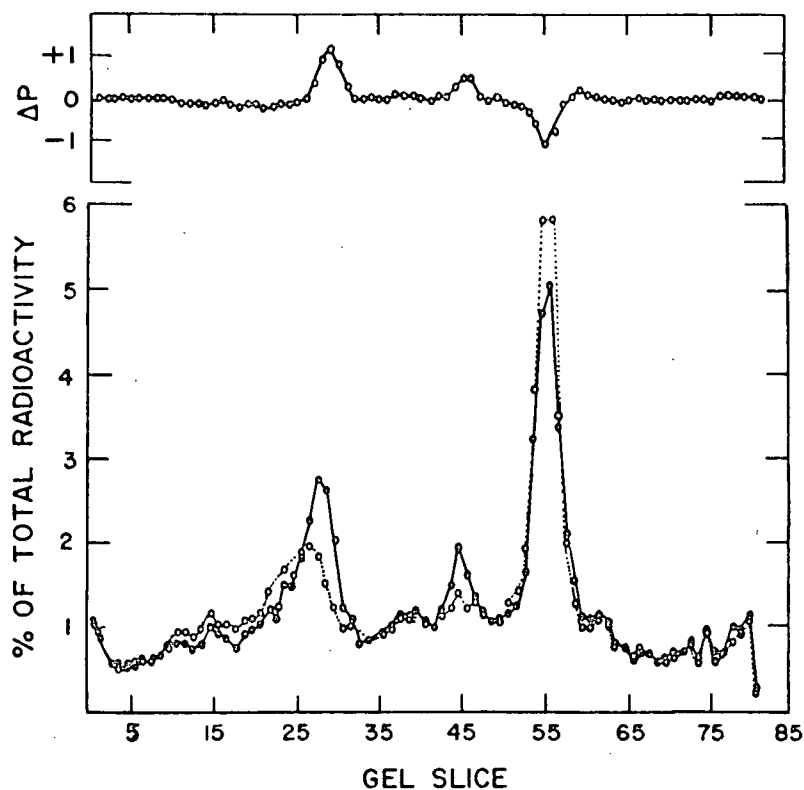


Figure 30.

Gel electrophoresis of cell envelope proteins prepared from *Escherichia coli* BUG-6 grown at 30 C and at 42 C. The cells were grown at 30 C for several generations with ^{14}C -amino acids (○) and at 42 C for 90 minutes with ^3H -amino acids (⊙) present for the last 60 minutes. The preparation of the proteins and gel electrophoresis were as described in "Materials and Methods". The radioactivity for each isotope in each gel slice is expressed as a percentage of the total radioactivity of that isotope which entered the gel. At the top of the graph, the ΔP represents the difference between the percentage of ^{14}C radioactivity and the percentage of ^3H radioactivity in each slice. A positive value indicates material synthesized more rapidly at 42 C than 30 C and a negative value indicates the opposite.

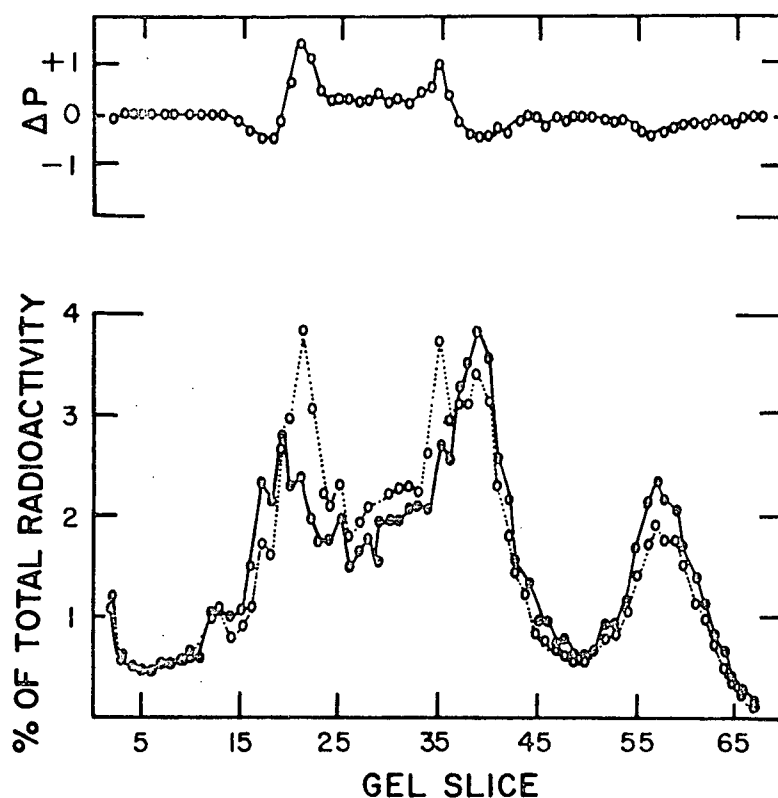


Figure 31. Gel electrophoresis of cell envelope proteins prepared from *Escherichia coli* BUG-6 grown at 30 C and 42 C. The same protocol as in Figure 30, but the ^{14}C -amino acids were incorporated at 42 C (O) and ^3H -amino acids (●) were incorporated at 30 C. The ΔP has been calculated so that positive and negative values indicate the same as in Figure 30.

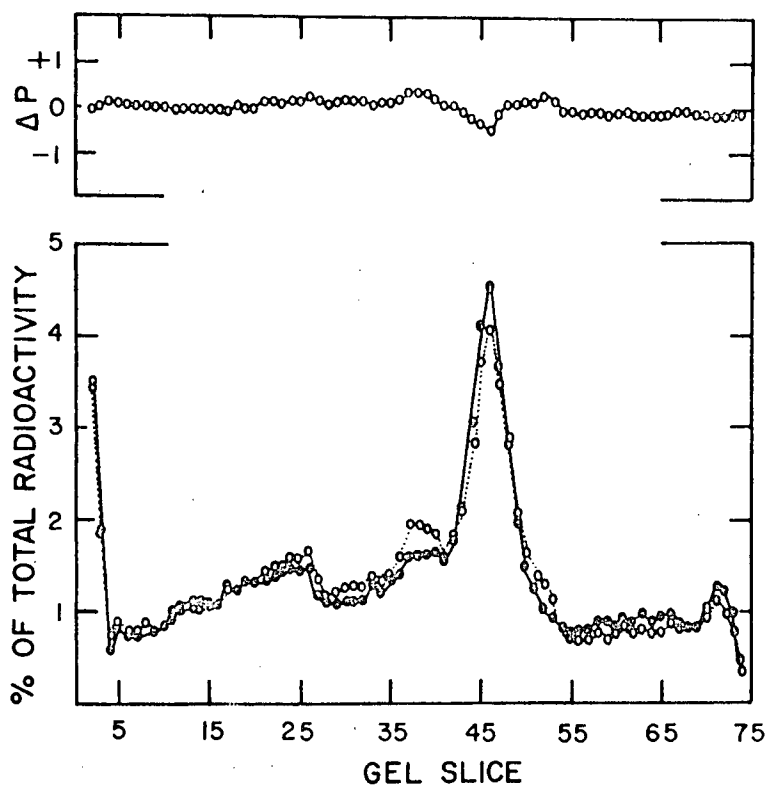


Figure 32. Gel electrophoresis of cell envelope proteins prepared from *Escherichia coli* AB1157 grown at 30 C and 42 C. The same protocol as in Figure 30 and the ΔP indicates the same.

Comparison of Figures 30, 31 and 32 indicate that only one positive ΔP area, i.e. increased synthesis at 42 C, can be attributed to the mutant which is not present in the wild type and which is not affected by the type of label used. This protein has a molecular weight of between 80,000 and 90,000 when its Rf is compared to the standard curve produced by running known molecular marker proteins (see Materials and Methods, section VIII-3) on a parallel gel of the same constitution. The marker proteins were prepared by exactly the same procedures as used for the unknown proteins (see Materials and Methods, section VIII-1 and 2).

The evidence obtained from studying the division kinetics of Escherichia coli BUG-6, with and without inhibitors, suggested that the "division potential" is "used-up" in the formation of septa and this suggested that a comparison of filaments allowed a period at 30 C to divide with filaments maintained at 42 C, might demonstrate an enrichment of a protein in the fragmented filaments, i.e. the "division potential" incorporated into the septa.

Escherichia coli BUG-6 was grown at 30 C for several generations, divided into two cultures, and both placed at 42 C for 90 minutes. After 30 minutes at 42 C, ^{14}C -amino acids were added to one culture and 60 minutes later that culture was stopped by rapid chilling. After 75 minutes at 42 C ^3H -amino acids were added to the second culture. At 90 minutes this culture was filtered from the radioactive medium, washed and resuspended in the same medium, minus the

radioactivity, at 30 C. Forty minutes later, this culture's growth was stopped by chilling and was mixed with the first culture. The cell membrane proteins were extracted and analyzed by disc-gel electrophoresis. In Figure 33 a positive ΔP would indicate material synthesized at 42 C but incorporated preferentially into cell septa at 30 C. There are no differences in the proteins labelled under the two conditions, suggesting that the cell membrane protein constitution was virtually the same 40 minutes after the 42 C \rightarrow 30 C shift as it was in the filaments at 42 C. To confirm this result, a comparison was made of the membrane proteins of 30 C grown cells with the membrane proteins of the fragmented filaments, labelled as described above (Figure 34). If, indeed, the proteins of the cell membrane do not change following the 42 C \rightarrow 30 C shift, then comparison of 30 C cells with the fragmented filaments should give the same result as in Figure 30 which is the comparison of 30 C cells and 42 C filaments. The ΔP of Figure 34 closely resembles the ΔP of Figure 30, ie. no gross changes are seen in the protein profile of fragmented filaments when compared with non-fragmented filaments, ie. "division potential" is not incorporated into septa or the amount incorporated is so small as not to be detectable.

The protein of molecular weight 80,000 - 90,000 which was made more rapidly at 42 C than at 30 C, may or may not be the product of the genetic lesion in Escherichia coli BUG-6. It could be a result of filamentation rather than a cause. An initial investigation of

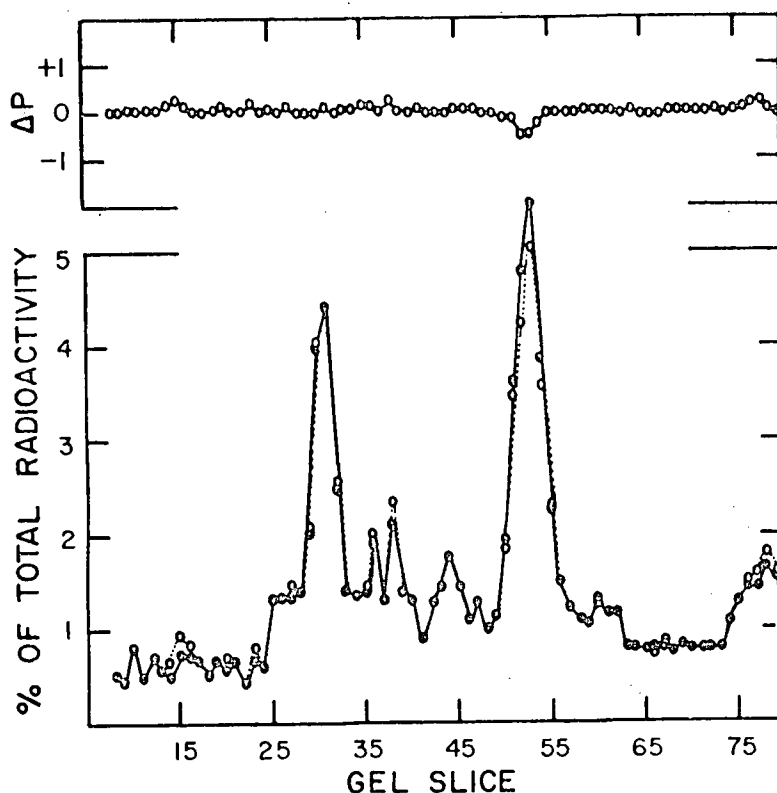


Figure 33. Gel electrophoresis of cell membrane proteins prepared from *Escherichia coli* BUG-6 grown at 42 C and at 42 C followed by recovery at 30 C. *Escherichia coli* BUG-6 was grown at 30 C and then placed at 42 C for 90 minutes. One subculture was treated with ^{14}C -amino acids for the last 60 minutes at 42 C (0), a second culture was treated with ^3H -amino acids for the last 15 minutes at 42 C and then placed at 30 C in the same medium minus the radioactive amino acids. The preparation of cell membrane proteins was by the technique of Shapiro *et al.* (98), electrophoresis as described in "Materials and Methods", and preparation of the graph as described in Figure 30. A positive ΔP indicates that material made at 42 C and preferentially incorporated in cell membrane at 30 C.

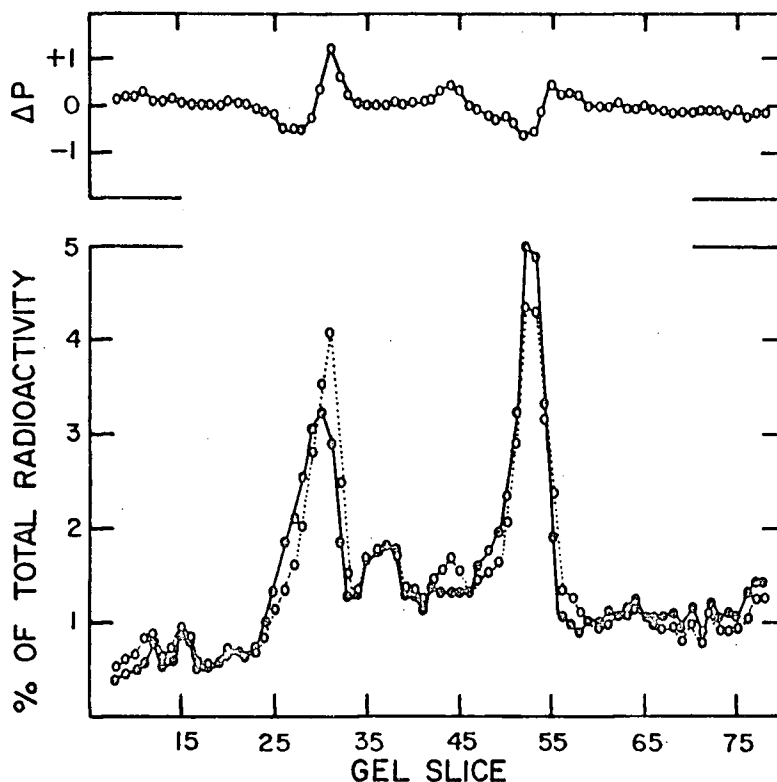


Figure 34.

Gel electrophoresis of cell membrane proteins prepared from *Escherichia coli* BUG-6 grown at 30 C and at 42 C followed by recovery at 30 C. *Escherichia coli* BUG-6 was grown for several generations in ^{14}C -amino acids at 30 C. A second culture was shifted to 42 C for 90 minutes with ^3H -amino acids present for the final 15 minutes, and then returned to 30 C in the same medium minus the radioactive amino acids. The same protocol was employed as for Figure 33. A positive ΔP indicates material was made at 42 C more rapidly than at 30 C. Changes from ΔP of Figures 30 and 31 would represent changes due to the period of recovery growth at 30 C not present in Figures 30 and 31.

this was attempted by production of filaments of Escherichia coli BUG-6 by an alternative method, ie. addition of nalidixic acid at 30 C. Cells growing at 30 C were labelled with ^{14}C -amino acids and compared to cells growing at 30 C, in the presence of 10 μg NAL/ml (final concentration), labelled with ^3H -amino acids. In Figure 35 the cell envelope profiles are compared, a positive $\Delta\Delta\text{P}$ indicates material made more rapidly in the presence of nalidixic acid. A positive peak does occur in the same region as that found when comparing 30 C cells with 42 C filaments, suggesting that this protein(s) is a product of filamentation and not vice-versa. Other ΔP changes are presumably related to the inhibition of DNA synthesis by nalidixic acid. Similar profiles after NAL treatment have been observed elsewhere (Shapiro. Personal communication. 1971).

2. Non-particulate proteins

No enrichment of "division potential" was found in septa although its involvement in septation was known. It was also known that a high level of potential must exist within filaments, therefore a comparison of the non-particulate proteins of Escherichia coli BUG-6 grown at 30 C and 42 C was made, using disc-gel electrophoresis.

In Figures 36 and 37, the profiles of these proteins are depicted, the labels being reversed from Figure 36 to 37. Comparison of these graphs indicate slight changes in the ΔP but because of the large number of proteins and necessity of adding a large amount of protein to these gels, the consistency of the ΔP is lacking. To continue

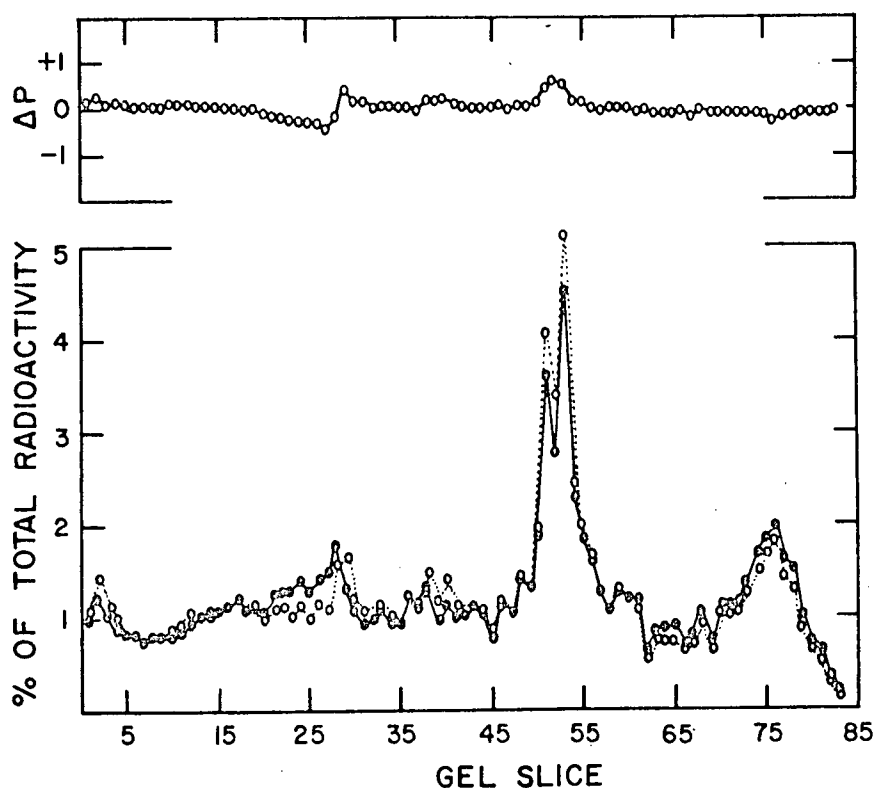


Figure 35. Gel electrophoresis of cell envelope proteins prepared from *Escherichia coli* BUG-6 grown at 30 C and grown at 30 C in nalidixic acid (10 $\mu\text{g/ml}$). One culture was grown at 30 C in ^3H -amino acids and the second at 30 C with 10 $\mu\text{g/ml}$ and ^{14}C -amino acids. The cell envelope proteins were prepared and the electrophoresis carried out as described in "Materials and Methods". A positive ΔP indicates material made preferentially in the presence of nalidixic acid.

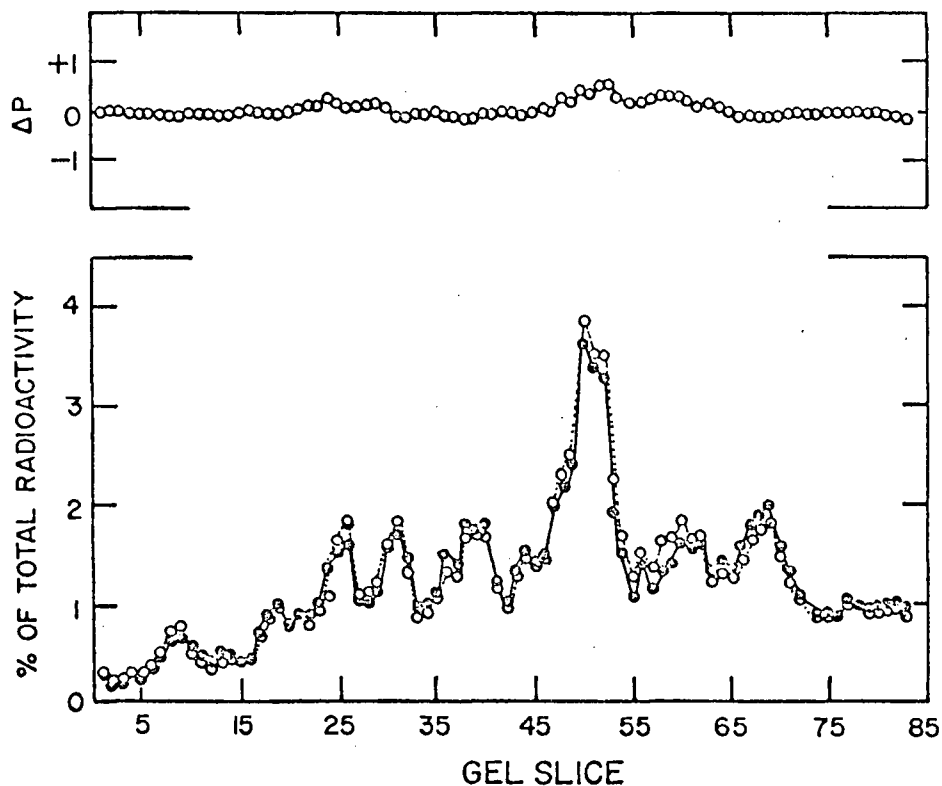


Figure 36. Gel electrophoresis of non-particulate proteins prepared from *Escherichia coli* BUG-6 grown at 30 C and at 42 C. One culture was labelled with ^{14}C -amino acids at 30 C. The second culture was grown at 30 C and then shifted to 42 C with ^3H -amino acids added for the last 60 minutes at 42 C. The non-particulate proteins were prepared and the electrophoresis carried out as described in "Materials and Methods". A positive ΔP indicates material synthesized preferentially at 42 C.

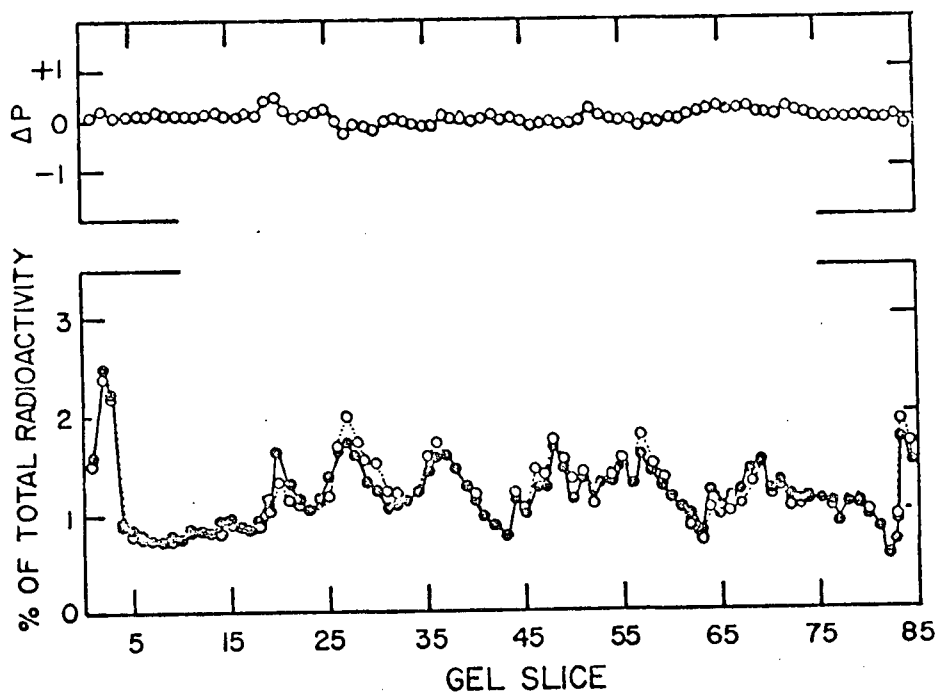


Figure 37. Gel electrophoresis of non-particulate proteins prepared from *Escherichia coli* BUG-6 grown at 30 C and at 42 C. The protocol was the same as in Figure 36 but the ^3H -amino acids were incorporated at 30 C and the ^{14}C -amino acids were incorporated at 42 C. A positive ΔP indicates material synthesized preferentially at 42 C.

this work would require the subdivision of the non-particulate fraction, eg. the column chromatography, before comparison by disc-gel electrophoresis.

DISCUSSION

A detailed analysis of the cell envelope biochemistry and cell division kinetics of a temperature-sensitive cell division mutant of Escherichia coli has been presented. Comparisons can now be made with similar published work.

Escherichia coli BUG-6 grows exponentially at both 30 C and 42 C and synthesizes protein, RNA, DNA and lipid apparently normally at both temperatures. However, it does not divide at 42 C. Other mutants have been described with apparently the same gross macro-molecular properties (44, 71, 110); however, these have not been investigated in detail and as yet no information is available with regard to their membrane biochemistry. The chemical fractionation of cells and chemical assay of the constituents indicated that the use of C^3H_3 -thymidine as a means of measuring DNA synthesis is unreliable immediately following a temperature shift. This confirms the same conclusion reached by Smith and Pardee (103). Recently, the use of thymidine by Escherichia coli as a direct precursor of normal DNA synthesis has itself been questioned (116). The chemical fractionation of the cells and the use of uracil-2- ^{14}C both suggest that the filaments, at 42 C, synthesize RNA slower, when compared to the rate of protein synthesis, than normal cells growing at 30 C. The presence of multiple genomes and therefore gene copies within

one cell may have an effect on the rate of transcription. It would be of interest to determine what kind(s) of RNA synthesis decreases when cell division stops.

The investigation of the phospholipid and fatty acid changes was undertaken because it was known that these cellular constituents should show changes when the temperature of incubation was changed (24, 27, 66, 101), and also they have been correlated directly with cell division (5, 69, 106). The fatty acids do, indeed, show the expected changes, ie. at the higher temperature of incubation the degree of saturation of the fatty acids increased when compared to the fatty acids extracted from cells grown at the lower temperature. The change from 42 C to 30 C is accompanied by a high rate of lipid synthesis and it has been shown that the fatty acid composition is rapidly changed from that typical of 42 C to that typical of 30 C. This is the first indication that Escherichia coli can accomplish this conversion so rapidly and suggests the presence of very active enzymes and a rapid supply of fatty acids. That the composition of this supply is also regulated is shown by the differences reported for the fatty acid contents of the individual phospholipids. This agrees with published results (24, 101). The most interesting result from the work on fatty acids was that the filaments of Escherichia coli BUG-6 contain measurable amounts of cyclic fatty acids which were not detected in normal cells at 30 C nor in the parent strain at 42 C (as stated in the "results", the amounts of

cyclic fatty acids reported should be regarded as minima as the extraction procedure would tend to degrade these compounds (24)).

The role of cyclic fatty acids is not understood but their importance is becoming increasingly apparent (24). Their role in cell division has not been investigated but the present work and that of Peypoux and Michel (79) both suggest that these components show marked differences when the fatty acids of dividing cells are compared to those of non-dividing filament producing cells. Changes have also been found in the cyclic fatty acid composition of an exponentially growing and dividing population of cells when compared to a population of the same organism in the stationary non-dividing phase (21,24).

The relative amounts of individual phospholipids have also been found to change dependent on the growth phase (21, 52,84) and growth media (73). However, the phospholipid composition and their relative rates of synthesis have also been correlated directly to cell division (5) or the lack of cell division (106). Starka and Moravová (106) produced filaments by treatment of Escherichia coli with low levels of penicillin and then allowed their division by addition of penicillinase. They found that during filamentation the cardiolipin content increased and phosphatidyl glycerol content decreased although the overall rate of phospholipid synthesis remained the same when dividing and non-dividing cells were compared. When the filaments were allowed to divide, their phospholipid composition eventually returned to that of the untreated Escherichia coli. Ballestra and Schaechter (5)

induced Escherichia coli to divide whilst other metabolic events were repressed by using a nutritional shift-down. They found that dividing cells increase their rate of phosphatidyl-ethanolamine and decrease their rate of phosphatidyl glycerol synthesis. They also found that lon⁻ cells after ultra violet irradiation, ie. whilst forming filaments, had a decreasing rate of phosphatidyl ethanolamine synthesis which increased when these filaments were induced to divide by a "shift down". The results with Escherichia coli BUG-6 differed from both the published reports. Since the cardiolipin content was not measured for technical reasons, a direct comparison with the results of Starka and Moravová (106) cannot be made. Unlike both published reports, no differences were found in the rates of synthesis of individual phospholipids when dividing and non-dividing filament producing cells were compared. When the filaments were induced to divide by returning the filaments to the permissive temperature, changes in the rates of the synthesis of individual phospholipids were apparent. The rate of synthesis of phosphatidyl glycerol increased and that of phosphatidylethanolamine apparently decreased when measured by a three minute incorporation of glycerol-2-³H. This result is the opposite of that reported by Ballestra and Schaechter (5) but the result may in fact be misleading because during this period, 20% of the radioactivity extracted from the cells as phospholipid, was not identified and could conceivably have been trapped in a precursor of phosphatidyl ethanolamine. An examination

of the biosynthetic pathways of phospholipids shows a divergence at the point of cytidine diphosphate-diglyceride in the pathway to phosphatidyl-ethanolamine and phosphatidyl glycerol/cardiophilin (53, 105). From this intermediate phosphatidyl-glycerol is produced by addition of glycerol and release of cytidine monophosphate. This step would increase the radioactivity in phosphatidyl glycerol if glycerol-2-³H were used to label the phospholipids. Phosphatidyl-ethanolamine is produced from the CDP-diglyceride via phosphatidyl-serine. This latter compound remains at the origin on the chromatography system employed in this work. Therefore, in a three minute pulse of radioactivity, combined with a much increased rate of synthesis, it is quite conceivable that the apparent decrease in rate of synthesis of phosphatidyl ethanolamine when compared to the rate of synthesis of phosphatidyl glycerol can be accounted for by radioactivity trapped in phosphatidyl serine. If this is true, then during the rapid phase of phospholipid synthesis, presumably due to septa synthesis, all phospholipids are synthesized more rapidly and no changes related to cell division can be demonstrated from this work. If the results are to be taken at face value, then during septa formation in this system, the rate of phosphatidyl glycerol synthesis is increased at the expense of phosphatidyl ethanolamine synthesis; and also a component(s) is synthesized which can be extracted by the Bligh and Deyar (11) extraction technique but has not been identified.

The protein composition of the cell envelope of Escherichia coli has been investigated, using sodium dodecyl sulphate disc-gel electrophoresis, in several laboratories, eg. (51, 74, 93, 95, 98), and the advantages and limitations of this and other techniques for membrane analysis have been reviewed (54). It has been possible to relate definite changes in the protein composition of the cell envelope with mutations causing an alteration in the resistance to antibiotics (74) and colicin E2 (93). Studies relating to mutants involving DNA replication and cell division have demonstrated that the proteins in the cell envelope do differ when dividing cells are compared with non-dividing cells (51, 98), whether the filaments are produced by mutation (49, 98) or by use of inhibitors (51). Inouye and Pardee (51) have demonstrated that a protein of molecular weight 39,000 is always enriched in the cell envelope of filaments and some techniques of producing filaments, eg. addition of nalidixic acid, also produce an enrichment for a protein of molecular weight 80,000. It was suggested that the latter protein may be a dimer of the 39,000 molecular weight protein. In the present study, the filaments were found to have a protein(s) of molecular weight 80,000 - 90,000 enriched in their cell envelope. The protein profiles of total cell envelope, eg. Figure 30 and purified cell membrane, eg. Figure 34, both demonstrated this enrichment and although there are differences in the overall shape of the profiles, there is little difference in the percentage change in the enriched protein peak. This suggests

that purification of the cell membrane from the cell envelope does not increase the purification of the enriched peak. However, as discussed in the review (54), and as can be seen in the published results (51, 98), quantitation is not really feasible by this technique. Unlike the results with antibiotic resistance (74), the increased protein in the cell envelope of filaments has not been directly related to the mutation in cell division, and the results of Inouye and Pardee (51) suggest that the enriched protein is a result of the inhibition of cell division and not vice-versa. The present results in which a similar enriched peak was found in nalidixic acid induced filaments would tend to support this conclusion.

In the present work, filamentous cells were also compared to the cells produced when the filaments had been induced to divide. It was known that the filaments would divide at the permissive temperature without protein synthesis, therefore the filaments which were induced to divide were labelled just before being placed at the permissive temperature. Thus the comparison would show if proteins, synthesized at the non-permissive temperature, were incorporated preferentially into the septa at the permissive temperature. No preferential incorporation was found and the results showed that the gross protein composition remained constant up to 40 minutes after the cells were returned to the permissive conditions (Figure 33). (The method of calculation used in analyzing these results would not detect a change in a minor protein constituent. Unfortunately

calculations which would detect such a change would also tend to exaggerate artifacts easily produced by double labelling techniques.) These results therefore indicate that the septa do not have a different protein constitution from the tubular part of the cell envelope. However, until improved methods of membrane analysis and assays for proteins involved in cell division are available, no definite conclusion can be made.

Experiments using cell wall inhibitors indicate that the block in division in Escherichia coli BUG-6 is associated with a terminal step in cell wall synthesis (Figure 15). The action of cycloserine blocks the addition of ala-ala to the N-acetyl-muramic acid residue (108). Vancomycin has been shown to competitively inhibit the insertion of N-acetyl-muramic acid-N-acetyl-glucosamine-pentapeptide from the phospholipid carrier to the acceptor in the cell wall (9). Penicillin acts at a step just one stage later; the joining of the pentapeptide units (108). The action of novobiocin is multifold; affecting DNA synthesis, RNA synthesis and membrane activity (70, 104). There is a suggestion that it also acts in cell wall synthesis at some stage between the formation of the N-acetyl-muramic acid-N-acetyl-glucosamine-pentapeptide and its insertion into the cell wall (108). As seen in Figure 15, cycloserine and novobiocin have little effect on the expression of the division step during the recovery period. These results suggest that novobiocin acts at a stage in cell wall synthesis which is prior to that of vancomycin and penicillin.

Evidently Escherichia coli BUG-6 cells contain cell wall precursors at the non-permissive temperature because when cell division is made possible by reversal of the temperature from 42 C to 30 C, division occurs in the presence of cycloserine. The insertion of the precursors into cross wall does not occur at 42 C since penicillin and vancomycin block division at 30 C indicating precursors are not in position when incubated at 42 C.

Cell division of Escherichia coli BUG-6 occurs during the recovery period in the presence of chloramphenicol if the period at 42 C does not exceed 110 minutes. The ability of filaments to divide without protein synthesis indicates that cells produce and contain a "division potential" at 42 C which can be expressed at 30 C. When protein synthesis is inhibited at the non-permissive temperature division potential decays and this decay rate has a half-life of approximately 0.5 minutes at 42 C. Extrapolation of the decay curve, Figure 17b, indicates an original amount of division potential of approximately 10^2 greater than that required to give division.

If division potential decays at 42 C and yet there is division potential at any time the cell is shifted from 42 C to 30 C, the rate of synthesis of division potential at 42 C must be greater than the rate of decay. Hence the doubling time of division potential at 42 C must be less than the half-life of 0.5 minutes. This is contrasted to the rate of accumulation of division potential at 30 C

equivalent to a doubling time of 20 minutes (Figure 17a), as calculated from the increase in final cell number for cells treated with chloramphenicol during the 30 C recovery period. It should be noted that the normal generation time of Escherichia coli BUG-6 at 30 C is 45 minutes, so that even at 30 C, division potential is synthesized faster than required and, as a constant division rate is found, it is necessary to postulate a control mechanism (see below).

A model which is consistent with these observations is shown in Figure 38. Division potential (d) necessary for the expression of cell division is synthesized as a result of metabolic activities and growth. The accumulation of substrate induces formation of d as in other standard inducible systems.

In Escherichia coli BUG-6 d is a temperature-sensitive component which operates at 30 C, but not at 42 C. At 42 C, d changes rapidly but reversibly to an inactive form (X_1). This conclusion is based on the observation that Escherichia coli BUG-6 stops dividing abruptly upon shift to 42 C (Figure 8a). Inactivation must be reversible since division potential is expressed as cell division at 30 C in the absence of protein synthesis at 30 C (Figure 16). At 42 C, X_1 decays irreversibly to X_2 , which cannot return to active d. The half-life of this conversion is about 0.5 minutes (Figure 17b).

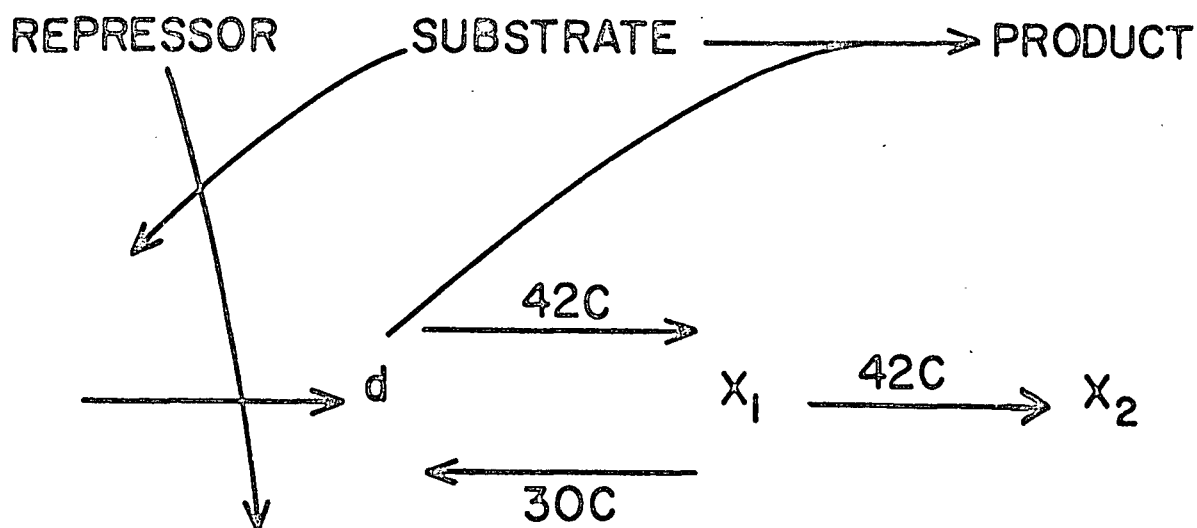


Figure 38. Model for production and interconversion of division potential in Escherichia coli BUG-6.

d = active division potential produced at 30 C.

X_1 = reversibly inactive division potential formed by placing d at 42 C.

X_2 = irreversibly inactive division potential formed by decay of X_1 at 42 C.

If d decays to X_2 through X_1 at a very rapid rate, and yet there is always excess division potential at 42 C in the absence of chloramphenicol, the formation of X_1 must be in excess of the formation of X_2 . Since the conversion of d to X_2 via X_1 at 42 C is about 0.5 minutes, the doubling time of d and X_2 is less than 0.5 minutes. This appears to be an extreme derepressed rate for the formation of d , since the doubling time for d at 30 C is about 20 minutes (Figure 17a).

As indicated in Figure 38, the synthesis of d depends upon the concentration and integrity of d . It is proposed that d is converted to X_1 at 42 C, which is an inactive unit with respect to feedback repression. Therefore, continued and derepressed rates of formation of d occur at the non-permissive temperature and it would be predicted that a high steady state level of X_1 would eventually be attained at the non-permissive temperature. This steady state may, however, be constant on a per cell basis, a gene dose basis or a concentration basis. The "amount" of division potential has been equated above to the number of residual divisions occurring at 30 C in the presence of chloramphenicol after 45 minutes at 42 C, but an increase in the length of time the cells were maintained at 42 C would give both a different estimate of the high internal level and of the decay kinetics (Figure 24), so that although the model fits well with one particular set of conditions, it must be considered with relevance to other conditions.

If indeed, a derepression mechanism is occurring then very brief periods at 42 C should cause, (a) not all d to be converted to X_1 ; and (b) not a maximum amount of X_1 as time is required for its synthesis following derepression. A pulse of 42 C if less than 1 minute gives little effect on division (Figure 2a), ie. sufficient d is still available for division. Pulses of 2, 3, and 4 minutes at 42 C stop division but the recovery of 30 C takes longer than the recovery of cells kept at 42 C for longer periods. This would be predicted if d were converted to X_1 and some to X_2 but the rapid derepressed rate of synthesis was not yet in effect. As the period at 42 C becomes longer, the rate of recovery becomes faster and the very rapid division phase becomes more evident (Figure 2 and 3). Approximately 10 minutes at 42 C gives a maximum rate of recovery. Presumably this is the period required to obtain a steady state level of X_1 or the minimum time required to express division potential. In this analysis, the time required for the 42 C pulsed cells to attain the cell number of the 30 C control cells is used as an assay of "division potential". These results substantiate the hypothesis of a derepression causing an accumulation of division potential.

One might anticipate that the time required to attain the control value would be independent of the length of the incubation period at the non-permissive temperature after the initial derepression stage. However, as seen in Figure 4, the time required to attain the control

value increases for incubation periods at 42 C in excess of 35 minutes. However, the kinetics of recovery are interesting in two respects: (1) there seems to be an initial burst of cell division which constitutes exactly one doubling at a constant of 15 minutes after the return to the permissive temperature; and (2) the rate of subsequent cell division is more rapid than that of a normal exponential culture at 30 C. Once the control value is attained and all cell equivalents have been expressed, the growth rate returns to the normal 30 C rate. From these results, it is obvious that the assay of "division potential" described for short 42 C pulses, does not apply to long periods at 42 C, suggesting its application to short pulses may also be incorrect. An alternative possible assay would be the amount of very rapid division occurring during the recovery phase. This becomes a constant as the period of filamentation at 42 C exceeds the time for one optical density doubling at 42 C, ie. 35 minutes. This constant is equivalent to exactly one doubling in the cell number over that maintained at 42 C. Using this as an assay of "division potential" then one must conclude that the filaments contain a steady state level at less than enough for two cell divisions and that it can only be used in unit amounts therefore only one division occurs. This constant of one division has also been reported for the recovery division of lon⁻ filaments (28) and filaments produced by thymine starvation (26). Because of this correlation with very different systems, it would seem

possible that the common act of dividing is controlling the amount of division rather than a supply of some division prerequisite, eg. separation of genomes which were previously interacting could feasibly affect the subsequent actions of the cells.

If a constant amount of division potential were available to be used at 30 C, then returning filaments of different lengths to 30 C in the presence of chloramphenicol should give a constant amount of division. This experiment is described in Figures 23 and 24; and no such constant amount of division occurred except over limited time periods of incubation at 42 C. In fact, the maximum amount of division gave approximately four times the number of cells at 42 C. With very short periods at 42 C, no division occurred at 30 C in the presence of chloramphenicol, again supporting the idea of a time required for the build-up of potential (Figure 24). No division occurred for cells kept at 42 C for more than 110 minutes when placed at 30 C in chloramphenicol. Cells treated the same way but replaced at 30 C in the absence of chloramphenicol would have divided once rapidly, then at a faster than normal rate until the 30 C control cell division rate and number were attained (Figure 4).

One explanation of this paradox was to assume a fixed amount of division potential per cell, enough for about four divisions. The single division at 30 C when no chloramphenicol was present would be explained by creating a further control system, ie. protein synthesis at 30 C caused the preferential completion of one division

site before a second was started. When protein synthesis was not allowed at 30 C, this control is lost and all potential division sites are available for expression of division potential. If a fixed amount of division potential per cell were available then an optimum cell length would occur when the fixed amount of potential was completely expressed by the number of available "division sites". As the number of sites increased over this optimum, the number of completed divisions would decrease as the fixed amount of potential became diluted throughout the filament and fewer sites obtained sufficient potential to divide. A filament length would be reached when no cell division occurred. This system described agrees with the information in Figure 24. If the number of division sites could be kept constant at the optimum, even though the filament increased in length, then on returning to 30 C, a fixed optimum number of divisions would be predicted assuming rapid, free mobility of division potential. Figure 26 is an attempt at such an experiment, assuming completed DNA replication would trigger the production of division sites. Nalidixic acid was added after 50 minutes at 42 C and then the cells returned to 30 C after a further 30 minutes at 42 C. The combined effect of nalidixic acid and chloramphenicol produced less division than filaments treated with only chloramphenicol and returned to 30 C at the same time. If, indeed, DNA replication does regulate the number of division sites, this result would indicate that division sites are not limiting. On the other hand,

the result may indicate division sites are not controlled by the amount of DNA replicated or that the idea of "division sites" as physical entities, is itself untenable.

The above discussion has demonstrated that the model proposed (Figure 38) for explanation of the cell division kinetics requires further assumptions, if it is to be extended to periods of filamentation at 42 C in excess of 45 minutes but other experimental results, using a 45 min period have been presented and should be accommodated by the model. When Escherichia coli BUG-6 is shifted from 30 C to 40 C, division does not cease immediately and then after a period of filamentation, division restarts spontaneously. Shifts from 30 C to 38 C or 36 C cause a decrease in the division rate followed by an increased rate before rates characteristic of 38 C and 36 C are obtained (Figure 1). These results can be incorporated into the model if the reaction $d \rightarrow X_1$ occurs more slowly as the "non-permissive" temperature is lowered. Initially, division takes longer to cease at 40 C, since the rate of conversion of $d \rightarrow X_1$ is slower than at 42 C and takes longer to reduce d below the threshold value. Once d is depleted, derepression will occur and rapid synthesis of d will ensue. Eventually, the level of d will exceed that required for division since the derepressed rate of formation of d is faster than the decay rate of $d \rightarrow X_1$ at 40 C. Division potential (d) continues to be made at the derepressed rate until all of the division sites or the substrate is exhausted. This

leads to the burst in cell division seen in Figure 1. When all division sites have been expressed, the substrate is depleted and there is normal repression of the synthesis of d . The steady state level of d , maintained by normal feedback controls, is sufficient to allow normal cell division. The rate of synthesis of d at 40 C would be anticipated to be faster than that observed at 30 C because of continual decay of d to the inactive forms X_1 and X_2 . At 36 C and 38 C, the same arguments apply, except the decay of d to X_2 is never complete, but is enough for derepression to be triggered and cause subsequent rapid division.

In order to accommodate the results of temperature shifts from 30 C to temperatures below 42 C, it was necessary to suggest the d is converted to X_1 at some finite rate. If this were true, then a rate of conversion of $d \rightarrow X_1$ should also occur at 42 C, and if cells could be produced with an excess of d , then on placing at 42 C cell division should occur until the available d is exhausted by usage in division and by conversion to X_1 . The model predicts that returning cells to 30 C after 45 minutes at 42 C will produce filaments with a high level of d (by reversion from X_1). In Figure 27 these cells were again shifted to 42 C after different periods at 30 C. If these cells had not been shifted to 42 C, they would have divided 15 minutes after the 42 C \rightarrow 30 C shift. As the length of 30 C incubation is increased before the cells are returned to 42 C, the available time at 42 C for $d \rightarrow X_1$ to occur before the cells are

due to divide is decreased. This predicts that with longer periods at 30 C, more residual division should occur at 42 C. This result was obtained. The same experiment was repeated but before the 42 C \rightarrow 30 C shift, a 3 minute pulse of chloramphenicol was given to the cells. This short inhibition of protein synthesis should, according to the model, reduce the division potential by 50% (Figure 17b). Comparing the amounts of residual division at 42 C for the chloramphenicol treated cells with the non-treated cells after the same period of 30 C incubation, demonstrated that the former completed very much less division (Figure 28). This result is consistent with a rate for $d \rightarrow X_1$ because a lower original amount of d would require less time at 42 C before the internal concentration of d fell below the threshold required for division. This result also supports the hypothesis that division potential decays at 42 C when protein synthesis is inhibited.

Filaments of Escherichia coli BUG-6 growing at 42 C divide when the external osmotic pressure is increased, however, this division requires de novo protein synthesis (Figures 12 and 19). The same result was found for a similar mutant (86). Osmotic remedial mutants have been investigated most thoroughly in yeast (7, 40) and from this work (7) it has been concluded that osmotic remedial conditions are required to aid either the polymerisation step in the formation of a protein from sub-unit polypeptides, or in the production of the correct tertiary configuration of nascent polypeptides facilitating

their conversion to an active gene product. The inactivation of division potential (d) could be due to separation of sub-units (X_1) and the addition of osmotic remedial conditions would enable de novo sub-units to combine at the non-permissive temperature and give division. That division occurs rapidly following the addition of NaCl suggests that the de novo sub-units could form active potential by combination with the (X_1) sub-units already present. The combination of de novo sub-units with X_1 sub-units as compared to the re-associated of only X_1 -sub-units could also explain the different division kinetics observed when cells kept at 42 C are returned to 30 C with or without the addition of chloramphenicol.

A comparison can now be made between the proposed model for control of cell division in Escherichia coli BUG-6 and models proposed for control of cell division in other systems. Cell division is dependent on DNA replication (16, 42), and this has been found to be true for Escherichia coli BUG-6 (Figure 20), although Figure 25b indicates that rate of DNA replication may oscillate and not be maintained at the same doubling rate as that found for the optical density. Donachie (26, 27, 28) has shown that filaments produced by inhibition of DNA replication do not divide until the DNA/mass ratio is returned to normal. This is not the case for Escherichia coli BUG-6. As shown in Figure 20, filaments can be produced with less DNA than normal, but on returning to the permissive temperature, these filaments divide. The number of divisions depends on the amount

of DNA within the filaments, i.e. in this system the DNA/protein ratio is not a direct control of cell division. The results with Escherichia coli BUG-6 do however confirm Donachie's observations that fragmenting filaments are limited to one rapid division (26, 28).

The model proposed here is virtually identical to that of Adler et al. (3). They suggested a "material essential for cell fission" which the cell produced during normal growth and which was "destroyed or otherwise inactivated as a function of time, fission occurs each time a critical level of this material is exceeded". It was suggested that filamentation occurred when this material never reached the critical level. In this respect lon⁻ cells were assumed to produce barely enough material and any injurious agent, eg. radiation, caused the level to drop below that needed for division. The lesion causing filamentation in lon⁻ has now been determined as an effect on DNA synthesis (35, 113), but, as this mutation can be suppressed (65), and the wild type lon⁺ is dominant in a merozygote (113), the involvement of a protein product must be necessary.

An alternative model for irradiation filamentation is that the irradiation blocks the synthesis of a repressor by affecting DNA integrity, which prevents the synthesis of an inhibitor of cell division (117). This is a negative control model and a similar model has also been suggested by Inouye (47) in which the division site is normally blocked by a division inhibitor (M) which is itself inactivated by a second agent (I). The division kinetics of Escherichia

coli BUG-6 could be explained if the (I) material were "division potential" which failed to recognize (M) at 42 C. The normal production of (I) would be cyclic, as suggested by Inouye (47), and in this respect, this model is exactly the same as the model of Adler et al. and the one proposed here. The localization of (M) could enable the preferential completion of one division as described for fragmenting filaments if the cell recognized the oldest (M) protein, i.e. the (M) present at the time of the 30 C \rightarrow 42 C shift. Paulton (77) has also described a system which requires the cell to control the localization and preferential completion of division sites.

There are several predictions for division control which could easily be tested on Escherichia coli BUG-6. For example, Bazill (8) has suggested filamentation is due to an altered outer cell wall configuration. Previc (81) suggested that the absence of an enzyme related to diamino-pimelic acid metabolism might cause filamentation. Also, Inouye and Pardee (50) showed that the polyamine content must be correct for cell division to occur in their system and claimed this as a prerequisite for division. The presence of a recA⁻ mutation has been found to allow division in three systems which normally would not be expected to divide (36, 48, 56). A valuable experiment would be the production of a strain of Escherichia coli BUG-6 containing the recA⁻ lesions to investigate whether division would then occur at 42.C.

In summary, Escherichia coli BUG-6 appears to be a valuable tool in the investigation of cell division in Escherichia coli. The present work has characterized the division kinetics of this mutant and a little of its biochemistry. The control of cell division has been shown to involve a temperature-sensitive protein which is produced at a very high rate at the non-permissive temperature. A protein has been identified in the cell envelope which is produced at a much higher rate at 42 C than 30 C and it is now necessary to determine whether or not this protein is the one controlling the division kinetics described in this work.

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