

Fall 1972

CHARACTERIZATION OF SEVERAL
THYMINE-REQUIRING MUTANTS OF
ESCHERICHIA COLIY MEL

JAMES HOWARD MARYANSKI

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by

JAMES HOWARD MARYANSKI

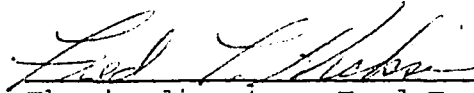
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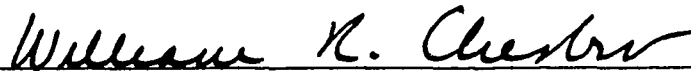
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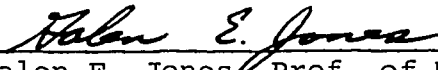
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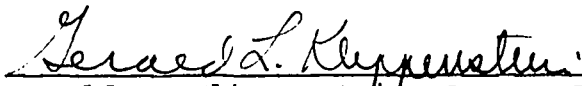
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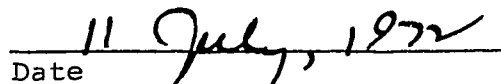
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This dissertation is dedicated to my loving wife,
Linda, and our children, Kathleen and David.

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ABSTRACT

CHARACTERIZATION OF SEVERAL THYMINE-REQUIRING MUTANTS OF ESCHERICHIA COLI Y MEL

by

JAMES H. MARYANSKI

Escherichia coli Y mel⁻, E. coli B27⁻, and E. coli B9⁻ were isolated from E. coli Y mel, E. coli B27, and E. coli B9, respectively, by trimethoprim selection. E. coli B27 and E. coli B9 were tryptophan deletion mutants derived from E. coli Y mel by Dr. I. P. Crawford. Mutants, E. coli Y mel⁻, E. coli B27⁻, and E. coli B9⁻ required 2-5 μ g of thymine/ml for aerobic growth on minimal agar and failed to utilize thymidine as a sole source of carbon. The inability to utilize thymidine indicated the presence of mutations in the genetic regions controlling the production of thymidylate synthetase (thy A) and the catabolism of deoxyribonucleosides. E. coli B9⁻ was sensitive to high (200 μ g/ml) concentrations of thymidine, a characteristic of deoxyriboaldolase negative (dra⁻) mutants. E. coli Y mel⁻ and E. coli B27⁻ were resistant to high concentrations of thymidine. Thymine, thymidine, and thymidylic acid supported growth on agar medium of all thymine-requiring mutants studied. Other nucleic acid bases, nucleosides, nucleotides, thymine analogues, orotic acid, deoxyuridine-5'-monophosphate, and other compounds failed to support growth when added to minimal agar lacking thymine.

Increased carbon dioxide tension (5%) supported colony-formation of E. coli Y melT⁻ and E. coli B27T⁻ but not of E. coli B9T⁻ on minimal agar lacking thymine.

E. coli B27T⁻ exhibited an unusual growth response to thymine starvation conditions. Growth of E. coli B27T⁻ in minimal broth lacking thymine resulted in a culture containing 2×10^9 cells/ml from an initial inoculum of 10^4 to 10^8 cells/ml. Thy⁺ revertants were not detected. Initial inocula of 10^3 cells/ml or less resulted in thymineless death, i.e. loss of colony-forming ability. E. coli Y melT⁻ lost colony-forming ability, exponentially, in minimal broth lacking thymine; after 4 hrs, 99.9% of the initial population (2×10^8 cells/ml) was unable to form colonies. E. coli B9T⁻ increased from 2×10^8 to 8×10^8 cells/ml (2 generations) in minimal broth lacking thymine, and a gradual loss of colony-forming ability followed. In minimal broth containing thymine, all thymine-requiring mutants attained a terminal concentration of 2×10^9 cells/ml. Thymine independent strains, E. coli Y mel, E. coli B27, and E. coli B9, attained a terminal cell concentration of 2×10^9 cells/ml in either minimal broth containing thymine or minimal broth lacking thymine.

Supernatant broth from cultures of E. coli B27T⁻ grown in minimal broth lacking thymine failed to prevent thymineless death of thymine-requiring mutants, E. coli Y melT⁻ and E. coli 15T⁻, although the onset of lethality was delayed. Thymineless death of E. coli B27T⁻ at low cell concentrations (10^3 cells/ml) was prevented by supernatant broth from a culture of E. coli B27T⁻ grown in minimal broth lacking thymine, although

an increase in the number of colony-forming units did not occur. Dilution of the initial inoculum (2×10^8 cells/ml) of E. coli B27T⁻ or starvation in physiological saline prior to resuspension in minimal broth lacking thymine did not prevent growth, indicating the absence of unusual intracellular pools of thymidine (or a derivative).

The deletion in the tryptophan operon of E. coli B27T⁻ did not influence the growth of this mutant in minimal broth lacking thymine, since trp⁺ transductants showed the same growth pattern as the trp⁻ parent. In addition, introduction of the F'ColVBM-K260, trp plasmid into E. coli B27T⁻ did not affect its growth in the absence of exogenous thymine.

Other properties of E. coli B27T⁻ were examined. Construction of a merodiploid of E. coli B27T⁻ harboring an F'ara plasmid which carried the markers associated with deoxyribonucleoside catabolism resulted in a 99.9% reduction in viable count during thymine starvation. Thymidylate synthetase activity was not detected in the crude cell extract of E. coli B27T⁻. In addition, E. coli B27T⁻ extract inhibited the thymidylate synthetase activity in extracts from E. coli Y mel and E. coli B27. Ultraviolet-absorbing compounds having the mobility (R_f) and absorption spectra of adenine, guanine, cytosine, and thymine were isolated from purified DNA preparations of E. coli B27T⁻ grown in minimal broth lacking thymine. The thymine requirement of E. coli B27T⁻ was cotransducible with arg A, indicating a lesion in or closely linked with thy A. The sparing effect of carbon dioxide was also cotransduced with the thymine requirement.

The data were consistent with a hypothesis that E. coli B27T⁻ possessed an alternate pathway, independent of thymidylate synthetase, for the biosynthesis of thymidine. Functioning of the alternate pathway appeared to be controlled by the growth conditions. Alternative explanations for growth of E. coli B27T⁻ in the absence of exogenous thymine included (1) biosynthesis of an analogue of thymine, (2) cross-feeding among the population at high cell density, and (3) a partially defective thymidylate synthetase protein which was active under permissive growth conditions. Mutants E. coli Y melT⁻ and E. coli B9T⁻ appeared to be thymine-requiring mutants with characteristics similar to mutants described by other investigators.

CHAPTER I

INTRODUCTION

The phenomenon of thymineless death was first described in 1954 by Barner and Cohen. Cells of the thymine-requiring bacterium, Escherichia coli 15T⁻, were observed to exponentially lose their ability to form colonies on nutrient agar medium following incubation in minimal broth lacking thymine. Loss of viability during thymine starvation was ascribed to unbalanced growth resulting from the inhibition of DNA synthesis while protein and RNA synthesis continued. The mechanism of thymineless death has eluded investigators since its discovery, although the lethal effect has been attributed to phage or colicin induction, protein or RNA synthesis, and damage to the bacterial chromosome.

Elucidation of the mechanism of thymineless death is of interest since the lethal event may be an important response of cells to chemotherapeutic agents such as folate antagonists (Cohen, 1971). Naturally occurring folate derivatives may serve as cofactors or inhibitors of both dihydrofolate reductase and thymidylate synthetase (Baugh and Krumdieck, 1971), enzymes which are involved in thymidylate biosynthesis. The relationship of thymineless death, inhibition of DNA synthesis, and folate derivatives has not been resolved. Research into various aspects of thymineless death may provide information with respect to macromolecular synthesis and regulation in addition to increased chemotherapeutic efficacy of folate antagonists.

Experiments designed to study genetic recombination between the tryptophan operons of the extrachromosomal element, F'ColVBM-K260, trp, and the bacterial chromosome of the thymine-requiring, tryptophan deletion mutant, E. coli B27T⁻, showed that this strain possessed several unusual properties in relation to its response to thymine deprivation. Gallant and Spottswood (1965) found that thymine starvation induced recombination between a plasmid (F'lac) and the bacterial chromosome. When similar experiments were attempted with merodiploid strain, E. coli B27T⁻/F'ColVBM-K260, trp, thymine starvation resulted in growth of the mutant, both in terms of cellular mass (as determined by an increase in culture turbidity) and an increase in the number of colony-forming units. The non-colicinogenic strain, E. coli B27T⁻, showed a similar response to thymine starvation, and routinely, 2×10^9 cells/ml were obtained from cultures containing, initially, 10^4 to 10^8 cells/ml. No thy⁺ revertants were detected on minimal agar lacking thymine on which E. coli B27T⁻ cells were plated. In contrast, initial cell concentrations of 10^3 cells/ml or less resulted in thymineless death. An investigation was initiated to determine the genetic and biochemical characteristics which permitted E. coli B27T⁻ to grow in liquid medium in the absence of exogenous thymine and from initial cell concentrations of above 10^4 cells/ml.

CHAPTER II

LITERATURE REVIEW

The phenomenon of thymineless death was discovered by Barner and Cohen (1954) during an investigation into the occurrence of the unusual pyrimidine, 5-hydroxymethylcytosine in T-even bacteriophages. Metabolic studies revealed that the thymine-requiring mutant, Escherichia coli 15T⁻, irreversibly lost the capacity to form colonies when cells of this strain were suspended in minimal medium lacking thymine but containing all other nutrients needed for growth. Under these conditions, E. coli 15T⁻ retained the ability to synthesize RNA and protein that resulted in cytoplasmic growth as demonstrated by an increase in culture turbidity (Barner and Cohen, 1954). The loss of colony-forming ability, which required a carbon and energy source (Barner and Cohen, 1954), was ascribed to unbalanced growth resulting from the specific inhibition of DNA synthesis while cytoplasmic growth (i.e., RNA and protein synthesis) continued (Cohen and Barner, 1954).

The cytotoxic effect caused by thymine deficiency was unique for thymine, since a uracil-requiring mutant of E. coli did not lose viability when resuspended in minimal medium lacking uracil (Barner and Cohen, 1954). At the same time, amino acid deficiency did not provoke a loss of viability as demonstrated by the retention of colony-forming ability of the multiple auxotroph, E. coli 15TAU, in minimal medium lacking arginine. However, Breitman, Finkleman, and Robinovitz (1971) reported methionineless death occurred when a methionine

auxotroph of E. coli 15 was deprived of methionine. In addition, histidine starvation caused a loss of viability in a mutant of Bacillus megaterium (Wachswan, Kemp, and Hogg, 1964), while Bacillus subtilis mutants, which required either tryptophan, uracil, or thymine, also lost colony-forming ability when deprived of any of these compounds (Pritikin and Romig, 1966).

All thymine-requiring strains of Escherichia coli and Bacillus subtilis studied were subject to thymineless death when the cells were starved for thymine (Luzzati, 1966). Thymineless death was also observed in such diverse organisms as Bacillus megaterium (Wachsmann, Kemp, and Hogg, 1964), Lactobacillus acidophilus (Reich and Soska, 1970), Diplococcus pneumoniae strain SIII-1 (Friedman and Ravin, 1972), and the pleuropneumonia-like organism Mycoplasma laidlawii (Smith and Hanawalt, 1968).

Thymine-requiring mutants of E. coli, in contrast to thymine-independent strains, were capable of using exogenous thymine for growth (Crawford, 1958), and the enzymes necessary for the incorporation of exogenous thymine into the DNA were present (Mantsavinos and Zemenhof, 1961). Utilization of exogenous thymine by thymine-requiring strains of E. coli appeared to be a function of the concentration of deoxyribosyl donors available for the synthesis of thymidine. When these donors were provided in the growth medium, particularly as purine deoxyribonucleosides, exogenous thymine was incorporated into DNA (Kammen, 1967). Thymine-requiring mutants of E. coli isolated by selection with aminopterin, amethopterin, or

trimethopterin (Okada, Yanagisawa, and Ryan, 1960; Okada, Homma, and Sonohara, 1962; Stacey and Simson, 1965) required high concentrations ($> 20 \mu\text{g/ml}$) of thymine for growth (Harrison, 1965). These mutants were designated thy⁻, tlr⁺ to signify thymine-requiring (thy⁻) mutants which have a high (tlr⁺) thymine requirement (O'Donovan and Neuhard, 1970). These mutants contained a defective thymidylate synthetase enzyme specified by the thy A locus located near arg A on the E. coli linkage map (Ishibashi, Sugino, and Hirota, 1964; Kitsujii, 1964; Alikhanian, Iljina, and Kaliaeva, 1966; Appendices 1 and 2). The requirement for high concentrations of thymine was related to the presence of thymidine phosphorylase (tpp, deoA), the first enzyme involved in the catabolism of deoxyribonucleosides that converts thymidine to thymine and deoxyribose-1-phosphate (Fangman and Novick, 1966; Appendix 2). Deoxyribose-1-phosphate is further catabolized to acetaldehyde and glyceraldehyde-3-phosphate by enzymes specified by the deo region (Appendix 2) located near thr on the E. coli linkage map (Appendix 1). Thymidine can be utilized as a sole source of carbon by strains prototrophic for the deo region (Fangman and Novick, 1966; Lomax and Greenberg, 1968). Mutants (thy⁻, tlr⁻), which require low concentrations of thymine (1-2 $\mu\text{g/ml}$), occur as spontaneous events and can be isolated directly from high requiring strains. These mutants were double mutants containing mutations in both the thy A and the deoxyribonucleoside regions of the chromosome (Breitman and Bradford, 1964; Harrison, 1965). Two types of tlr⁻ mutants have been described. In one type (sensitive) growth was inhibited by high concentrations

(200 μ g/ml) of deoxyribonucleosides (tds), and the second type (resistant) was capable of growing in the presence of high concentrations of deoxyribonucleosides (Alikhanian et. al., 1966; Munch-Petersen, 1968). Both types of tlr⁻ mutations were linked to thr and unlinked to thy A (Alikhanian et. al., 1966; Okada et. al., 1966).

Several consequences of thymineless death were observed in bacteria subjected to thymine starvation. Induction of prophage λ was reported in thymine-requiring mutants of E. coli K12 (Korn and Weissback, 1962; Melechen and Skarr, 1962), as well as the induction of colincinogenic factors (Luzzati and Chevallier, 1964). Mutagenicity increased during thymine starvation (Barner, Cohen, and Kanazir, 1958); the number of his⁺ revertants of E. coli 15T⁻his⁻ increased during thymine starvation (Coughlin and Adelberg, 1956). Intracellular accumulation of deoxyadenosine triphosphate (dATP) was observed by Munch-Peterson and Neurath (1964). Pritchard and Lark (1964) reported premature initiation of replication at the origin of the chromosome in E. coli 15T⁻ during thymine starvation. The addition of thymine resulted in simultaneous replication of the chromosome from both the origin and the growth point present prior to starvation. Recombination was induced by thymine starvation in the F'lac system employed by Gallant and Spottswood (1965), while thymine starvation stimulated the production of an endonuclease in a thymine-requiring mutant of E. coli K12 (Freifelder and Levine, 1972).

The mechanism of thymineless death has eluded investigators since its discovery (Cohen, 1971). Several investigators

have suggested that the loss of viability was associated with the production of a colicin or a phage. Ryan, Fried, and Mukai (1955) found that ultraviolet irradiation followed by incubation of the cells in a growth-supporting medium led to lysis of E. coli 15 cells and a concomitant release of an antibacterial agent. E. coli 15T⁻, being colicinogenic (col 15⁺), led Mennigmann (1964) to postulate that colicinogeny was a major factor responsible for thymineless death. From the results of an electronmicroscopic investigation of Mitomycin C-induced cultures of E. coli 15, Endo et. al. (1965) suggested that the col 15⁺ particle was a bacteriophage. Mennigmann (1965) isolated a phage from E. coli 15 which was designated ψ and obtained electronmicrographs of phage ψ particles which showed a polyhedric head (600 \AA diameter) and a tail (1100 x 170 \AA) containing a base plate with at least three spikes. This phage was similar in structure to the T-even coliphage, although the phage head was smaller. In addition, the electronmicrographs of phage ψ showed a large number of tails without heads which Mennigmann (1965) interpreted as evidence that phage ψ was defective. Ishibashi and Hirota (1965) obtained hybrid strains from crosses of E. coli 15T⁻ and strains of E. coli K12. Using these strains under thymine starvation conditions, they showed that strains which produce the colicin but were insensitive to its lethal effect were less susceptible to thymineless death than E. coli 15T⁻, which both produces and was sensitive to the colicin. The former strains, on the other hand, were more susceptible to thymineless death than a strain

which could no longer produce colicin and was sensitive to the colicin. These results led Ishibashi and Hirota (1965) to postulate that although colicinogeny was an important factor responsible for thymineless death, the rate of loss of colony-forming ability was affected by other genetic characteristics as well. Medoff and Swartz (1969) demonstrated that E. coli 15T⁻, grown in the presence of thymine and an antibacterial agent such as Mitomycin-C, nalidixic acid, or hydroxyurea, showed an increased amount of 6-methyladenine in the extractable DNA and an increased level of the virus-induced enzyme, DNA methylase. Similar results were observed to occur during thymine starvation but not following treatment with penicillin, phenethyl alcohol, or novobiocin. Thus, the induction of a defective phage appeared to be associated with the action of the antibacterial agents which led to increased methylation. E. coli 15T⁻_R was isolated as a Mitomycin C-resistant strain of E. coli 15T⁻ and did not show increased production of phage, methylase activity, or 6-methyl-adenine following treatment with various agents. Medoff and Swartz (1969) postulated that the rapid killing during thymine starvation of E. coli 15T⁻ was secondary to the induction of a defective phage. More recently Medoff (1972) compared thymineless death in lysogenic and nonlysogenic bacteria and found that lysogenic strains were very sensitive to thymine starvation and were killed more rapidly than nonlysogenic strains. This suggested that the phenomenon of thymineless death was separable from phage induction.

A circular plasmid was detected by Cozzarelli, Kelly, and Kornberg (1968) in a col 15⁻ strain of E. coli 15T⁻ that was susceptible to thymineless death. Ikeda, Inuzuka, and Tomizawa (1970) isolated three plasmids from E. coli 15. The molecular length of these extrachromosomal elements was 32.9, 53.9, and 0.9 μm with the 32.9 μm plasmid being largely homologous to phage P1 DNA. These investigators also found two different inducible phages in E. coli 15. One phage was identified as the col 15 particle, while the second phage carried DNA different from both the P1-like plasmid and col 15 DNA. Thus the effect of phage and colicins on the course of thymineless death has not been fully elucidated.

Several investigators provided evidence that RNA synthesis was intimately involved in thymineless death. Barner, Cohen, and Kanazir (1958) showed that inhibition of RNA synthesis through uracil deprivation of a uracil-requiring strain of E. coli 15T⁻ concomitant with thymine starvation retarded thymineless death. Thymineless death was also inhibited by fluorouracil (Cohen *et. al.*, 1958), a compound which Champe and Benzer (1962) demonstrated to act at the messenger RNA level. Hanawalt (1963) conducted experiments which also suggested that mRNA synthesis was particularly necessary for thymineless death. Using E. coli TAU-bar, which has a stringent uracil requirement, a very slow loss of viability was demonstrated when thymine and uracil were removed. However, the removal of any or all amino acids required by this strain as well as thymine and uracil resulted in an increased killing rate. Since amino acid deficiency in a

stringent bacterium represses ribosomal RNA (rRNA) and transfer RNA (tRNA) synthesis, but not messenger RNA (mRNA) synthesis (Noumora et. al., 1962), Hamawalt (1963) postulated that inhibition of rRNA synthesis, which accounted for 98% of the cellular RNA, allowed more uracil to be made available by RNA turnover to be incorporated into mRNA. This mRNA, in turn, may specify the synthesis of a colicin.

Further evidence that RNA synthesis was involved in thymineless death was presented by Gallant and Suskind (1962) who found that thymineless death in thymine-requiring mutant, E. coli B3, could be prevented by the inhibition of RNA synthesis through phosphate starvation. The extent of thymineless death in this strain correlated with the unbalanced synthesis of RNA at 37 C. However, at 25 C, thymineless death was not observed although RNA synthesis was unbalanced. In subsequent experiments at 25 C, Gallant (1962) found that sublethal damage occurred during thymine starvation, and shifting the cells to 37 C resulted in a decrease in the lag phase prior to the onset of thymineless death. The decrease in lag phase was correlated with RNA synthesis since, after RNA synthesis ceased, little further decrease in the lag phase was observed.

Rosenkranz, Carr, and Rose (1965) suggested that the bacteriostatic action of phenethyl alcohol on E. coli resulted from inhibition of mRNA synthesis. These workers found that this bacteriostatic concentration of phenethyl alcohol affected the course of thymineless death. When a high (0.5%) concentration of phenethyl alcohol was added to a culture during the

initial thymine starvation period, an immediate lethal effect was observed. This effect was unusual, since all cells at this time could be rescued by thymine. However, the addition of a low (0.05%) concentration of phenethyl alcohol during the initial thymine starvation phase resulted in protection from thymineless death. During the exponential die-off phase, a high (0.5%) concentration of phenethyl alcohol was required to rescue the surviving population from thymineless death. Rosenkranz, Carr, and Rose (1965) suggested that mRNA synthesis was selectively inhibited by 0.05% phenethyl alcohol, while high concentrations of this compound arrested all macromolecular synthesis.

During thymine starvation of E. coli 15T⁻, Luzzati (1966) demonstrated that the rate of synthesis of mRNA decreased exponentially in parallel with the viability of the cells. Luzzati (1966) also found that the ability of cell-free extracts from starved cells of E. coli 15T⁻ to incorporate ribonucleotide triphosphates into RNA was inferior to the incorporation by extracts from unstarved cells. The decreased RNA polymerase activity needed for incorporation was due to the modification of the DNA molecule that decreased its ability to serve as a template (Luzzati, 1966). The decrease was postulated to result from either a decreased number of RNA polymerase molecules bound to the DNA, or a decreased growth rate of mRNA chains, or an increased number of stop signals on the template. The base compositions of rapidly labeled RNA extracted from thymine starved and unstarved cultures were similar, indicating that no extensive alteration occurred

during thymineless death (Sicard, Simonnet, and Astrachan, 1967).

Evidence supporting the involvement of protein synthesis in the lethal event during thymine starvation was presented by several investigators. Lark and Lark (1964) and Lark and Lark (1966) suggested that at least two types of proteins affected the course of thymineless death. These investigators postulated that a structural protein, required for the initiation of a new round of DNA replication, did not accumulate in the absence of thymine since synthesis of this regulatory protein depended upon the completion of a round of chromosome replication which could not occur when DNA synthesis was blocked. Lark and Lark (1964) suggested that normal DNA synthesis terminated upon the synthesis of a new structural site which could serve as the point of initiation for the replication of a new daughter chromosome. These results were in agreement with the model of replication proposed by Jacob, Brenner, and Cuzin (1963) that was based on studies of chromosome and episome replication and included a structural site for replication that might be a part of the cell membrane. The second protein involved in replication, the initiator, was believed to be required for the activation of replication at the structural site (Lark and Lark, 1964). A requirement of protein synthesis for the initiation of new replication was proposed by Maaloe and Hanawalt (1961) and Hanawalt et. al. (1961) who found that, in the absence of arginine and the presence of thymine, E. coli 15TAU synthesized the amount of DNA equivalent to the completion of one round of replication. The addition of

arginine was essential for initiation of a new round of replication.

Cummings and Kusy (1969) demonstrated that the addition of chloramphenicol and thymine after thymineless death led to a rapid and almost complete recovery of E. coli B strains. The addition of low concentrations (10-20 $\mu\text{g/ml}$) of chloramphenicol during thymine starvation led to the survival of a fraction of the population, while concentrations of chloramphenicol greater than 25 $\mu\text{g/ml}$ caused increased sensitivity to thymine starvation. The recovery data showed that a certain combination of thymine and chloramphenicol was essential for protection which these investigators interpreted as an indication of the induction of a substance necessary for thymineless death. Thus, the addition of thymine and chloramphenicol stopped the induction, and the concomitant inhibition of protein synthesis caused the depletion of the inhibitory substance. This induction mechanism agreed with the "internal inducer" hypothesis proposed by Goldthwaite and Jacob (1964) and Sicard and Devoret (1962).

Thymineless death in limiting concentrations of thymine was studied by Deutch and Pauling (1971). Their data indicated that, as the concentration of thymine was decreased to less than 0.5 $\mu\text{g/ml}$, protein and RNA synthesis was inhibited. At these concentrations of thymine, the cells first began to lose colony-forming ability. These results were interpreted as an indication that RNA and protein macromolecules were involved in the lethal event.

One of the earliest indications that thymine starvation resulted in damage to bacterial DNA was the demonstration by Coughlin and Adelberg (1956) that the number of his⁺ revertants in E. coli 15T⁻ his⁻ increased during thymine starvation. Barner, Cohen, and Kanazir (1958) also observed a high mutagenic rate during thymine starvation. McFall and Magasanik (1962) were the first to suggest that periods of thymine starvation could damage DNA. From experiments with Bacillus subtilis 168, Mennigmann and Szybalski (1962) found that thymine starvation resulted in an increased sensitivity to shear, decreased viscosity, and partial loss of transforming activity of the DNA. They suggested that such changes were the result of single strand breaks due to action similar to that of Exonuclease II on native transforming DNA.

Other types of damage to bacterial DNA were also observed in early experiments on thymine starvation. Gold and Hurwitz (1963) suggested that excessive methylation of the bacterial DNA of E. coli 15T⁻ resulted in the impairment of DNA synthesis. Pritchard and Lark (1964) found that E. coli 15T⁻ initiated replication prematurely at the chromosome origin during thymine starvation. However, the presence of this second replicating point per se was not lethal, since replication returned to normal after one generation. Nonconservative replication was observed by Pauling and Hanawalt (1965) to occur in a small fraction of the cellular DNA after thymine starvation. Schaiberger, Giegel, and Sallman (1967) found DNA polymerase activity was unchanged by thymine starvation and purified DNA from a thymine-requiring mutant showed

an increased resistance to nucleolytic enzymes. Green, Douch, and Greenberg (1970) showed that a rec A strain of E. coli, which was sensitive to nalidixic acid and fluorodeoxyuridine, was resistant to thymine starvation. Rec A strains degraded considerable amounts of DNA spontaneously, which led to the suggestion by these authors that degradation resulted in the release of nucleotides which, in turn, allowed DNA synthesis to proceed at a level essential for survival.

Damage to the chromosome during thymine starvation was observed in Bacillus subtilis. Decreased transforming activity of DNA from B. subtilis 168T⁻ was not due to abnormal penetration of the host cell (Sicard, 1969) which suggested a failure, after uptake, of the DNA to undergo genetic recombination. A thyme-requiring mutant of B. subtilis lost 10-15% of its DNA during 4 hours of thymine starvation (Reiter and Ramareddy, 1970). This degradation appeared to be initiated at the replication point and to proceed away from the replication point, since the amount of label released was inversely proportional to the length of the chromosome labeled.

The first definitive experiments suggesting damage to DNA during thymine starvation were performed by Freifelder (1969) using a thymine-requiring strain of E. coli harboring the F'lac plasmid. Experiments using zone centrifugation in alkaline sucrose gradients showed that the circular plasmid was lost at a constant rate during thymine starvation. Omission of glucose or phosphate from the minimal medium lacking thymine prevented both thymineless death and loss of the plasmid. Freifelder (1969) suggested that single strand breaks

were a normal process and that thymine starvation resulted in the production of a substance which inhibited the bacterial ligase, thus preventing repair of the DNA. More recently Freifelder and Katz (1971) found that Okazaki fragments accumulated during thymine starvation of the thymine-requiring mutant, E. coli DF3. However, covalently closed circles of phage λ could still be found in starved cells of E. coli DF3 lysogenic for phage λ . This led Freifelder and Katz (1971) to suggest that only very low levels of DNA ligase activity were required for λ conversion. Recently endonuclease activity was demonstrated by Freifelder and Levine (1972) to be present in extracts of thymine starved cells of E. coli K12-DF4, but not detected in the extracts of unstarved cells. DNA ligase activity was unaffected by thymine deprivation. Freifelder and Levine (1972) postulated that the endonuclease activity during thymine starvation was responsible for damage to the DNA in contrast to the earlier hypothesis (Freifelder and Katz, 1971) that damage was due to the inhibition of ligase activity. Reichenbach, Schaiberger, and Sallman (1971) showed that E. coli JG151, a strain of E. coli 15T⁻ cured of the col 15 plasmid, could recover colony-forming ability during growth under thymine starvation at a certain ratio of culture volume to flask volume (0.4) but not at another ratio (0.08). During thymine starvation, extracted DNA showed a decreasing sedimentation velocity. DNA of cells incubated in cultural conditions leading to the spontaneous recovery of viability showed an increase in sedimentation velocity approaching the value obtained for unstarved cells. It was

suggested that single strand breaks occurred. However, the damage was not irreversible, since the average number of breaks decreased during the recovery phase indicating repair.

In spite of the evidence for damage to DNA during thymine starvation, several investigators have suggested that extensive damage to the DNA does not occur. Luzzati and Revel (1962) found that all of the physical and chemical parameters of DNA isolated from thymine starved cells such as viscosity, molecular weight, density in cesium chloride, melting curves, and sedimentation constants of native and denatured DNA were identical with the corresponding values from unstarved cells. Extensive fragmentation of thymine starved DNA was not observed during measurements of viscosity and sedimentation designed to detect single strand breaks and interstrand cross-links (Smith and Burton, 1965). Recently Baker and Hewitt (1971) studied the effects of thymine starvation on single strand DNA isolated from E. coli C thy-321. The molecular weight of the DNA was determined by sedimentation through alkaline sucrose gradients. Growth in minimal medium lacking thymine for up to 150 min did not reduce the molecular weight of the extracted DNA below the value of $2.4 \pm 0.3 \times 10^8$ daltons obtained for unstarved DNA. Incubation of cells of this strain in minimal medium lacking thymine following their exposure to ultraviolet light or gamma rays did not appear to block the rejoining of single strand breaks associated with irradiation. Baker and Hewitt (1971) concluded that thymine starvation did not significantly inhibit DNA repair enzymes such as DNA ligase.

Elucidation of the mechanism of thymineless death is of interest since the lethal event may be an important response of cells to folic acid antagonists (Cohen, 1971). Such studies could increase the chemotherapeutic efficacy of these drugs. Reduced rates of DNA synthesis are characteristic of some pathological states such as megaloblastic anemia as well as some processes of normal cellular differentiation. In addition, folate antagonists have been used effectively in the treatment of some types of tumor growth, childhood leukemia, bacterial and protozoal infections as well as to suppress the immune response in autoimmune diseases and tissue grafts (discussed in Cohen, 1971). Most of these antagonists react with dihydrofolate reductase. Chemotherapy has been made effective by adjusting the dose schedule and using leucovorin (N^5 -formyltetrahydrofolate, citrovorum factor) to rescue normal cells (Bertino *et. al.*, 1971). Amethopterin (Methotrexate) inhibits dihydrofolate reductase and kills proliferating cells more rapidly than non-proliferating cells (Cohen, 1971). Aminopterin, on the other hand, inhibits both thymidylate synthetase and dihydrofolate reductase and, in addition, produces chromosome breaks. The chemotherapeutic efficacies of these drugs were ascribed to their inhibition of dihydrofolate reductase or thymidylate synthetase that resulted in thymineless death of animal cells (Cohen, 1971). Thymineless death may result from chemotherapeutic use of the antitumor agent, 5-fluorouracil, since this drug inhibited DNA synthesis in animal cells (Heidelberger *et. al.*, 1957) and invoked thymineless death in thymine-independent strains of E. coli

(Cohen et. al., 1958). Cohen and coauthors also found that the deoxyribonucleotide of 5-fluorouracil was a potent inhibitor of bacterial thymidylate synthetase.

In conclusion, investigators have been interested in describing the lethal effect of thymine starvation in bacteria as a method of studying chromosome replication and other aspects of macromolecular synthesis and regulation. Similar studies in animal cells may result in the development of folate antagonists with increased chemotherapeutic efficacy.

CHAPTER III

MATERIALS AND METHODS

1. Bacterial Strains

Thymine-requiring mutants referred to in this thesis were derivatives of Escherichia coli K12 Y mel. Relevant genetic markers are presented in Tables 1 and 2. E. coli B9 and E. coli B27 were isolated by I. P. Crawford as trp B deletion mutants of E. coli Y mel (I. P. Crawford, personal communication, 1970). A genetic map of these deletions is presented in Fig. 1. Thymine-requiring (T^-) derivatives of E. coli B9 and E. coli B27 were obtained using trimethoprim (Stacey and Simson, 1965) and penicillin selection (Gorini and Kaufman, 1960). E. coli Y mel T^- was isolated using the same technique, but penicillin selection was not applied. Following initial isolation, mutants of E. coli Y mel T^- , E. coli B9 T^- and E. coli B27 T^- were selected which required low concentrations (2 to 5 μ g/ml) of thymine for aerobic growth on minimal agar plates. This was accomplished by successive streaking on minimal agar supplemented with 40, 20, 10 and finally 5 μ g of thymine/ml. Isolated colonies obtained on the latter medium were restreaked twice on the same medium and on minimal agar containing no thymine in order to insure purity and to check for thy⁺ revertants of the strains.

E. coli B307c and E. coli B840c were isolated by F. Kaudewitz and were obtained from G. R. Greenberg. E. coli ara B24/F'ara B24 (strain 306) was isolated by D. Sheppard and was also obtained from G. R. Greenberg. Bacteriophage

Table 1. Bacterial strains derived from E. coli Y mel

<u>Escherichia coli</u> Strain	Thy ^a	TdR ^b	TdR Sensitivity ^c	DeoA ^d	DeoB ^e	DeoC ^f	Phage λ ^g
K12 Y mel	+	+		+	+	+	R
B27	+	+		+	+	+	R
B9	+	+		+	+	+	R
Y melT ⁻	-	-	R				R
B27T ⁻	-	-	R				R
B9T ⁻	-	-	S				R
B9T ⁺	+	-	S				R
B9T ⁻ Trp ⁺	-	-	S				R
B27T ⁺	+	-	R				R
B27T ⁻ Trp ⁺	-	-	R				R
B27 ⁻ Thr	-	-					R
B27T ⁻ Thr ⁻ /F'ara B24	-	-					R
B27T ⁻ /F'ara B24	-	+					R
B9T ⁻ /F'ColVBM-K260, trp	-	-					R
B27T ⁻ /F'ColVBM-K260, trp	-	-					R

a. Ability (+) to grow in the absence of exogenous thymine.

b. The ability to grow on thymidine as a sole source of carbon.

c. Sensitivity (S) or resistance (R) to a high concentration of thymidine.

d., e., f. Presence (+) or absence (-) of enzymes of the deoxyribonucleoside region: Thymidine phosphorylase (deoA, tpp), deoxyribomutase (deoB, drm), deoxyriboaldolase (deoC, dra).

g. Sensitivity (S) or resistance (R) of bacterial strain to infection by phage λ .

Table 1 (cont.)

<u>Escherichia coli</u> Strain	Trp	Thr	Leu	Pro	His	Arg	Met	Lys	Ser	Thi	Lac	Ara
K12 Y mel	+	+	+	+	+	+	+	+	+	+	+	+
B27	-	+	+	+	+	+	+	+	+	+	-	+
B9	-	+	+	+	+	+	+	+	+	+	-	+
Y melT ⁻	+	+	+	+	+	+	+	+	+	+	+	+
B27T ⁻	-	+	+	+	+	+	+	+	+	+	-	+
B9T ⁻	-	+	+	+	+	+	+	+	+	+	-	+
B9T ⁺	-	+	+	+	+	+	+	+	+	+	-	+
B9T ⁻ Trp ⁺	+	+	+	+	+	+	+	+	+	+	-	+
B27T ⁺	-	+	+	+	+	+	+	+	+	+	-	+
B27T ⁻ Trp ⁺	+	+	+	+	+	+	+	+	+	+	-	+
B27T ⁻ Thr ⁻	-	-	+	+	+	+	+	+	+	+	-	+
B27T ⁻ Thr ⁻ /F'ara B24	-	+	+	+	+	+	+	+	+	+	-	+
B27T ⁻ /F'ara B24	-	+	+	+	+	+	+	+	+	+	-	+
B9T ⁻ /F'ColVBM-K260, trp	+	+	+	+	+	+	+	+	+	+	-	+
B27T ⁻ /F'ColVBM-K260, trp	+	+	+	+	+	+	+	+	+	+	-	+

+, represents the ability (-, inability) to synthesize an amino acid or vitamin or the ability (-, inability) to utilize a sugar. Trp, tryptophan; Thr, threonine; Leu, leucine; Pro, proline; His, histidine; Arg, arginine; Met, methionine; Lys, lysine; Ser, serine; Thi, thiamine; Lac, lactose; Ara, arabinose.

Table 1 (cont.)

<u>Escherichia coli</u> Strain	Str ^a	ColVBM ^b	Sex ^c	Origin
K12 Y mel	S	-	F ⁻	K12
B27	R	-	F ⁻	K12 Y mel
B9	S	-	F ⁻	K12 Y mel
Y melT ⁻	S	-	F ⁻	Y mel
B27T ⁻	R	-	F ⁻	B27
B9T ⁻	S	-	F ⁻	B9
B9T ⁺	S	-	F ⁻	B9T ⁻
B9T ⁻ Trp ⁺	S	-	F ⁻	B9T ⁻
B27T ⁺	R	-	F ⁻	B27T ⁻
B27T ⁻ Trp ⁺	R	-	F ⁻	B27T ⁻
B27T ⁻ Thr ⁻	R	-	F ⁻	B27T ⁻
B27T ⁻ Thr ⁻ /F'ara B24	R	-	F'	B27T ⁻ Thr ⁻ x ara B24/F'ara B24
B27 ⁻ /F'ara B24	R	-	F'	B27T ⁻ x ara B24/F'ara B24
B9T ⁻ /F'ColVBM-K260, trp	S	+	F'	B9T ⁻ x JC411T ⁻ /F'ColVBM-K260
B27T ⁻ /F'ColVBM-K260, trp	S	+	F'	B27T ⁻ x JC411T ⁻ /F'ColVBM-K260

a. Str, strptomycin; Sensitivity, (S) or Resistance (R).

b. Presence (+) or absence (-) of determinants for the production of colicins V, B, and M. r, resistance to colicin.

c. F', presence of plasmid possessing male determinants in addition to other genetic markers. F⁻, female strain.

Table 2. Supplemental bacterial strains used

<u>Escherichia coli</u> Strain	Thy ^a	TdR ^b	TdR Sensitivity ^c	DeoA ^d	DeoB ^e	DeoC ^f	Phage λ ^g
B307c	-	-	R	+	-	+	S
B840c	-	-	S	+	+	-	S
ara B24/F'ara B24 (Strain 306)	+	+		+	+	+	S
JC411T ⁻ /F'ColVBM- K260, trp	-	-		+	+	+	S
C600	+						S
AB151	+						R
AB259	+						S
YS40/EB	+						R
YS40/V	+						R
RG274	+						R
15T	-	-					

- a. Ability (+) to grow in the absence of exogenous thymine.
b. The ability to grow on thymidine as a sole source of carbon.
c. Sensitivity (S) or resistance (R) to a high concentration of thymidine.
d., e., f. Presence (+) or absence (-) of enzymes of the deoxyribonucleoside region:
Thymidine phosphorylase (deoA, tpp), deoxyribomutase (deoB, drm), deoxyriboaldolase
(deoC, dra).
g. Sensitivity (S) or resistance (R) of bacterial strain to infection by phage λ .

Table 2 (cont.)

<u>Escherichia coli</u> Strain	Trp	Thr	Leu	Pro	His	Arg	Met	Lys	Ser	Thi	Lac	Ara
B307c	+	+	+	+	+	+	+	+	+	+	+	+
B840c	+	+	+	+	+	+	+	+	+	+	+	+
ara B24/F'ara B24 (Strain 306)	+	+	+	+	+	+	+	+	+	+	+	-
JC411 T/F'ColVBM- K260, trp	+	+	-	+	-	-	-	+	+	+	-	+
C600	+	-	-	+	+	+	+	+	+	-	+	+
AB151	+	-	-	-	-	+	+	+	+	-	-	+
AB259	+	+	+	+	+	+	+	+	+	-	+	-
YS40/EB	+	+	+	-	-	+	+	+	+	+	-	
YS40/V	+	+	+	-	-	+	+	+	+	+		
RG274	+	+	+	+	-	-	+	+	+	+		
15T												

+, represents the ability (-, inability) to synthesize an amino acid or vitamin or the ability (-, inability) to utilize a sugar. Trp, tryptophan; Thr, threonine; Leu, leucine; Pro, proline; His, histidine; Arg, arginine; Met, methionine; Lys, lysine; Ser, serine; Thi, thiamine; Lac, lactose; Ara, arabinose.

Table 2 (cont.)

<u>Escherichia coli</u> Strain	Str ^a	ColVBM ^b	Sex ^c	Origin
B307c	S	-	F ⁻	B
B840c	S	-	F ⁻	B
ara B24/F'ara B24 (Strain 306)	S	-	F'	
JC411T ⁻ /F'ColVBM- K260, trp	R	+	F'	JC411 x <u>P. mirabilis</u> T ⁻ /F'ColVBM- K260
C600	R	-	F ⁻	
AB151	R	-	F ⁻	
AB259	S	-	F ⁻	
YS40/EB	S	ColB ^r	F ⁻	YS40/E
YS40/V	S	ColV ^r	F ⁻	YS40
RG274		-	F ⁻	
15T ⁻		-	F'	15

a. Str, strptomycin; Sensitivity, (S) or Resistance (R).

b. Presence (+) or absence (-) of determinants for the production of colicins V, B, and M. r, resistance to colicin.

c. F', presence of plasmid possessing male determinants in addition to other genetic markers. F⁻, female strain.

Fig. 1. Genetic map indicating the region of the tryptophan deletion in E. coli B9 and E. coli B27.

A tryptophan synthetase A
B tryptophan synthetase B
C indoleglycerolphosphate synthetase
CDRP 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate
CYS B cysteine biosynthesis
D phosphoribosyltransferase
E anthranilate synthetase
O tryptophan operator
P tryptophan promoter
PRA phosphoribosylanthranilate
TON B phage T-one resistance/sensitivity
TRP tryptophan

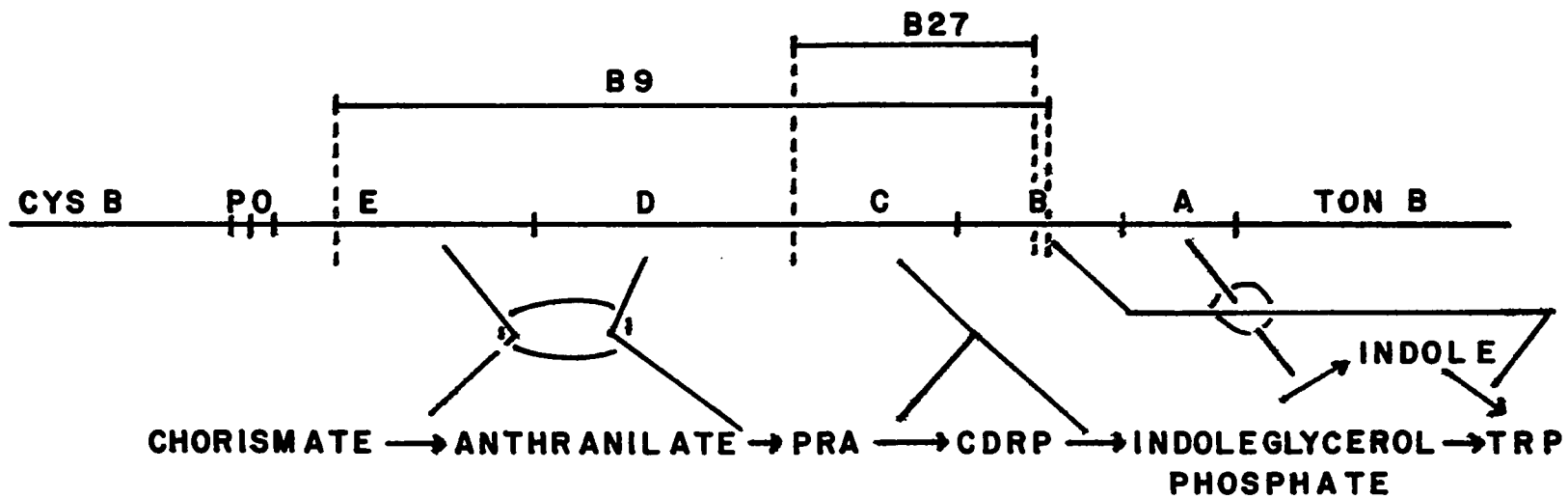


Fig. 1. Genetic Map of the Tryptophan Deletion Mutants *E. coli* B27 and *E. coli* B9^a.

a. I. P. Crawford personal communication, 1970.

Plkc and Shigella dysenteriae strain SH-16 were obtained from C. Yanofsky. E. coli strains Y mel, C600, AB151, AB259, AB1157, YS40/EB, YS40/V and JC411T⁻/F'ColVBM-K260, trp were obtained from D. R. Helinski. E. coli RG274 was obtained from W. K. Maas, and E. coli 15T⁻ was obtained from G. Medoff.

2. Maintenance of Stock Cultures

E. coli cultures were maintained at 4 C on Enriched Nutrient Agar slants and were transferred every two months. Shigella dysenteriae strain SH-16 was maintained at room temperature on L agar slants and was transferred weekly.

3. Cultural Media

a. Enriched Nutrient Broth (ENB) contained Difco nutrient broth, 8.0 g; KH_2PO_4 , 1.5 g; Na_2HPO_4 , 3.5 g; NaCl, 5.0 g; dextrose, 1.0 g; and Bacto-peptone, 5.0 g per liter distilled water. Difco agar (1.5%) was added to make Enriched Nutrient Agar.

b. Minimal medium lacking thymine was described by Vogel and Bonner (1956) and contained per liter sodium citrate (10%, W/V), 5.0 ml; Bacto-casamino acids (technical), 5.0g; concentrated inorganic salts (25x), 40.0 ml; dextrose (40%), 12.5 ml; and L-tryptophan (2 mg/ml), 10.0 ml. Minimal medium containing thymine was prepared by the addition of 2.5 ml of thymine (2 mg/ml) to the above medium. Concentrated inorganic salts (25x) consisted of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g; citric acid $\cdot \text{H}_2\text{O}$, 50.0 g; K_2HPO_4 (anhydrous), 250.0 g; $\text{Na}(\text{NH}_4)\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 87.5 g/l distilled water. The concentrated inorganic salts, dextrose, thymine and tryptophan solutions were prepared

separately, autoclaved, and added to the remaining medium following autoclaving. The pH following sterilization was 7.1. To make plates of the above media, 1.5% agar was added.

c. Penassay broth was Difco antibiotic medium number 3.

d. L broth (Luria et. al., 1960) consisted of tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 5.0 g; and dextrose, 1.0 g per liter distilled water. The pH was adjusted to 7.0 using 10N NaOH. Agar (10 g/l) was added when required for making L-bottom agar. Following autoclaving 5.0 ml of sterile CaCl_2 (0.5M) was added per liter. L top agar contained 5 g agar per liter.

4. Chemicals

Tris (hydroxymethyl) aminomethane (Trizma base, reagent grade); 2-mercaptoethanol; dl, L-tetrahydrofolic acid (grade III); albumin, bovine (Fraction V); 2'-deoxyuridine 5'-monophosphoric acid, sodium salt; maleic acid; thymidine; uridine; DL-pantoyl lactone; deoxyadenosine; and 6-azauracil were obtained from Sigma Chemical Company. Penicillin G, potassium salt, B grade; cytosine; 5-methylcytosine; thymidine-5'-monophosphate sodium $\cdot 3\frac{1}{2}\text{H}_2\text{O}$, A grade; orotic acid $\cdot \text{H}_2\text{O}$; and folic acid were obtained from Calbiochem. L-tryptophan; disodium ethylenediaminetetraacetate, certified A.C.S.; formaldehyde; and MgCl_2 were obtained from Fisher Scientific Co. Uracil, guanine $\cdot 2\text{H}_2\text{O}$, adenine sulfate $\cdot 2\text{H}_2\text{O}$, and D,L serine were obtained from Eastman. Guanosine $\cdot 2\text{H}_2\text{O}$, cytidine (hemi) sulfate, 5-hydroxymethylcytosine, 2-aminopurine (nitrate), and 6-amino-thiouracil were obtained from California Corporation for

Biochemical Research. Calcium pantothenate (dextrorotatory) was obtained from Nutritional Biochemical Corporation. Thymine was purchased from K&K Laboratories, Inc. N-methyl-N'-nitro-N-nitrosoguanidine was purchased from Aldrich Chemical Co., Inc. Ribonuclease was purchased from Worthington Biochemical Corporation. Thymidine-methyl-C¹⁴ was purchased from New England Nuclear. Trimethoprim was a gift of Burroughs Wellcome Co.

5. Method for the Isolation of Thymine-Requiring Mutants

Trimethoprim [2,4 diamino-5 (3', 4', 5'-trimethoxy) benzyl pyrimidine] selection was used to obtain thymine-requiring mutants from a predominantly thymine-independent population (Stacey and Simson, 1965). Since thymine-independent cells cannot utilize appreciable amounts of exogenous thymine for DNA synthesis (Mantsavinos and Zamenhof, 1961; Crawford, 1958), cells grown in the presence of trimethoprim and thymine continue to attempt to synthesize thymine using the tetrahydrofolate pathway. However, since trimethoprim inhibits dihydrofolate reductase which converts dihydrofolic acid to tetrahydrofolic acid, the synthesis of thymine ceases and the thymine-independent cells die from lack of thymine. On the other hand, the spontaneous thymine-requiring mutants in the population fail to synthesize thymine through the tetrahydrofolate pathway, but utilize the exogenous thymine in the medium. These mutants thus become the dominant viable population.

Prototrophic cells were grown 18 hr with aeration in minimal broth containing thymine at 37 C. Four tubes were prepared as follows:

- a. minimal broth, 10 ml; thymine, 60 μ g/ml.
- b. minimal broth, 10 ml; thymine, 60 μ g/ml; 18 hr culture, 0.2 ml.
- c. minimal broth, 10 ml; trimethoprim, 10 μ g/ml; 18 hr culture, 0.2 ml.
- d. minimal broth, 10 ml; trimethoprim, 10 μ g/ml; thymine, 60 μ g/ml; 18 hr culture, 0.2 ml.

The cultures were incubated statically for 96 hours at 37 C at which time the turbidity of the tube d. was greater than that of tube c. This was due to the development of the thymine-requiring population and subsequent growth of these mutants on the exogenously supplied thymine. Cells from tube d. were then spread on minimal agar plates containing 50 μ g of thymine per ml, and the plates were incubated at 37 C. Additional selective pressure was required for the isolation of thymine-requiring mutants of E. coli B27 and E. coli B9. Thus following exposure to trimethoprim, cells of these strains from tube d. were treated with penicillin (see method for penicillin selection). Thymine-requiring mutants so isolated require high concentrations of thymine (> 20 μ g/ml). Thymine mutants which require low concentrations (2-5 μ g/ml) of thymine result from a second mutation which occurs as a spontaneous event (Lomax and Greenberg, 1968). These were isolated following successive streaking on minimal agar plates

supplemented with decreasing concentrations (50, then 20, then 10, and finally 5 μ g/ml) of thymine.

6. Method for Penicillin Selection

The method of selecting auxotrophic mutants through penicillin selection was that described by Gorini and Kaufman (1960). Complete minimal broth was inoculated from a stock ENB slant, and the culture was grown at 37 C with aeration for 18 hr. One ml of the 18-hr culture was used to seed 50.0 ml of fresh complete minimal broth contained in a 125 ml Erlenmeyer flask. The culture was then shaken at 37 C until a concentration of 2×10^8 cells/ml was attained. Ten ml of these cells were pelleted by centrifugation for 30 sec at 27,000 x g at 4 C. The pellet was washed once by resuspension in 10.0 ml of sterile saline, again pelleted by centrifugation and finally resuspended in 10.0 ml of sterile saline. This saline-culture was shaken at 37 C for 30 min, and the cells were then pelleted by centrifugation under the same conditions as before. The pellet was then resuspended in 9.0 ml of minimal broth containing 20% sucrose but lacking the compound for which the auxotrophic mutant was being selected (e.g. thymine). The culture was shaken 3.0 hr at 37 C during which time the culture grew several generations. One ml of penicillin was then added to give a final concentration of 2000 units/ml, and the culture was incubated statically for an additional 60 min at 37 C. Following the incubation with penicillin, the culture was chilled in ice and the cells were pelleted by centrifugation for 30 sec at 27,000 x g at 4 C. The pellet was resuspended in 10.0 ml of sterile water to lyse the osmotically unstable cells. Again the cells were

pelleted by centrifugation as above and finally resuspended in 10.0 ml of sterile saline. Cultures were then diluted and spread-plated on complete minimal agar so as to obtain individual colonies. Plates were incubated at 37 C. Mutant colonies could then be isolated by replica plating (Lederberg and Lederberg, 1952) on complete minimal agar and on minimal agar complete except for the compound for which mutants were being selected (e.g. thymine). Isolated mutant colonies which grew on the plates containing thymine but not on plates containing no thymine were streaked twice on complete minimal agar to insure purity and stability.

7. Method for Mutagenesis with Nitrosoguanidine

The method described by Adelberg et. al. (1965) for the selection of bacterial mutants by treatment with N-methyl-N'-nitro-N-nitrosoguanidine was employed for the isolation of certain auxotrophic strains. One ml of an 18-hr culture in Enriched Nutrient Broth was used to inoculate 50 ml of fresh ENB. The culture was grown to 2×10^8 cells/ml by shaking at 200 R.P.M. in a waterbath at 37 C. Cells from 10.0 ml of this culture were collected on a Millipore filter membrane (pore size $0.45 \mu\text{m}$). Cells were washed once by passing 10.0 ml of sterile tris-maleic acid buffer 0.5 M tris (hydroxymethyl) aminomethane and 0.05 M maleic acid, pH 6.0 through the filter and resuspended in 9.0 ml of sterile tris-maleic acid buffer by agitating the membrane plus buffer in a 100 ml sterile beaker on a Vortex Junior Mixer. The cell suspension was then pipetted into a sterile 125 ml Erlenmeyer flask covered with aluminum foil for protection from light. One ml of

nitrosoguanidine (1.0 mg/ml) was added to give a final concentration of 100 μ g/ml. Next, the culture was shaken at 200 R.P.M. in a waterbath for 30 min at 37 C. Cells from 5.0 ml of nitrosoguanidine-treated culture were collected by membrane filtration and were washed once by passing 10.0 ml of ice-cold minimal broth through the filter. Cells were resuspended in 10.0 ml of minimal broth by agitating the filter plus the broth in a 100 ml beaker on a Vortex Junior Mixer. The cell suspension was placed in a test tube (10 x 170 mm), and shaken for 4 hr in a waterbath at 37 C to allow for segregation and phenotypic expression. Following incubation, the cells were subjected to penicillin selection (see above) and plated by the dilution-spread plate technique on complete minimal agar so as to obtain individual colonies. Plates were incubated at 37 C. Mutant colonies could then be isolated by replica plating (Lederberg and Lederberg, 1952) on complete minimal agar and on minimal agar complete except for the compound for which mutants were being selected. Mutant colonies were streaked twice on complete minimal agar to ensure purity and stability.

8. Cultural Conditions for Growth Experiments

Experiments to determine the turbidity and the viable cell count were carried out by the following procedure: Isolated colonies were inoculated into 5.0 ml of minimal broth containing thymine. Cultures were then shaken in a reciprocal waterbath for 18 hr at 37 C. One ml of the 18-hr culture was used to seed 100 ml of fresh minimal medium containing thymine contained in a sterile 250 ml Erlenmeyer

Klett sidearm flask. Cultures were then incubated in a Metabolyte rotary shaking waterbath (New Brunswick Scientific Co., In., N. J.) at 200 R.P.M. and 37 C. Turbidity increases were followed using a Klett-Sommerson colorimeter equipped with a number 54 (green) filter. Cultures were allowed to attain a cell density equivalent to 60 Klett units ($1-2 \times 10^8$ cells/ml). Cells from 50 ml of this early logarithmic phase culture were then washed by one of the two following methods. Method I: The culture (50 ml) was chilled in ice, and the cells were pelleted by centrifugation at 16,000 x g at 4 C for 5 min. The cell pack was washed once by resuspension in 50 ml of minimal broth lacking thymine and dextrose and recentrifugation. Following the second centrifugation, the cells were resuspended in 50 ml of minimal broth lacking thymine that had previously been equilibrated to 37 C. Method II: Culture (50 ml) was immediately centrifuged at room temperature (25-30 C) at 16,000 x g for 5 min. Cell pack was washed once at room temperature by resuspension in 50 ml of minimal broth lacking thymine and dextrose. Cells were again pelleted by centrifugation at 16,000 x g at room temperature for 5 min. Finally, cells were resuspended in 50 ml of minimal broth lacking thymine previously equilibrated to 37 C. Method II was employed for all growth experiments unless otherwise stated. The cell suspension from each method was then placed in a 250 ml Erlenmeyer Klett sidearm flask and shaken at 200 R.P.M. in a rotary waterbath at 37 C. Viable cell counts were determined by serially diluting a portion of the culture in minimal broth lacking dextrose, and isolated

colonies were obtained for enumeration on Enriched Nutrient Agar plates by the spread plate technique. Plates were incubated 24 hours at 37 C, and the colonies were counted.

9. Effect of E. coli B27T⁻ Supernatant Medium on Growth of Thymine-Requiring Mutants

The culture used to prepare supernatant medium was grown to early logarithmic phase (see section on cultural conditions for growth experiments) in minimal medium containing thymine. Cells were removed by centrifugation at 10,000 x g at 4 C for 10 min, and the supernatant fluid was sterilized by Millipore membrane filtration (pore size 0.45 μ m). Fresh nutrients were added to the sterile supernatant medium to yield final concentrations equivalent to those of the original minimal broth. This was accomplished by adding sterile inorganic salts (25x), 4.0 ml; dextrose (40%), 1.25 ml; tryptophan (2mg/ml), 1.0 ml; and casamino acids (125 mg/ml), 4.0 ml to 100 ml sterile supernatant medium. Supplemented supernatant medium was then streaked on ENB agar as a sterility test.

Experiments were performed using the supplemented supernatant medium in order to determine whether a thymine-requiring strain normally susceptible to thymine starvation (e.g. E. coli Y melT⁻) would be protected from starvation by protective substances contained in the supplemented supernatant medium. Cultures to be tested were grown to 2×10^8 cells/ml in 100 ml of minimal broth containing thymine in a rotary-shaking waterbath (200 R.P.M.) at 37 C. The logarithmic phase cultures (50 ml) were then centrifuged for 5 min at

16,000 x g at room temperature after which the pellets were resuspended in 50 ml of room temperature minimal broth lacking thymine and dextrose. These cells were then recentrifuged and resuspended in 50 ml of supplemented supernatant medium equilibrated to 37 C. The cultures were transferred to 250 ml Erlenmeyer Klett sidearm flasks and were shaken at 200 R.P.M. in a 37 C waterbath. Growth was measured by viable cell counts and turbidity as described in section 8, Cultural Conditions for Growth Experiments.

10. Colony-Forming Ability of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ on Agar Plates Containing Various Concentrations of Thymine

The ability of thymine-requiring mutants to grow on minimal agar plates supplemented with various concentrations of thymine was measured as the percentage of the population which was capable of forming colonies as compared with the maximum colonial population which arose on minimal agar plates containing 50 μ g of thymine/ml. Isolated colonies of E. coli Y melT⁻, E. coli B9T⁻ and E. coli B27T⁻ were inoculated into minimal broth containing thymine and grown to a cell concentration of 2×10^8 cells/ml (as described in section 8). Cells (10 ml) were washed once at room temperature by centrifugation at 27,000 x g for 30 sec, and then resuspended in 10 ml sterile room temperature saline. Cells were again centrifuged, resuspended in 10 ml sterile room temperature saline and serially diluted in sterile saline to a concentration of approximately 2×10^3 colony-forming units/ml. One-tenth of a ml of the diluted suspension was then spread on duplicate minimal

agar plates supplemented with various concentrations of thymine. Enriched Nutrient Broth (ENB) agar plates were spread at the same time in order to obtain the total viable cell population. Plates were incubated at 37 C for 2-5 days and then counted. Equivalent counts were obtained on ENB and thymine (50 μ g/ml) plates. The counts obtained on the latter medium were allowed to equal 100%.

11. Conjugation of Bacterial Strains

Strains to be crossed by conjugation were first streaked on ENB agar plates and incubated at 37 C until isolated colonies appeared. A colony was then picked and inoculated into 5.0 ml of penassay broth. The culture was shaken in a reciprocal shaking waterbath at 37 C for 18 hr. One ml of the 18 hr culture was used to seed 50 ml of penassay broth contained in a 125 ml Erlenmeyer flask. The donor and recipient cultures were then grown until early logarithmic phase (2×10^8 cells/ml) in a reciprocal shaking waterbath at 37 C. The donor (0.2 ml) and the recipient (0.6 ml) cultures were then mixed in 10 ml of fresh penassay broth. Conjugation was allowed to proceed for 2 hr with slow shaking (setting 5.5) in a Warner-Chilcott reciprocal waterbath at 37 C. When the percentage of plasmids being transferred to the recipient strain was low, longer mating times were employed. Following conjugation, cultures were chilled in ice, and the cells were pelleted by centrifugation at 27,000 x g at 4 C for 30 sec. The pellet was washed once by resuspension in 10 ml sterile, room temperature saline, centrifuged again and finally resuspended in 10 ml sterile saline. Recipient cells were isolated

as individual colonies by the dilution-spread plate technique using minimal agar which was selective against the donor strain (e.g. streptomycin). Recipient cells were tested for relevant genetic markers characteristic of the plasmid involved, such as the Col V or Col B markers of the F'ColVBM-K260, trp plasmid or the thr⁺ marker of the F'araB24 plasmid.

12. Introduction of the F'araB24 Plasmid into E. coli B27T⁻.

Several methods of conjugation were employed to form the merodiploid, E. coli B27T⁻/F'araB24. All of these employed E. coli araB24/F'araB24 (strain 306) as the donor and E. coli B27T⁻ as the recipient. Initially, the donor and recipient colonies were differentiated on the basis of their colony size on minimal agar plates containing thymine (50 μ g/ml) and tryptophan (20 μ g/ml) but substituting L-arabinose (0.5%) for dextrose as a sole carbon source. Colonies of E. coli B27T⁻ (ara⁺) were larger in diameter than those of E. coli araB24/F'araB24 (ara⁻/ara⁻). This procedure failed since, following conjugation, only a low proportion (< 1%) of the recipients were ara⁺, and, of these, none were capable of utilizing thymidine as a sole carbon source. Also, the F'araB24 plasmid was not transferred by this method to either E. coli B307c or E. coli B840c. Thus, other selection procedures were employed in an attempt to increase the probability of isolating merodiploid strains.

One such procedure employed a threonine-requiring mutant of E. coli B27T⁻ isolated following nitrosoguanidine treatment (Methods section 7) and penicillin selection. The mutant, E. coli B27T⁻thr⁻ (sm^T), so isolated was crossed by

conjugation with E. coli ara B24/F'araB24 (sm^S) (Methods section 11), and thy⁻ trp⁻ thr⁺ isolates were selected on Vogel-Bonner minimal medium (casamino acids omitted) lacking threonine and supplemented with thymine (50 μ g/ml), tryptophan (20 μ g/ml), and streptomycin (250 μ g/ml). Such isolates were streaked twice as a check for purity and stability and then stored on ENB stock agar slants. They were designated as E. coli B27T⁻thr⁻/F'araB24.

In another method an E. coli B27T⁻/F'araB24 strain was isolated following mating of E. coli ara B24/F'araB24 (sm^S) with E. coli B27T⁻ (sm^F) and subsequent plating on Vogel-Bonner minimal agar (without casamino acids) but supplemented with streptomycin (250 μ g/ml) and tryptophan (20 μ g/ml) and in which thymidine (500 μ g/ml) was substituted for dextrose as the sole source of carbon. Approximately 10^8 recipient cells were spread on each plate, and an average of 1 colony was found/plate. Isolates which were thr⁺, thy⁻, trp⁻ were streaked twice as a check for purity and stability and stored on ENB stock agar slants. These were designated as E. coli B27T⁻/F'araB24.

13. Effect of Limiting Concentrations of Thymine on the Turbidity of E. coli B27T⁻ and E. coli B27T⁻/F'araB24

Comparison of the concentration of thymine required for maximum growth of E. coli B27T⁻ and B27T⁻/F'araB24 was determined by measuring the terminal turbidity for cultures grown in minimal broth supplemented with various concentrations of thymine. Isolated colonies were inoculated into 5 ml of minimal broth supplemented with 50 μ g of thymine/ml and grown

in a shaking waterbath for 18 hr at 37 C. A high concentration of thymine (50 μ g/ml) was utilized in order to avoid the curing of the F'araB24 plasmid which was found to occur when the threonine-requiring merodiploid, E. coli B27T⁻thr⁻/F'araB24, was grown in minimal broth lacking thymine. One ml of the 18-hr culture was used to seed 100 ml of fresh minimal medium supplemented with 50 μ g of thymine/ml, and the culture was shaken at 37 C (as described in Section 8) until a cell concentration of 2×10^8 cells/ml was attained. The cells were washed (as described in Methods section 8) and resuspended in an equal volume of minimal broth lacking thymine and dextrose. One-tenth of a ml (10^6 cells/ml) of this cell suspension was then added to each of 8 tubes containing 10 ml of minimal broth plus one of the following concentrations of thymine/ml - 0, 0.5, 1.0, 2.0, 5.0, 10, 20, or 50 μ g. In another experiment, the cells were serially diluted in minimal broth lacking dextrose to a final concentration of 10^5 cells/ml, and 0.1 ml of this suspension was added to each tube of varying concentrations of thymine to give an initial cell concentration of 10^3 cells/ml. Duplicate determinations were made for each concentration of thymine. Tubes were shaken in a reciprocal waterbath at 37 C for 24 hr, and the turbidity was measured using a Klett Sommerson colorimeter equipped with a number 54 (green) filter.

14. Effect of Nucleic Acid Bases, Nucleosides, Nucleotides and Other Compounds on the Growth of Thymine-Requiring Mutants

The ability of various chemicals to support the growth of thymine-requiring mutants on agar plates was tested using a

filter paper disc assay technique (Lomax and Greenberg, 1968). Isolated colonies of the strains to be tested were inoculated into 10.0 ml of minimal broth containing thymine, and the cultures were shaken in a reciprocal waterbath for 18 hr at 37 C. The culture was then pelleted by centrifugation for 30 sec at 27,000 x g at room temperature, and the cells were resuspended in 10.0 ml of room temperature sterile saline. The culture was again centrifuged and resuspended in 10.0 ml sterile saline. One-tenth of a ml of cell suspension was spread on minimal agar plates lacking thymine. A sterile filter paper disc (Schleicher and Schuell Co. No. 740-E, 12.7 mm diameter) was placed on each plate. The compound to be tested, which previously had been dissolved in sterile distilled water at a concentration of 1.0 mg/ml, was placed on the disc at a final concentration of 100 μ g/disc and allowed to dry. Plates were incubated at 37 C for 48 hr.

15. Effect of Thymidine as a Sole Carbon Source on Thymine- Requiring Mutants

The ability of thymine-requiring mutants to utilize thymidine as a sole carbon source was tested by the method of Greenberg (personal communication, 1971). Minimal agar, in which dextrose and casamino acids were omitted, was prepared and supplemented with 500 μ g of thymidine/ml. The stock thymidine solution (25 mg/ml) used in these experiments was filter sterilized and added to the sterile minimal medium. Cultures to be examined for their ability to utilize thymidine as a sole carbon source were inoculated from isolated colonies into 5.0 ml of minimal broth containing thymine and shaken in

a reciprocal shaking waterbath for 18 hr. The cells from the 18-hr cultures were pelleted by centrifugation for 30 sec at 27,000 x g at 4 C and resuspended in an equal volume of room temperature, sterile saline. The cells were then recentrifuged, resuspended in 5 ml of room temperature, sterile saline and streaked on minimal agar plates containing thymidine. Plates were incubated for one week at 37 C.

16. Effect of Deoxyribonucleosides on the Growth of Thymine-
Requiring Mutants

The method of Lomax and Greenberg (1968) was employed to determine the effect of high concentrations of deoxyribonucleosides on the growth of thymine-requiring mutants. Isolated colonies (3) of each strain to be tested were picked from plates of minimal agar containing thymine and resuspended in 0.5 ml of sterile saline. The cell suspension was then streaked on minimal agar plates supplemented with 200 μ g of thymidine/ml. Plates were incubated 4 days at 37 C. Controls consisted of thymine-independent strains streaked on the same medium.

17. Effect of Increased Carbon Dioxide Tension on Colony-
Forming Ability of Thymine-Requiring Mutants

The effect of increased carbon dioxide tension upon the growth of thymine-requiring mutants on minimal agar plates lacking thymine was investigated. Isolated colonies were used to seed 5.0 ml of minimal broth containing thymine. The cultures were incubated 18 hr at 37 C in a shaking waterbath. Cultures were centrifuged at 27,000 x g for 30 sec at room temperature, and the cells were resuspended in 5.0 ml of

minimal broth lacking thymine and dextrose. The cells were again centrifuged and resuspended in 5.0 ml of the same medium. The cell suspensions were streaked on minimal agar lacking thymine and on minimal agar containing thymine. Plates were incubated 72 hr at 37 C either in a walk-in incubator, or in a candle jar, or in an anaerobic incubator (National Appliance Co., Model 3640) which contained a mixture of hydrogen (95%) and carbon dioxide (5%).

In an experiment to determine if all viable cells of E. coli Y mel and E. coli B27T⁻ were capable of growing on minimal agar lacking thymine in the presence of carbon dioxide (5%), cultures were grown in minimal broth containing thymine and washed as described in the preceding paragraph. The washed cells were diluted in minimal broth lacking thymine and dextrose and spread on plates of minimal agar lacking thymine and minimal agar containing thymine so as to obtain isolated colonies. The plates were incubated 72 hr at 37 C either in a walk-in incubator, or in a dessicator in which the air was replaced by nitrogen, or in an anaerobic chamber in which 5% of the air was evacuated and replaced with carbon dioxide.

18. Presence of Phage λ in Bacterial Lysates of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻

Isolated colonies of E. coli Y mel, E. coli Y melT⁻, E. coli B9, E. coli B9T⁻, E. coli B27 and E. coli B27T⁻ were inoculated into 5.0 ml ENB and grown in a shaking waterbath for 18 hr at 37 C. Two ml of each 18 hr culture were then treated with 0.2 ml of chloroform. Cellular debris was removed by centrifugation at 3,000 x g at 4 C for 10 min. The

supernatant fluid was spotted on three ENB agar plates previously spread with 0.1 ml of an 18 hr culture of indicator bacteria grown in ENB. One plate was seeded with E. coli C600 λ^S , the second with AB259 λ^S , and the third with AB151 λ^R . A lysate of phage λ obtained from Dr. F. T. Hickson was employed as a positive control. The plates were incubated 18 hr at 37 C and examined for plaques.

19. Effect of Pantoyl Lactone on the Cell Morphology of E. coli B27 and E. coli B27T⁻

DL-pantoyl lactone (0.08M) caused filamentous cells of E. coli to divide normally following their exposure to ultraviolet irradiation (Adler and Hardigree, 1964). The effect of pantoyl lactone on strains derived from E. coli Y mel was studied by incorporating 0.08M DL-pantoyl lactone into minimal broth lacking thymine. DL-pantoyl lactone (0.8M) was filter sterilized and added to sterile minimal medium lacking thymine to give a final concentration of 0.08M.

Photomicrographs of the cells were obtained using a Zeiss WL Research Microscope (Carl Zeiss) equipped with a Polaroid Land Instrument Camera (Model ED-10) equipped with Polaroid Land Pack black and white film (#107). The cells, in a broth culture, were photographed as wet mounts under oil immersion (800 x magnification).

20. Preparation of Phage Plkc Lysates

Lysates containing phage Plkc were prepared by the confluent lysis method (Swanstrom and Adams, 1951, and Lennox, 1955). Stock phage Plkc (7×10^{10} plaque forming units/ml)

was diluted to 7×10^7 plaque forming units/ml in room temperature L broth. One-tenth ml of this dilution was mixed with 0.2 ml of an 18-hr suspension of E. coli Y mel, E. coli B27T⁻, or E. coli B9 which had been grown in L broth at 37 C in a shaking waterbath. Three ml of L-top agar were then added, and the suspension was poured over a plate containing L-bottom agar and allowed to solidify. Plates were incubated at 37 C for 24 hr. The top layer was removed by pipetting 3.0 ml of L broth onto the agar surface and gently scrapping off the soft agar layer with a pipette. Suspension thus obtained was pipetted into a test tube and chilled in ice. Chloroform (0.2 ml) was added, and the suspension was mixed vigorously on a Vortex Junior Mixer. Cellular debris was removed by centrifugation at 3,000 x g at 4 C for 10 min. Supernatant fluid was then stored over chloroform at 4 C. The phage titer was determined by the following assay: Shigella dysenteriae strain SH16 from a stock L agar slant was inoculated into 10 ml of L broth and grown not more than 18 hr at 37 C in a shaking waterbath. The phage lysate was serially diluted (using 1/100 dilutions) in L broth, and 0.1 ml of each dilution of the lysate was mixed in a test tube with 0.2 ml of S. dysenteriae strain SH16 cell suspension. This mixture was incubated statically 10 min at 37 C to allow for adsorption of the phage. Three ml of soft L agar was then added to the mixture, and the suspension was poured on the surface of a plate containing L-bottom agar. When the top layer was solidified, the plates were incubated at 37 C for 24 hr, and the

plaques were counted. Phage lysates containing 10^{11} plaque-forming units/ml were obtained using the above procedure.

21. Introduction of the Tryptophan Operon into Tryptophan Deleted, Thymine-Requiring Mutants Using Transduction with Phage Plkc

The deletions in the tryptophan operons of E. coli B9T⁻ and E. coli B27T⁻ were repaired using phage Plkc transduction and employing the method of Yanofsky and Lennox (1959) for the transduction of tryptophan markers. A lysate of phage Plkc grown on E. coli Y mel was prepared by confluent lysis by the method described in the preceding section. Cultures of E. coli B9T⁻ and B27T⁻ in L broth containing 5 μ g thymine/ml were grown 18 hr at 37 C in a shaking waterbath. Cells were mixed with phage in a ratio of 20 plaque forming units/cell in a total volume of 0.2 ml of L broth. Phage were allowed to adsorb 10 min at 37 C. Two ml of sterile saline containing CaCl₂ (2.5×10^{-3} M) were added. The cells were removed by centrifugation at 3,000 x g at 4 C for 10 min and resuspended in 2.0 ml of sterile saline containing CaCl₂. Finally, the cells were centrifuged again and resuspended in an equal volume of sterile saline containing CaCl₂. One-tenth of a ml of this cell suspension was spread on each of two minimal agar plates containing thymine but lacking tryptophan. Plates were incubated for 48 hr at 37 C. Tryptophan-independent colonies arising on this medium were restreaked twice more on the same medium for purity. Strains which were now tryptophan-independent and thymine-dependent were then streaked on stock ENB agar slants.

22. Transduction of the Thymine Requirement of E. coli B27T⁻ to E. coli RG274

Co-transduction of the thy A and arg A markers was determined using phage Plkc. Arg A is located near the thy A region of the E. coli linkage map (Appendix 1). A lysate of phage Plkc grown on E. coli B27T⁻ or E. coli B27 (a control) was used to infect E. coli RG274 (argA⁻, thyA⁺). The phage lysates were prepared and titered according to the procedure described in section 20. Lysates prepared from E. coli B27T⁻ and E. coli B27 contained 8.0×10^9 and 1.3×10^{11} plaque-forming units/ml, respectively. Subsequent reinfection of E. coli B27T⁻ with the phage Plkc lysate prepared on E. coli B27T⁻ failed to produce a lysate of higher titer.

E. coli RG274 was inoculated into 10 ml of L broth from an isolated colony, and the culture was grown for 18 hr at 37 C in a reciprocal shaking waterbath. This yielded a culture containing 1.3×10^9 colony-forming units/ml. A portion of the E. coli RG274 culture was diluted in L broth to give a cell concentration of 1.3×10^8 colony-forming units/ml prior to mixing it with the B27T⁻ phage lysate. Bacterial cells were mixed with the phage lysate of either E. coli B27 or E. coli B27T⁻ in a ratio of 20 plaque-forming units/cell in a total volume of 0.2 ml of L broth. The suspensions were incubated statically at 37 C for 10 min to allow for adsorption of the phage. Two ml of sterile saline containing CaCl₂ (2.5×10^{-3} M) were added. The cells were removed by centrifugation at 3,000 x g at 4 C for 10 min and resuspended in 2.0 ml of sterile saline containing CaCl₂. Finally, the cells were

centrifuged again and resuspended in an equal volume of sterile saline containing CaCl_2 . The cell suspension was then spread on the following media so as to obtain isolated colonies:

1. Complete medium (C) consisted of Vogel-Bonner minimal agar without casamino acids but supplemented with thymine ($50\mu\text{g/ml}$) and a mixture of amino acids - L-glycine, L-leucine, L-aspartic acid, L-glutamic acid, L-glutamine, L-tyrosine, L-cysteine, L-proline, L-tryptophan, L-histidine, L-lysine, and L-arginine ($20\mu\text{g/ml}$); D,L-threonine, D,L-alanine, D,L-methionine, D,L-isoleucine, D,L-phenylalanine, D,L-asparagine, and D,L-serine ($40\mu\text{g/ml}$).
2. Medium lacking arginine (A^-) consisted of complete medium but L-arginine was omitted.
3. Medium lacking lysine (L^-) consisted of complete medium but L-lysine was omitted.
4. Medium lacking arginine and lysine (A^-L^-) consisted of complete medium but L-arginine and L-lysine were omitted.

The plates were incubated for 48 hr at 37 C and, the colonies were counted. The total viable count was obtained from the complete medium, while the number of arg⁺ and arg⁺ lys⁺ transductants were obtained from the appropriate plates. Transductant colonies were examined for their ability to synthesize thymine (thy A) by picking 40 isolated colonies with sterile toothpicks and placing them on plates containing complete medium, complete medium lacking thymine, medium lacking arginine, and medium

lacking both arginine and lysine. The plates were incubated 48 hr at 37 C, and the number of arg⁺thy⁺ transductants was determined.

23. Preparation of Cell-Free Extracts from E. coli Y mel, E. coli B27, and E. coli B27T⁻

The method described by Breitman and Bradford (1967) was employed in preparing cell-free extracts for the assay of thymidylate synthetase. Cells were picked from an isolated colony and inoculated into minimal broth containing thymine, and the culture was shaken in a waterbath at 37 C for 18 hr. One ml of the 18 hr culture was then used to seed 100 ml of the same medium contained in a 250 ml Erlenmeyer Klett side-arm flask. The culture was incubated at 37 C in a rotary shaking waterbath at 200 R.P.M. until a cell concentration of 2×10^8 colony-forming units/ml was attained. Half of the cells (50 ml) from this culture were pelleted by centrifugation at 16,000 x g at room temperature for 5 min and were resuspended in minimal broth lacking thymine and dextrose. The cells were recentrifuged, resuspended in minimal broth lacking thymine (50 ml), and transferred to a 250 ml Erlenmeyer Klett side-arm flask. The culture was grown to mid-logarithmic phase (200 Klett units using a No. 54 filter) which was equivalent to about 1×10^9 cells/ml for E. coli Y mel and E. coli B27 and about 6×10^8 cells/ml for E. coli B27T⁻. Cells were washed once by centrifugation at 16,000 x g for 5 min at 4 C and were resuspended in an equal volume of cold minimal broth lacking thymine and dextrose. Following recentrifugation the pellet was resuspended in 5.0 ml of cold 0.05 M Tris

(hydroxymethyl) aminomethane containing 0.01M 2-mercaptoethanol and 0.001M disodium ethylenediaminetetraacetate which had previously been adjusted to pH 7.4 with hydrochloric acid (5N). The concentrated cell suspension was subjected to sonication for 1 min using a Measuring and Scientific Equipment, Ltd. Ultrasonic Disintegrator (100 watts) operating at 20 kilocycles/sec. The cell suspension, during sonication, was cooled in saturated NaCl-ice water (0 C). Cellular debris was removed by centrifugation at 30,000 x g for 15 min at 4 C. The supernatant fluid was chilled in ice and assayed for thymidylate synthetase activity (Freidkin, 1963). Protein concentration was determined by the method of Lowry *et. al.* (1951) using bovine serum albumin as the standard.

24. Assay of Thymidylate Synthetase Activity from Crude Extracts of E. coli Y mel, E. coli B27, and E. coli B27T⁻

The spectrophotometric procedure for assaying thymidylate synthetase activity was described by Freidkin (1963) and measures the spectral change which occurs during the oxidation of tetrahydrofolate to dihydrofolate. Since thymidylate synthetase converts deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP) in the presence of Mg⁺⁺ and N⁵, N¹⁰-methylene tetrahydrofolate, the change from tetrahydrofolate to dihydrofolate can be measured and used as an indication of thymidylate synthetase activity. The assay reaction mixture contained 0.9 ml cell-free extract (section 21) and 0.3 ml of Plus Mix. Plus Mix contained 6.0 ml 1M mercaptoethanol previously adjusted to pH 7.4 with KOH, 1.8 ml of tetrahydrofolate solution (5 mg of dl, L-tetrahydrofolate/ml

of 1 M mercaptoethanol previously adjusted to pH 7.4 with KOH), 3.0 ml of 0.3 M HCHO, 3.0 ml of 0.5 M MgCl₂, 3.0 ml of 0.001 M dUMP and 1.2 ml of water. A reference cuvette contained 0.9 ml of the enzyme and 0.3 ml of Minus Mix. Minus Mix contained all of the reagents described for Plus Mix except dUMP. The reaction was measured at room temperature in a Bausch and Lomb 600 spectrophotometer as the difference in absorbance at 340 nm for the Plus Mix over the Minus Mix.

25. Extraction and Purification of DNA from E. coli Y mel, E. coli B27 and E. coli B27T⁻

Bacterial cultures for DNA extraction were grown as follows: An isolated colony was used to inoculate 10 ml of minimal broth containing thymine and grown in a reciprocal shaking waterbath at 37 C for 18 hr. This culture (10 ml) was used to seed 1 liter of the same medium contained in a 2800 ml Fernbach flask. The culture was shaken in a rotary waterbath at 200 R.P.M. at 37 C until a cell concentration of 2×10^8 colony-forming units/ml was attained. Cells were centrifugated at room temperature at 16,000 x g for 5 min and were resuspended in 1 liter of room temperature, minimal broth lacking thymine and dextrose. The cells were again centrifuged and resuspended in 10 ml of minimal broth lacking thymine contained in a 2800 ml Fernbach flask. The culture was rotary shaken at 200 R.P.M. at 37 C until a cell concentration of 1 to 3×10^9 colony-forming units/ml was attained. Cells were harvested by centrifugation at 16,000 x g at 4 C for 5 min, and the wet weight of the cell pack was determined. About 6g wet weight of cells were obtained for the thymine-

independent cultures and 7-8_g for the thymine-dependent cultures (Tables 15 and 23). The cells were resuspended in 100 ml of saline-EDTA (0.15 M NaCl plus 0.1 M ethylenediamine-tetra-acetate, pH 8.0). Cultures were streaked on minimal agar plates lacking thymine to check for thy⁺ revertants when thymine-requiring strains were employed. Minimal agar plates lacking tryptophan but containing thymine were also streaked when the trp deletion mutants were employed to check for trp⁺ revertants.

The method of Marmur (1961) was followed closely for the extraction and purification of bacterial DNA. The purified DNA was dissolved in 5mM NaOH (pH 8.5) and stored over chloroform at 4 C. The concentration of DNA was estimated using the diphenylamine reaction (Burton, 1968) and absorbance at 260 nm (Tables 16 and 24). The purity of the DNA preparation (Tables 16 and 24) was estimated from the ratios of extinctions, 260:230:280 nm, for which typical ratios for DNA should be 1.0:0.450:0.515 (Marmur, 1961).

26. Hydrolysis and Chromatography of Purified DNA

The method of hydrolysis of DNA and chromatographic separation of DNA bases was described by Lin and Maes (1963). One ml of stock DNA solution (for concentration, see Tables 16 and 24) contained in a Pyrex tube (6mm inside diameter) was freeze-dried in vacuo for 36 hr. Dry DNA was dissolved in 0.5 ml of formic acid (88%) for every 700 μ g of DNA. Tubes were sealed under CO₂ in an oxygen-gas flame, and the samples were hydrolyzed in a muffle furnace (Blue M Electric Co.,

Blue Island, Ill.) at 175 C for 30 min. Tubes were cooled to room temperature and opened by heating the sealed tip in an oxygen-gas flame. The hydrolysates were then dried in vacuo at 4 C for 18 hr. Dry hydrolysates were dissolved in 20 μ l N HCl and spotted on Whatman No. 1 chromatographic paper. Hydrolysis tubes were rinsed with 10 μ l N HCl which was also applied to the chromatogram. An equivalent amount (30 μ l) of N HCl was spotted on the chromatogram as a blank control. Control samples of DNA consisted of the following dissolved in 5 mM NaOH: (a) a mixture of adenine sulfate, cytosine, guanine hydrochloride, and thymine, 80 μ g/ml of each base; (b) Escherichia coli DNA (Worthington DNA AEC 9KA), 500 μ g/ml; and (c) Salmon sperm DNA (Calbiochem, A grade), 380 μ g/ml.

Descending chromatography was performed using a solvent system consisting of either redistilled isopropanol (325 ml plus conc. HCl (83.5 ml) made up to 500 ml with distilled water (Wyatt, 1951) or 86% (v/v) aqueous n-butanol (Markham and Smith, 1949). The solvent front was allowed to run for at least 30 cm at room temperature. The chromatogram was dried at room temperature, and the nucleic acid spots were visualized with a short wave ultraviolet light (Ultra-Violet Products, Inc. UVSL-25). Spots were cut out and eluted by shaking in 5.0 ml of 0.1N HCl for 2 hr at room temperature. Eluates were centrifuged at 27,000 x g for 10 min at 4 C to remove debris, and ultraviolet absorbance spectra were obtained for the eluates using a Bausch and Lomb 600 spectrophotometer. The concentration (μ moles) of eluted bases were determined from the absorption maxima according

to the method described by Bendich (1957). The absorbance at the maximum was multiplied by the volume of eluate (5 ml) and the number of micromoles of base/O.D. unit (0.0794, 0.100, 0.0901, and 0.126 for adenine, cytosine, guanine, and thymine, respectively).

27. Incorporation of Thymidine-methyl- C^{14} into DNA of E. coli Y mel and E. coli B27T⁻

Cultures of E. coli Y mel and E. coli B27T⁻ were grown in the presence of thymidine-methyl- C^{14} in order to study the incorporation of labeled thymidine into bacterial DNA. An isolated colony was used to inoculate 10 ml of minimal broth containing thymine, and the culture was grown 18 hr at 37 C in a reciprocal shaking waterbath. The 18 hr culture (5.0 ml) was used to seed 500 ml of minimal broth supplemented with 0.375 g of deoxyadenosine (Boyce and Setlow, 1962), 1500 g of thymidine, and 25 μ Ci of thymidine-methyl- C^{14} (specific activity 54.7 m Ci/mM, New England Nuclear) contained in a 2800 ml Fernbach flask. Prior to inoculation, 1.0 ml of broth was removed and pipetted into a scintillation vial to use as the initial isotope count. The initial cell concentration was determined by plating on ENB agar as being 2.6×10^7 colony-forming units/ml. The cultures were then rotary shaken at 37 C at 200 R.P.M. until a cell concentration of about 1×10^8 colony-forming units/ml was attained. Three ml of the cell suspension were filtered through a Millipore membrane (0.45 μ m), and 1.0 ml of the filtrate was employed to obtain the isotope count for the supernatant, thymidine-methyl- C^{14} broth. The cells were then pelleted by centrifugation

at 16,000 x g for 5 min at room temperature and resuspended in an equal volume of minimal broth lacking thymine and dextrose. The washed cells were recentrifuged and resuspended in 10 ml of minimal broth lacking thymine and dextrose. This cell suspension (10 ml) was then used to seed 1 liter of minimal broth lacking thymine contained in a 2800 ml Fernbach flask. Ten ml of cell suspension in the Fernbach flask was filtered through a Millipore membrane (0.45 μ m), after which the membrane was placed in a scintillation vial and counted. One ml of the filtrate was also placed in a scintillation vial and counted. The cultures contained in the Fernbach flasks were shaken at 200 R.P.M. at 37 C until the population reached early stationary phase (1 to 3 x 10⁹ cells/ml). Ten ml of the cell suspension was then filtered through a Millipore membrane (0.45 μ m), and the filter was placed in a scintillation vial and counted. One ml of the filtrate was also placed in a scintillation vial and counted. The DNA was extracted, purified and chromatographed as described previously (sections 25 and 26). Ten ml of scintillation fluid (Aquasol, New England Nuclear) was added to each vial, and the number of counts per minute (CPM) was obtained for each sample. Isotopic counts were obtained using a Packard Tri-Carb liquid scintillation Spectrometer (Model 3320).

CHAPTER IV

RESULTS

1. Characteristics of Thymine-Requiring Mutants of Escherichia coli Y mel

The physiological properties of thymine-requiring mutants, E. coli Y mel⁻, E. coli B9T⁻, and E. coli B27T⁻, isolated following treatment with trimethoprim were compared with the properties of parental strains E. coli Y mel, E. coli B9, and E. coli B27 as well as with deoxyribonucleoside negative (deo⁻) mutants, E. coli B307c and E. coli B840c. Experiments were conducted to determine if the E. coli Y mel derivatives were double mutants of the thy A⁻ deo⁻ type. Mutants of the thy A⁻ genotype require high concentrations of thymine (> 20 μg/ml) for growth due to the catabolism of thymidine by enzymes specified by the deo region (Lomax and Greenberg, 1968). These enzymes degrade nucleosides and deoxynucleosides to the free base and deoxyribose-1-phosphate. The latter is further degraded to glyceraldehyde-3-phosphate and acetaldehyde. Thus, in a thy A⁻ deo⁻ strain, compounds such as thymidine can be utilized as a sole source of carbon. Deo⁻ mutants, then by definition, are unable to utilize nucleosides or deoxynucleosides as sole sources of carbon, and thus double mutants of the thy A⁻ deo⁻ genotype require low concentrations of thymidine (< 20 μg/ml) for growth. The following experiments suggest that E. coli Y mel⁻, E. coli B9T⁻, and E. coli B27T⁻ are double mutants of the thy A⁻ deo⁻ genotype.

a. Colony-Forming Ability of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ on Agar Plates Containing Various Concentrations of Thymine

E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ mutants were isolated as strains which grew aerobically on streaked, minimal agar plates containing thymine but would not grow on minimal agar lacking thymine at 37 C. In order to more clearly define the growth of these mutants on agar plates, the effective concentration of thymine which would allow the thymine-requiring population to grow was investigated. Table 3 shows the type of response of thymine-requiring derivatives of E. coli Y mel to various concentrations of thymine. The results showed that E. coli Y melT⁻ had maximal colony formation on concentrations of thymine as low as 5 μ g/ml. In addition, this strain grew well (87% recovery) on 2 μ g/ml. Greater than 90% of the E. coli B27T⁻ population was capable of forming colonies on 5 μ g/ml, but only a fraction of the population (0.6%) could form colonies on 2 μ g/ml. In contrast, E. coli B9T⁻ was capable of attaining maximum growth (96% recovery) on as little as 1 μ g of thymine/ml, and 74% of the population formed colonies on 0.5 μ g/ml. The data suggested that E. coli Y melT⁻ and E. coli B9T⁻ could survive on lower concentrations of thymine than could E. coli B27T⁻. However, a concentration of 5 μ g/ml appeared to be sufficient to support growth of greater than 90% of the population of the mutants studied, which indicates that these strains have a low requirement (< 20 μ g/ml) for thymine.

Table 3. Colony-forming ability of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ on agar plates containing various concentrations of thymine

Thymine Concentration ^a	Colony-Forming Units, %		
	Y melT ⁻	B9T ⁻	B27T ⁻
0	0	0	0
0.1	0	0	0
0.5	1.2	74	0
1.0	11	96	0
2.0	87	96	0.6
5.0	100	96	94
20	92	91	100
50	100	100	100

Cultures were grown to early logarithmic phase (2×10^8 cells/ml) in minimal broth containing thymine, washed, and resuspended in sterile, room temperature saline to a concentration of approximately 2×10^3 cells/ml. One-tenth of a ml of the diluted suspension was then spread on duplicate minimal agar plates supplemented with various concentrations of thymine. Plates were incubated 2-5 days at 37 C and counted. The counts obtained on minimal agar containing 50 μ g/ml of thymine were allowed to equal 100% recovery.

a. Concentration of thymine added to minimal agar in micrograms per milliliter

- b. Inability of E. coli Y mel^{T⁻}, E. coli B9^{T⁻}, and E. coli B27^{T⁻} to Grow on Thymidine as a Sole Source of Carbon

The ability of thymine-requiring mutants derived from E. coli Y mel to utilize thymidine as a sole source of carbon was examined in order to determine if there was a mutation in the deo region in these strains. Cultures of parental strains E. coli Y mel, E. coli B9, and E. coli B27 were compared with their respective thymine-requiring mutants E. coli Y mel^{T⁻}, E. coli B9^{T⁻}, and E. coli B27^{T⁻} for the ability to grow on agar plates containing 500 ug of thymidine as a sole source of carbon. The results (Table 1) indicated that parental strains E. coli Y mel, E. coli B9, and E. coli B27 were capable of utilizing thymidine as a sole source of carbon while the thymine-requiring derivatives of these strains were unable to grow on this deoxyribonucleoside. Thymine-independent revertants of E. coli B9^{T⁻} (B9^{T⁺}) and E. coli B27^{T⁻} (B27^{T⁺}) were unable to utilize thymidine as a sole source of carbon indicating that the mutation affecting the catabolism of deoxyribonucleosides was not a mutation at the thy A locus of the chromosome but probably a deo mutation. Tryptophan-independent revertants, E. coli B9^{T⁻}trp⁺ and E. coli B27^{T⁻}trp⁺, isolated following phage Plkc mediated transduction of the trp operon into E. coli B9^{T⁻} or E. coli B27^{T⁻}, also were not capable of growing on thymidine as a sole carbon source, nor were the colicinogenic strains E. coli B9^{T⁻}/F'ColVBM-K260,trp and E. coli B27^{T⁻}/F'ColVBM-K260,trp. The mutation in the deo region, was introduced into E. coli B27^{T⁻} by conjugation. Thus, the thymine-requiring mutants isolated from E. coli Y mel

contained mutations in the genetic region which specifies the enzymes involved in the catabolism of deoxyribonucleosides, and in E. coli B27T⁻, the mutation was overcome by the presence of the F'ara B24 plasmid.

c. Effect of a High Concentration of Thymidine on Thymine-Requiring Mutants

Lomax and Greenberg (1968) found that a high concentration (200 μ g/ml) of all deoxyribonucleosides, except deoxycytidine, inhibited the growth of thy A⁻ deo C⁻ (dra⁻) mutants such as E. coli B840c. Mutants carrying the thy A⁻ deo B⁻ (drm⁻) genotype were not inhibited. The effect of a high concentration of thymidine (200 μ g/ml) on the aerobic growth of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ was compared with the effect of this deoxyribonucleoside on the parental strains E. coli Y mel, E. coli B9, and E. coli B27 as well as E. coli B307c (thy A⁻ deo B⁻ deo C⁺) and E. coli B840c (thy A⁻ deo B⁺ deo C⁺). The results (Table 1) indicated that E. coli B9T⁻ was sensitive to a high concentration of thymidine suggesting that this strain may be similar to mutants of the thy A⁻ deo C⁻ genotype. E. coli Y melT⁻ and E. coli B27T⁻ were resistant to a high concentration of thymidine, as were the parental strains E. coli Y mel, E. coli B9, and E. coli B27 also were resistant to a high concentration of thymidine. Thymine and tryptophan-independent revertants, E. coli B9T⁺ and E. coli B9T trp⁺, were sensitive to thymidine supporting the evidence obtained in the previous section which showed that the mutation in the deo region was independent of the thy⁻ and trp⁻ mutations. Revertants, E. coli

B27T⁺ and E. coli B27T⁻trp⁺, retained their resistance to a high concentration of thymidine, a characteristic of the strains from which they were derived.

d. Effect of Nucleic Acid Bases, Nucleosides, Nucleotides, and Other Compounds on the Growth of Thymine-Requiring Mutants

The specificity of the requirement for thymine by thymine-requiring derivatives of E. coli Y mel was investigated by surveying a series of chemicals to determine which ones would support the growth of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ aerobically on minimal agar plates lacking thymine. The data presented in Table 4 show that, of the compounds studied, only thymidine and thymidine-5'-monophosphate in addition to thymine could support growth of these mutants. Other nucleic acid bases, nucleosides, and nucleotides were ineffective as were 5-methylcytosine and 5-hydroxymethylcytosine. Thymine analogues 6-amino thiouracil, 6-azauracil and 2-aminopurine were also ineffective. Orotic acid, a precursor in nucleic acid biosynthesis; folic acid and serine, compounds involved in one carbon transfer in the biosynthesis of thymidylate, were ineffective. Pantoic lactone, a precursor of pantothenic acid, was examined as a potential growth factor; since this compound reverses inhibition of cell division in species of Erwinia (Grula and Grula, 1962) and E. coli B (Cummings and Mondale, 1967) and was found to prevent cellular elongation in E. coli B27T⁻ (Results section, 2,h). Pantothenic acid also was ineffective in supporting growth.

Table 4. Effect of nucleic acid bases, nucleosides, nucleotides, and other compounds on the growth of thymine-requiring mutants

Compound	Growth Y melT ⁻	Response of E. coli B9T ⁻	Mutants B27T ⁻
Thymine	+	+	+
Thymidine	+	+	+
Thymidine-5'-mono- Phosphate	+	+	+
Uracil	-	-	-
Uridine	-	-	-
d-uridine-5'-mono- Phosphate	-	-	-
Cytosine	-	-	-
Cytidine	-	-	-
Adenine	-	-	-
Adenosine	-	-	-
Guanine	-	-	-
Guanosine	-	-	-
5-methylcytosine	-	-	-
5-hydroxymethyl cytosine	-	-	-
6-amino thiouracil	-	-	-
6-azauracil	-	-	-
2-aminopurine	-	-	-
Orotic acid	-	-	-
Pantothenic acid	-	-	-
Folic acid	-	-	-
Serine	-	-	-

Filter paper discs containing 100 μ g of the compound to be tested were placed on minimal agar plates spread with a lawn of the mutant to be tested. Plates were then incubated 48 hr at 37 C.

+ = growth

- = no growth

e. Effect on Growth of Increased Carbon Dioxide Tension

Experiments described later (Results section 2,c) demonstrated that E. coli B27T⁻ was capable of initiating growth in minimal broth lacking thymine from an inoculum as low as 10⁴ colony-forming units/ml but not aerobically on minimal agar plates lacking thymine. This suggested the possibility that partial anaerobic conditions might be necessary to support growth of this mutant in the absence of exogenous thymine. Growth of thymine-requiring mutants on minimal agar plates lacking thymine was investigated under several gaseous environments. In an experiment in which thymine-requiring mutants were streaked on minimal agar lacking thymine and minimal agar containing thymine, and growth was compared under several gaseous atmospheres (Table 5), it was apparent that E. coli B27T⁻ and E. coli Y melT⁻ were capable of growing on minimal agar lacking thymine in both a candle jar and a chamber containing hydrogen (95%) and carbon dioxide (5%). In contrast, other thymine-requiring mutants failed to grow on minimal agar lacking thymine either aerobically or anaerobically. Under the gaseous environments examined, all strains were capable of growing on thymine supplemented agar plates. The data in Table 5 did not determine if growth in anaerobic conditions resulted from reduced oxygen tension or increased carbon dioxide tension. Also, the possibility was not eliminated that only a fraction of the E. coli Y melT⁻ and E. coli B27T⁻ populations was capable of growing anaerobically on minimal agar plates lacking thymine.

Table 5. Effect of anaerobic incubation on growth of thymine-requiring mutants

Thymine-requiring mutants were grown to early logarithmic phase in minimal broth containing thymine, washed, and resuspended in minimal broth lacking thymine and dextrose. Final suspensions were streaked on the indicated minimal agar plates. Plates were then incubated at 37 C for 48 hr in the atmospheres shown.

- Thy = minimal agar lacking thymine
- + Thy = minimal agar containing thymine
- ND = not determined
- + = growth
- = no growth

<u>E. coli</u> Strain	Medium	Growth on Agar Plates in Atmospheres of		
		Air	CO ₂ (candle jar)	H ₂ (95%) + CO ₂ (5%)
Y melT ⁻	-Thy	-	+	+
	+Thy	+	+	+
B9T ⁻	-Thy	-	-	-
	+Thy	+	+	+
B27T ⁻	-Thy	-	+	+
	+Thy	+	+	+
B307c	-Thy	-	-	ND
	+Thy	+	+	ND
B840c	-Thy	-	-	ND
	+Thy	+	+	ND
<u>JC411T⁻/</u> <u>F' ColVBM-</u> <u>K260, trp</u>	-Thy	-	-	ND
	+Thy	+	+	ND
15T ⁻	-Thy	-	-	ND
	+Thy	+	+	ND

Another experiment (Table 6), in which 5% of the air in an anaerobic chamber was replaced with carbon dioxide, demonstrated that mutants E. coli Y melT⁻ and E. coli B27T⁻ require increased carbon dioxide tension for growth on minimal agar plates lacking thymine. The number of colony-forming units on each type of media was also determined. The results show that the entire viable populations of E. coli Y melT⁻ and E. coli B27T⁻ were capable of growing on minimal agar lacking thymine in an environment supplemented with 5% carbon dioxide. None of the mutants were capable of growth on minimal agar lacking thymine under a nitrogen atmosphere, which shows that carbon dioxide was required for growth on minimal agar lacking thymine.

- f. Presence of Phage λ in Bacterial Lysates of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻

Escherichia coli Y mel is normally lysogenic for phage λ (I.P. Crawford personal communication, 1970). Evidence for the presence of phage λ in the bacterial strains derived from E. coli Y mel was obtained by spotting lysates of the bacterial strain on lawns of bacterial strains which were sensitive or resistant to infection by phage λ . The data (Table 7) indicated that the E. coli Y mel derivatives employed in this study released phage λ when treated with chloroform.

2. Growth of Thymine-Requiring Mutants in Minimal Broth Lacking Thymine

- a. Thymineless Death of Thymine-Requiring Mutants of E. coli

Thymine-requiring mutants of Escherichia coli lose colony-forming ability exponentially when placed in minimal

Table 6. Effect of increased carbon dioxide tension on colony-formation of thymine-requiring mutants

<u>E. coli</u> Strain	Medium	Number colony-forming units/ml in atmospheres of:		
		Air	Nitrogen	Air + CO ₂ (5%)
Y melT ⁻	-Thy	50	70	1.0 x 10 ⁹
	+Thy	1.2 x 10 ⁹	1.1 x 10 ⁹	1.1 x 10 ⁹
B9T ⁻	-Thy	0	0	0
	+Thy	0.9 x 10 ⁹	0.9 x 10 ⁹	1.0 x 10 ⁹
B27T ⁻	-Thy	0	0	0
	+Thy	1.9 x 10 ⁹	2.0 x 10 ⁹	1.9 x 10 ⁹

Thymine-requiring mutants were grown to early stationary phase in minimal broth containing thymine, washed, and resuspended in minimal broth lacking thymine and dextrose. Suspensions were serially diluted in minimal broth lacking thymine and dextrose, and 0.1 ml was spread on the indicated minimal agar plates in order to obtain total counts. Plates were incubated at 37 C for 48 hr in the atmospheres shown.

-Thy = Minimal agar lacking thymine

+Thy = Minimal agar containing thymine

Table 7. Presence of phage λ in bacterial lysates of E. coli Y mel^{T-}, E. coli B9^{T-}, and E. coli B27^{T-}

<u>E. coli</u> Lysate	Bacterial Indicator Strain		
	C600 λ^S	AB259 λ^S	AB151 λ^R
C600 (λ)	+	+	-
Y mel	+	+	-
Y mel ^{T-}	±	±	-
B9	+	+	-
B9 ^{T-}	+	+	-
B27	+	+	-
B27 ^{T-}	+	+	-

Chloroformed lysates were spotted on a lawn of the indicator strain spread on ENB agar. The presence of a clear zone, following overnight incubation at 37 C, was indicative of lysis of the host cells.

+ = lysis; ± = weak lysis; - = no lysis

medium which is complete except for thymine (Barner and Cohen, 1954). The growth response of several thymine-requiring mutants of E. coli in minimal medium lacking thymine is presented in a composite graph (Fig. 2). E. coli B (T^-) is sensitive to thymine starvation and loses colony-forming ability immediately following resuspension in minimal medium lacking thymine (Cummings and Taylor, 1966). E. coli 15 T^- exhibits a lag period of 50-60 min before the onset of thymineless death (Barner and Cohen, 1954). E. coli 15 T_R^- is a Mitomycin-C resistant derivative of E. coli 15 T^- and neither grows nor loses colony-forming ability under conditions of thymine starvation. E. coli THU, a derivative of E. coli 15 T^- , requires histidine and uracil in addition to thymine and is more resistant to thymine starvation than E. coli 15 T^- .

b. Growth of E. coli Y mel T^- , E. coli B9 T^- , and E. coli B27 T^- in Minimal Broth Lacking Thymine

Growth curves for E. coli Y mel and its derivatives, upon their resuspension in minimal broth lacking thymine were determined (Fig. 3). Strains E. coli Y mel, E. coli B27, and E. coli B9 exhibited similar growth curves in minimal medium lacking thymine. Thymine-requiring mutant E. coli Y mel T^- responded to thymine starvation in a manner similar to that of E. coli 15 T^- . Following a lag period of approximately 60 min, E. coli Y mel T^- exponentially lost colony-forming ability until the number of viable cells was reduced by about 99.8% after 5 hr. Subsequently, E. coli Y mel T^- regained colony-forming ability. This growth phase was shown to be the result of spontaneous thymine-independent revertants (Fig. 12).

Fig. 2. Growth of thymine-requiring mutants of E. coli in minimal broth lacking thymine. A composite graph of data showing the growth curves for several strains of E. coli upon resuspension in minimal broth. E. coli 15T⁻ (Barnes and Cohen, 1954), ●; E. coli B(T⁻) (Cummings and Taylor, 1966), O; E. coli THU, X; and E. coli 15T_R⁻, ▼. T⁻ indicates minimal broth lacking thymine; T indicates minimal broth containing thymine.

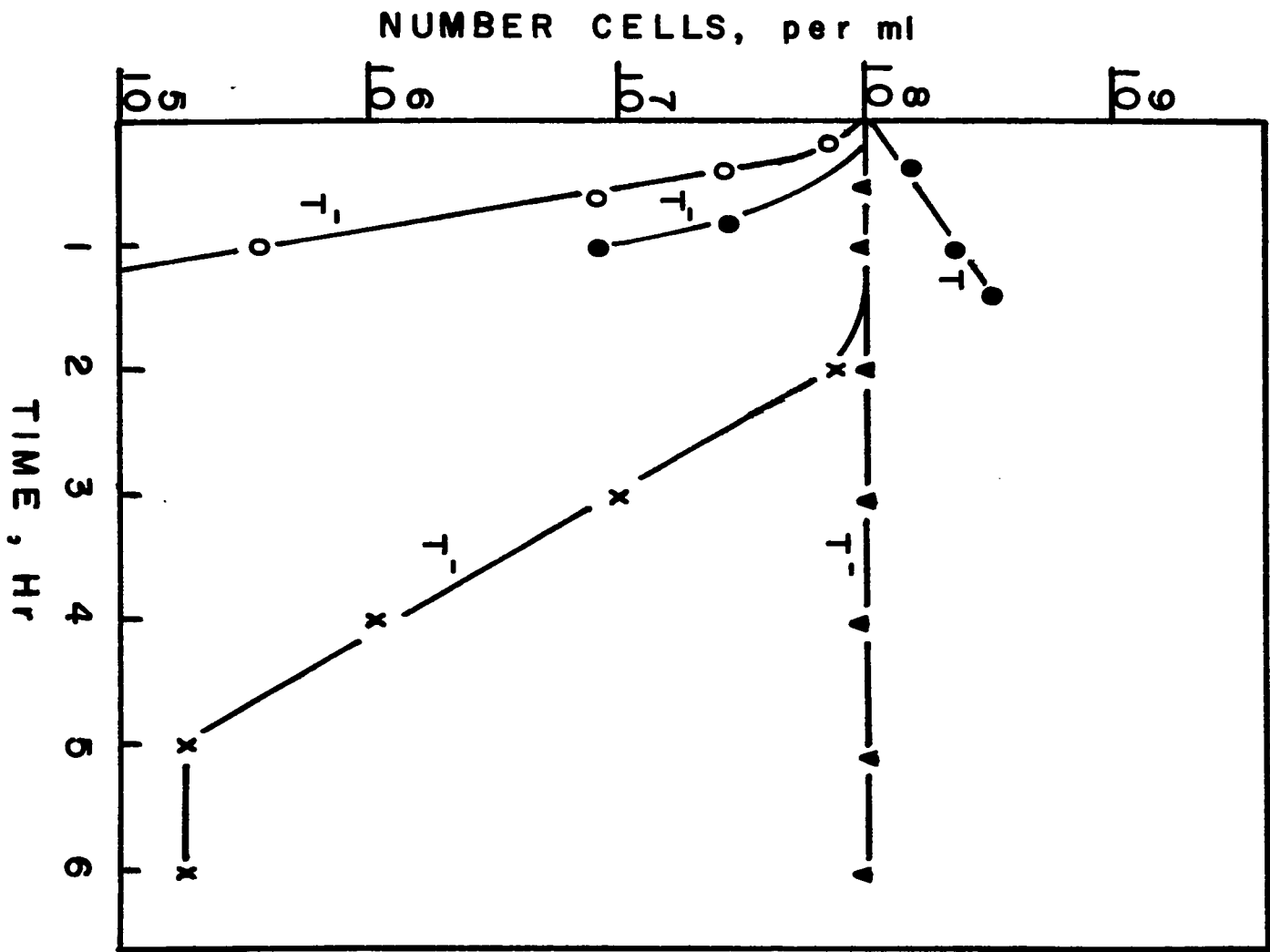
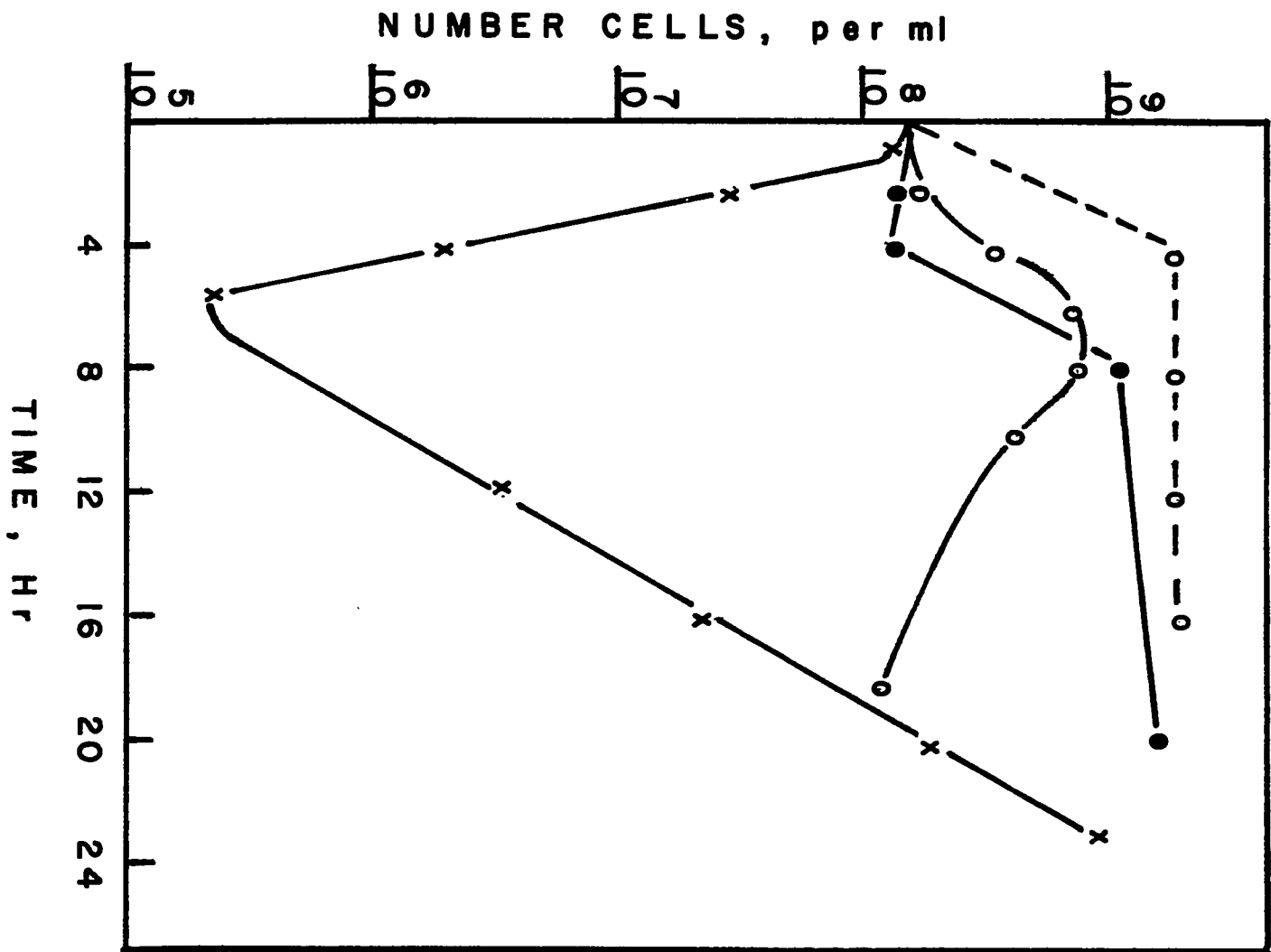


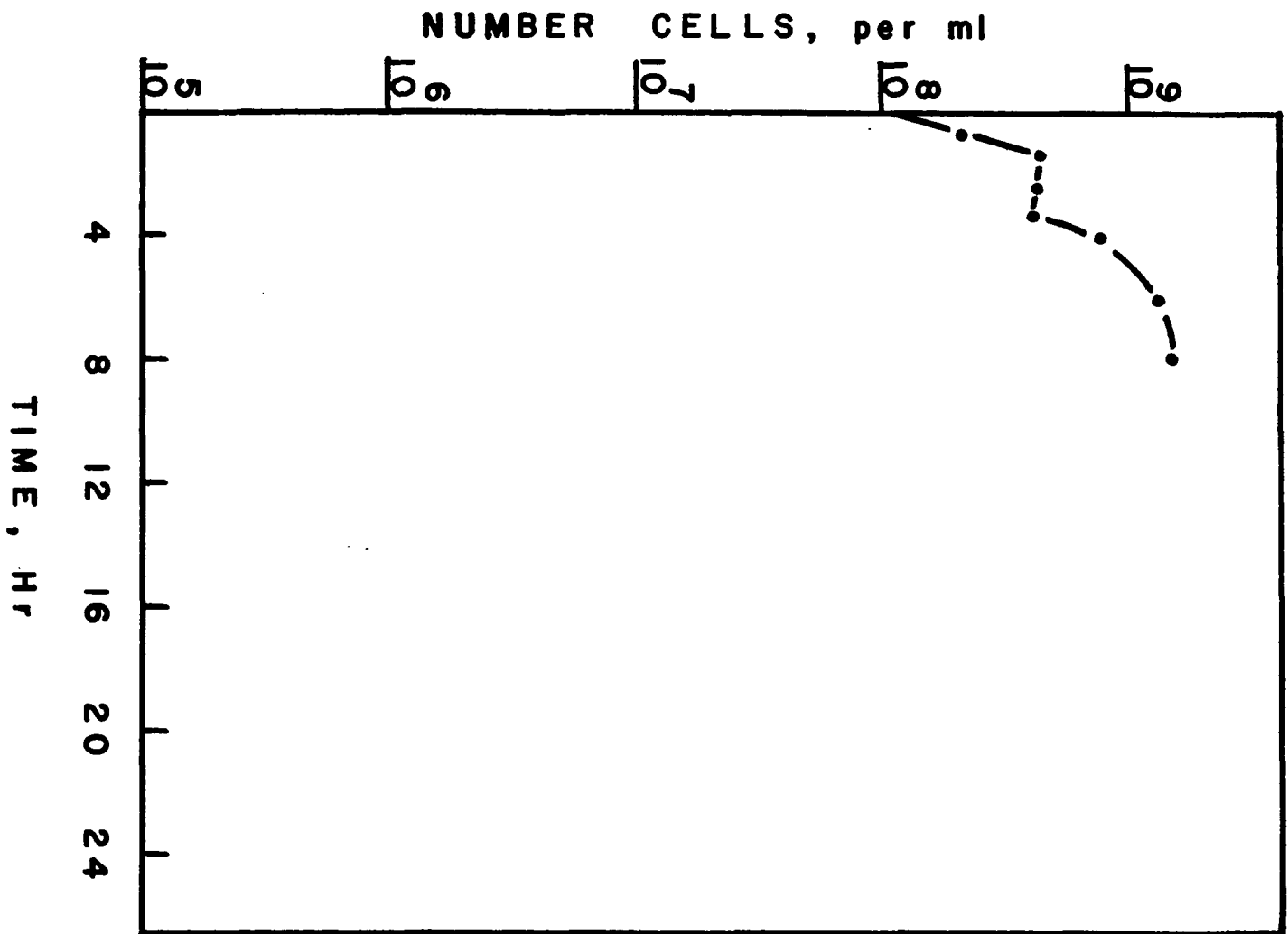
Fig. 3. Growth of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ in minimal broth lacking thymine. Cultures were grown to early logarithmic phase in minimal broth containing thymine, centrifuged, washed, and then resuspended in minimal broth lacking thymine. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Prototrophic strains E. coli Y mel, E. coli B9, and E. coli B27 (- - -). E. coli Y melT⁻, X; E. coli B9T⁻, O; and E. coli B27T⁻, ● .



Escherichia coli B27T⁻ exhibited a multiphasic growth curve in minimal broth lacking thymine (Fig. 3). Initially upon resuspension in minimal medium lacking thymine, the number of colony-forming units increased by about one generation during the first 15 minutes. This initial phase was followed by a false stationary phase lasting for from one to four hr during which time the number of colony-forming units remained constant or decreased slightly. Mutant E. coli B27T⁻ then regained colony-forming ability, and the population increased to about 2×10^9 cells/ml. The initial part of the growth phase could be altered by varying the concentration of thymine in which the cells were grown prior to their resuspension in minimal medium lacking thymine. Growth in 50 μ g of thymine/ml, prior to resuspension in minimal medium lacking thymine, resulted in an increase of colony-forming units in the initial phase equivalent to 2.3 generations (Fig. 4). No thymine-independent revertants were detected on minimal agar plates lacking thymine. Thus, growth of E. coli B27T⁻ was not the result of a revertant population nor of cross-feeding of some of the mutant cells by other cells in the population.

Upon resuspension in minimal broth lacking thymine, E. coli B9T⁻ (Fig. 3) showed an initial growth phase equivalent to about two generations. After about 7 hr incubation in this medium, E. coli B9T⁻ cells began to lose colony-forming ability in a manner similar to that expected for a thymine-requiring mutant. Thymine-independent revertants were not detected on minimal agar plates lacking thymine.

Fig. 4. Effect of a high concentration of thymine on subsequent growth of E. coli B27T⁻ in minimal broth lacking thymine. E. coli B27T⁻ was grown to early logarithmic phase in minimal broth containing 50 μ g of thymine/ml, centrifuged, washed, and resuspended in minimal broth lacking thymine. The culture was shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).



Growth curves of thymine-requiring mutants resuspended in minimal broth containing thymine (Fig. 5) showed that mutants E. coli Y melT⁻ and E. coli B27T⁻ grew normally in the presence of thymine. Strain E. coli B9T⁻ grew at approximately the same rate as E. coli Y melT⁻ and E. coli B27T⁻ for about 6 hr (4 generations). However, loss of colony-forming ability ensued after 6 hr resulting in a gradual decrease in the number of viable cells.

The multiphasic growth curve characteristic of E. coli B27T⁻ growing in minimal broth lacking thymine was further investigated. Viable cell counts and turbidity measurements were determined at 15 min intervals during the initial 4 hr of incubation in minimal broth lacking thymine and in minimal broth containing thymine. The data (Fig. 6) showed that E. coli B27T⁻ lost colony-forming ability (50% survival) rapidly during the first hr of incubation in both the presence and absence of exogenous thymine. Colony-forming ability was then regained for a short period of time after which a second phase occurred between 2 and 3 hr when E. coli B27T⁻ again lost colony-forming ability. The reduction in viable count was more pronounced for cells grown in minimal broth lacking thymine than for the comparable culture incubated in the presence of exogenous thymine. Growth in terms of cellular mass, measured as the increase in turbidity of the culture (Fig. 7), was continuous throughout the growth phase.

Experiments were performed in an attempt to minimize the multiphasic nature of the growth of E. coli B27T⁻ in minimal broth lacking thymine. Experiments showed that when

Fig. 5. Colony-forming ability of E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ grown in minimal broth containing thymine. Cultures were grown to logarithmic phase in minimal broth containing thymine, centrifuged, washed, and resuspended in fresh minimal broth containing thymine. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). E. coli Y melT⁻, ●; E. coli B9T⁻, O; E. coli B27T⁻, X.

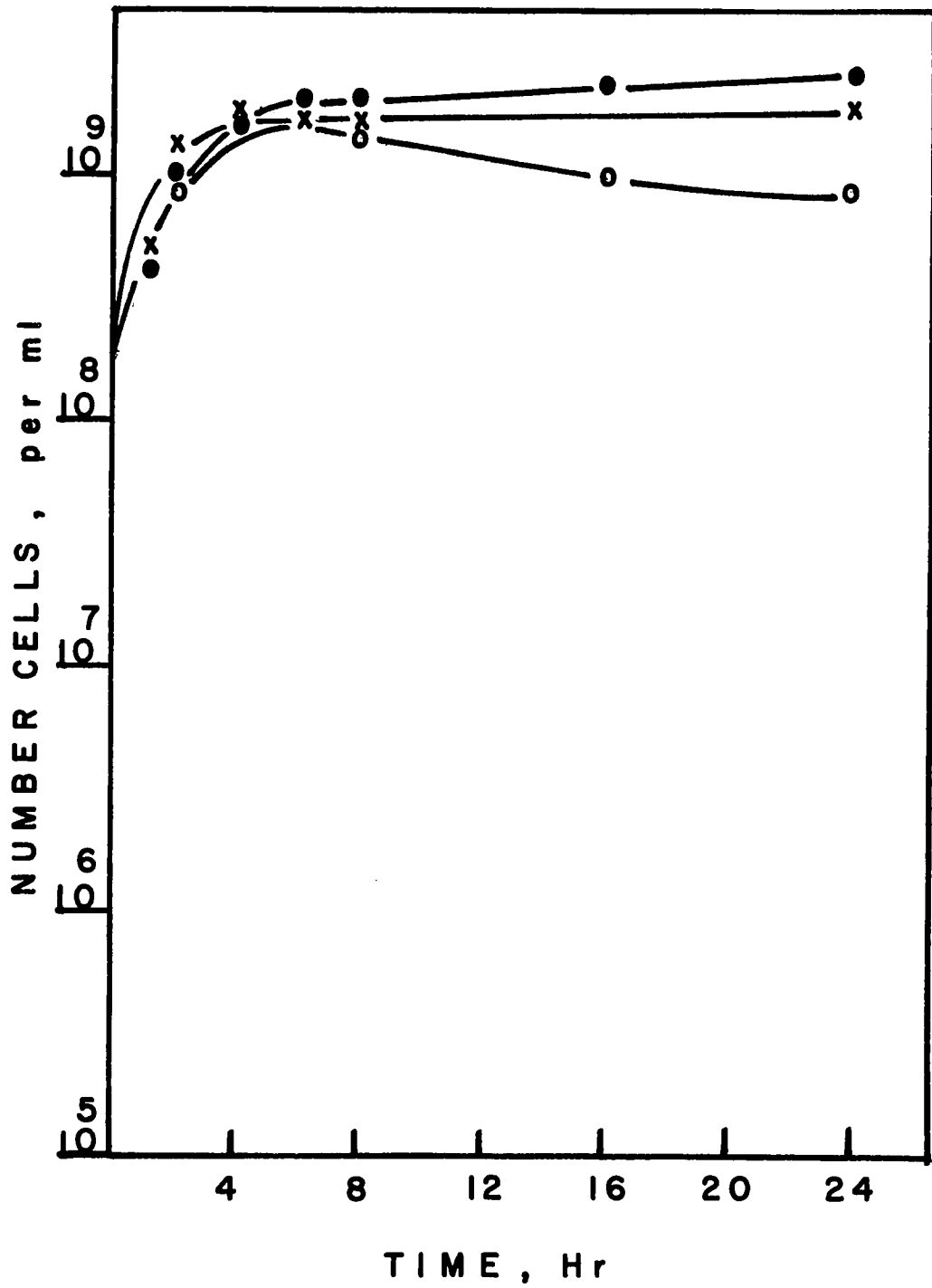


Fig. 6. Cold sensitivity of E. coli B27T⁻ upon resuspension in minimal broth lacking thymine. Cells were grown to early logarithmic phase in minimal broth containing thymine, washed by the cold method (Method I), and resuspended in minimal broth. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Minimal broth containing thymine, ●; minimal broth lacking thymine, ○.

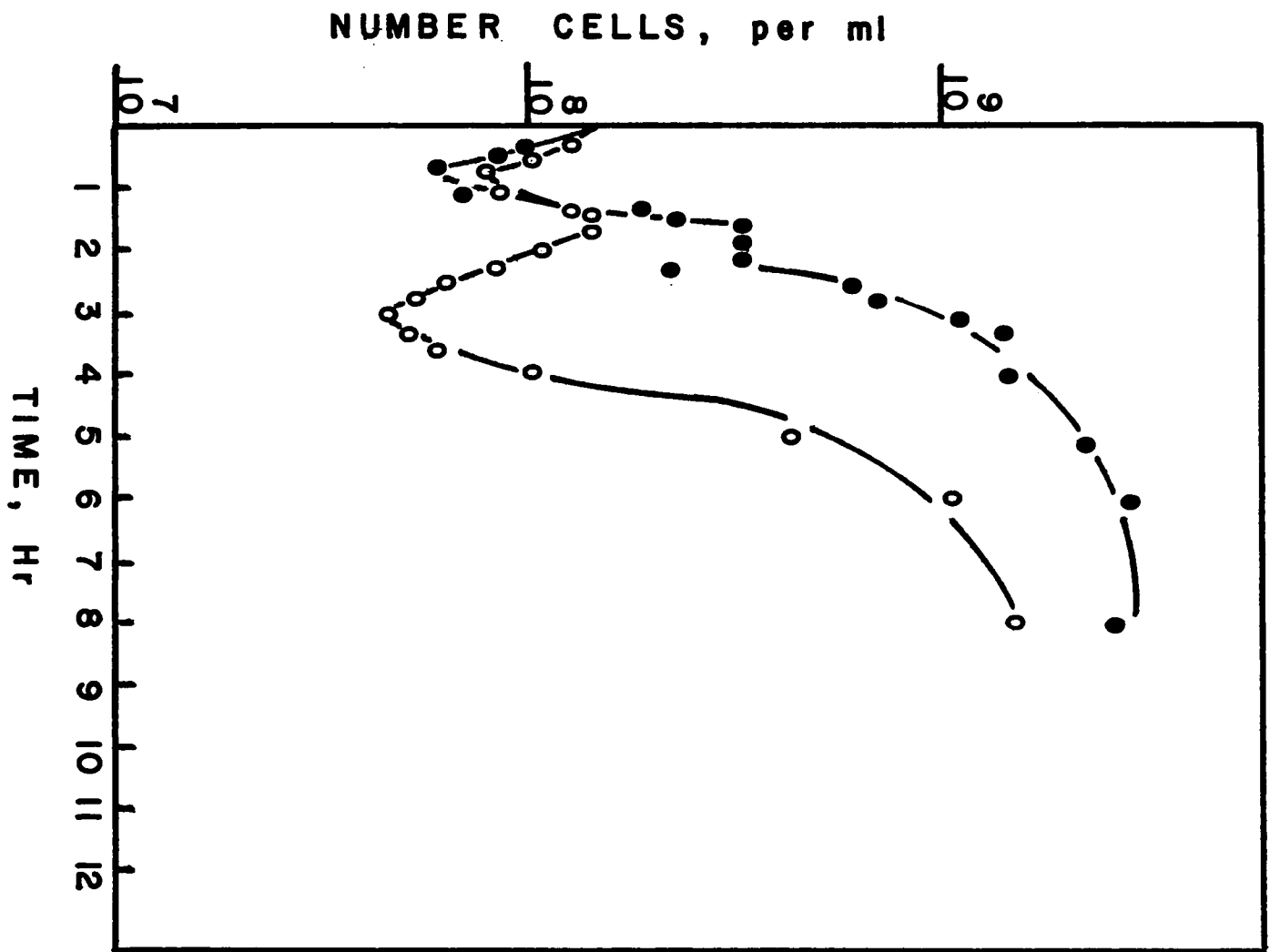
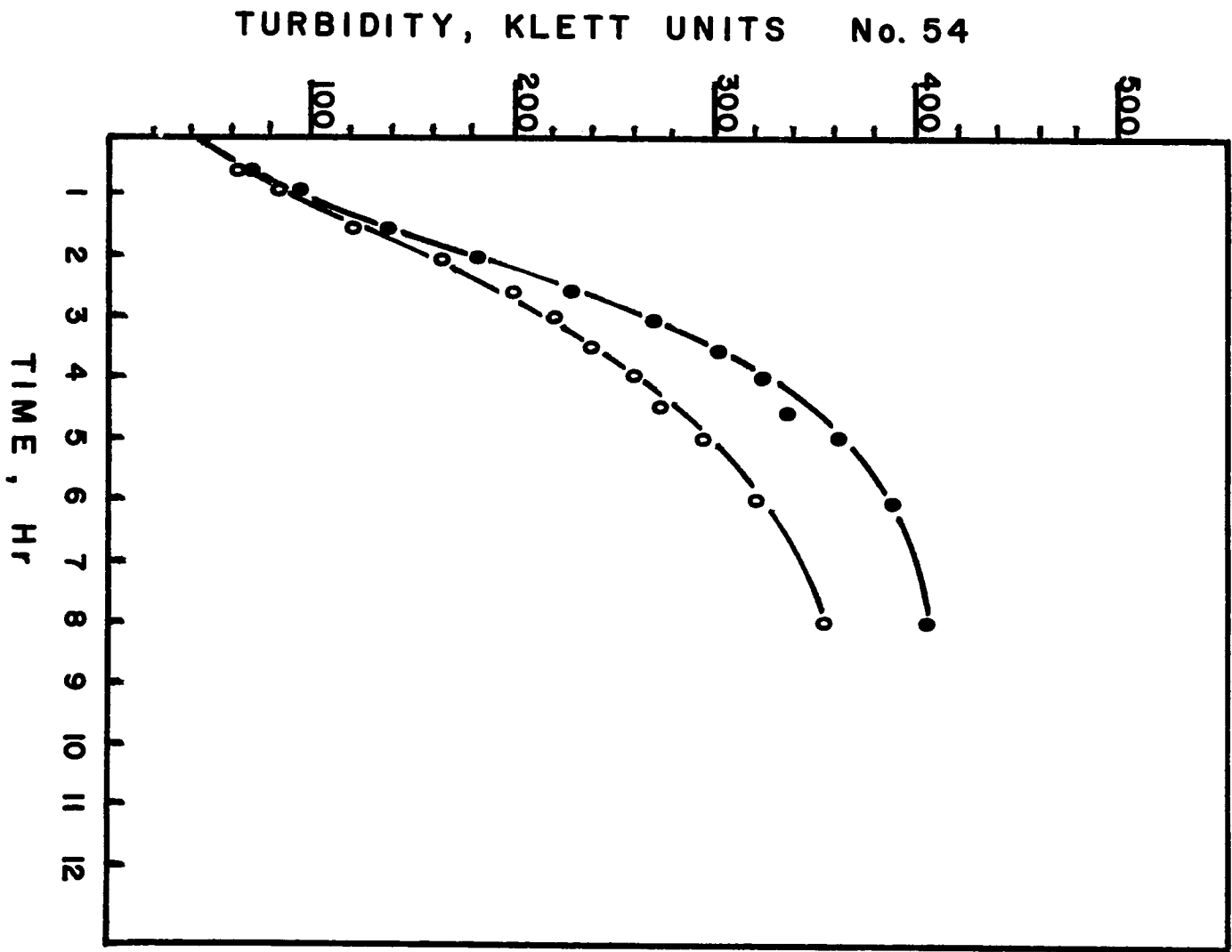


Fig. 7. Turbidity measurements of cold sensitized E. coli B27T⁻ grown in minimal broth lacking thymine. Cells were grown to early logarithmic phase in minimal broth containing thymine, washed by the cold method (Method I), and resuspended in broth. Cultures were shaken in a 37 C waterbath, and increases in turbidity were followed using a Klett-Sommerson colorimeter equipped with a No. 54 filter. Minimal broth containing thymine, ●; minimal broth lacking thymine, ○.

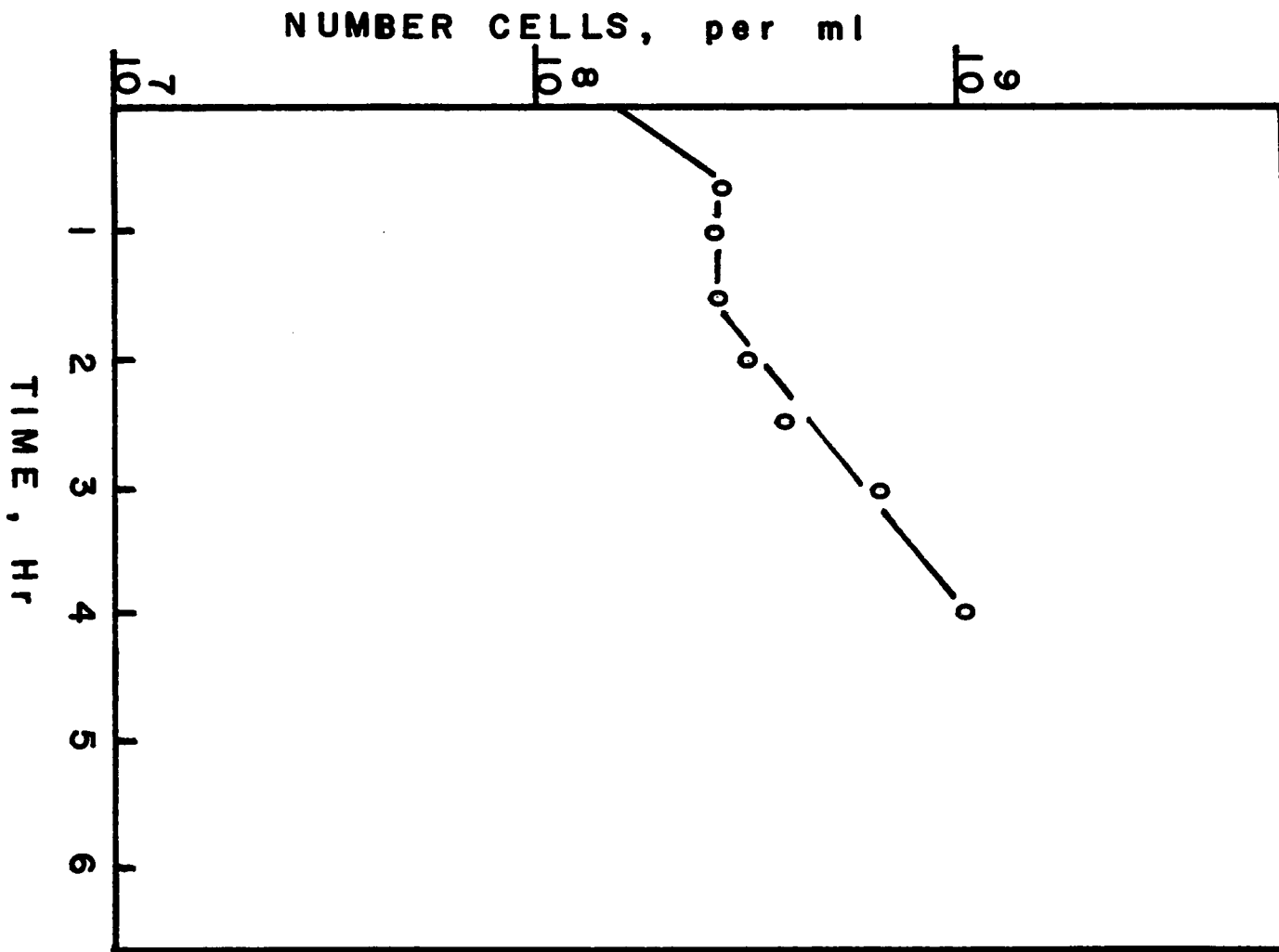


the cells of E. coli B27T⁻ were washed at room temperature (Methods section 8, Method II) prior to resuspension in minimal broth lacking thymine, the growth in terms of colony-forming ability became more uniform (Fig. 8). The loss of colony-forming ability did not occur. However, where the second phase of reduction in viable count should have taken place, there occurred, instead, a stationary phase. The length of the stationary phase was found to be variable with each experiment. Subsequently Method II was employed for washing the cells prior to resuspension in the growth medium.

c. Effect of Inoculum Size on Growth of E. coli B27T⁻ in Minimal Broth Lacking Thymine

The concentration of E. coli B27T⁻ cells used to inoculate minimal broth lacking thymine was varied to determine the effect of inoculum size on the growth of strain B27T⁻ under conditions of thymine starvation. If growth was the result of cross-feeding among the population, dilution of the initial population may prevent growth and result in thymine-less death. Also, if E. coli B27T⁻ cells possessed intracellular pools of thymine used for DNA synthesis in the absence of exogenous thymine, dilution of the initial inoculum in minimal medium lacking thymine may permit the culture to grow only for the number of generations equivalent to growth at a higher initial inoculum. A terminal cell concentration of 2×10^9 cells/ml would not be attained under these conditions. Cells were grown and resuspended in minimal medium lacking thymine concentration of 2×10^8 cells/ml. The culture was diluted in minimal medium lacking thymine to yield

Fig. 8. Effect on colony-forming ability of warm washing on E. coli B27T⁻ cells. E. coli B27T⁻ was grown to early logarithmic phase in minimal broth containing thymine, centrifuged, washed by the room temperature method (Method II), and resuspended in minimal broth lacking thymine. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).



cultures containing an initial concentration of 10^5 , 10^4 , 10^3 and 10^2 cells/ml, and the cultures were shaken at 37 C. Growth curves were obtained (Fig. 9) indicating that E. coli B27T⁻ grew from initial concentrations of anywhere from 10^4 to 10^8 cells/ml to a terminal concentration of 2×10^9 cells/ml. In contrast, a culture of E. coli B27T⁻ containing, initially, 10^3 (or 10^2) cells/ml lost colony-forming ability rapidly after a growth period of less than one generation. Additional information relating to culture dilution was obtained by making a 10^{-5} dilution into minimal broth lacking thymine of a culture (Fig. 9, curve a) previously grown for 9.5 hr. The diluted culture (Fig. 9, curve f) was shaken at 37 C, and the number of colony-forming units was observed to increase at the same rate as the parent culture. A terminal concentration of 2×10^9 cells/ml was attained. However, a 10^{-6} dilution (Fig. 9, curve g) of the parent culture (Fig. 9, curve a) resulted in thymineless death following a brief lag phase. The results showed that E. coli B27T⁻ did not contain unusual intracellular pools of thymine (or a derivative) and that cells of this mutant did not adapt in an irreversible manner to growth in the absence of exogenous thymine. Additional evidence that pools were not involved was obtained by starving E. coli B27T⁻ in saline for various lengths of time at 37 C prior to resuspension in minimal medium lacking thymine (Fig. 10). Starved cultures grew equally as well as the unstarved control. Starvation, however, appeared to decrease the initial loss of colony-forming ability. In contrast, dilution experiments employing the parent strain, E. coli B27, showed that

Fig. 9. Effect of initial cell concentration on the growth of E. coli B27T⁻ in minimal broth lacking thymine. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed, resuspended in minimal broth lacking thymine at 10^8 cells/ml (curve a), and shaken in a 37 C waterbath. Serial ten-fold dilutions in minimal broth lacking thymine were prepared from this culture (curve a) to yield cultures containing 10^5 , 10^4 , 10^3 , and 10^2 cells/ml (curves b, c, d, and e, respectively). Curve f was obtained from a 10^{-5} dilution in minimal broth lacking thymine of the culture (curve a) which was grown for 9.5 hr. At the same time, a 10^{-6} dilution (curve g) was made of the same culture (curve a). Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).

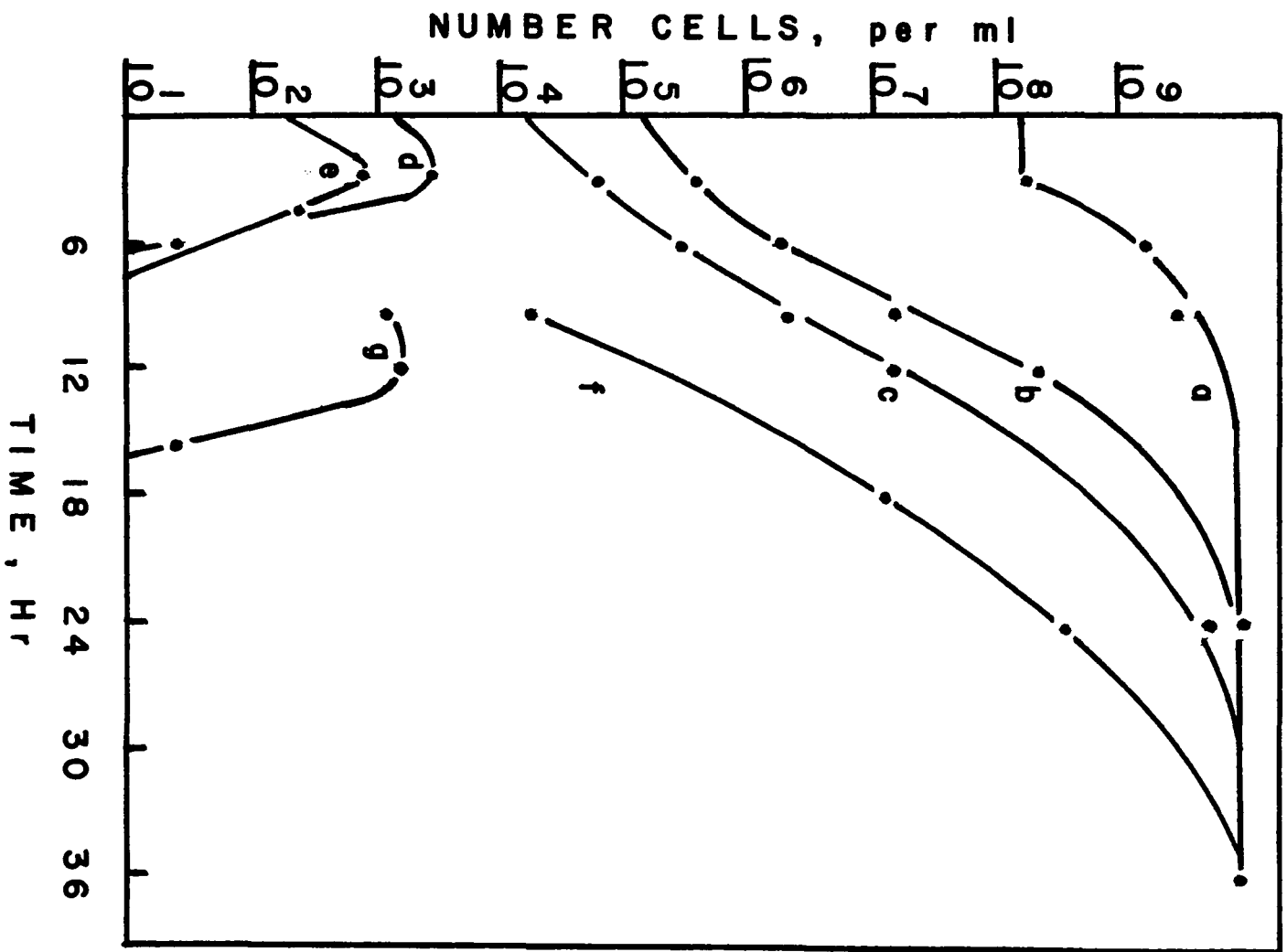
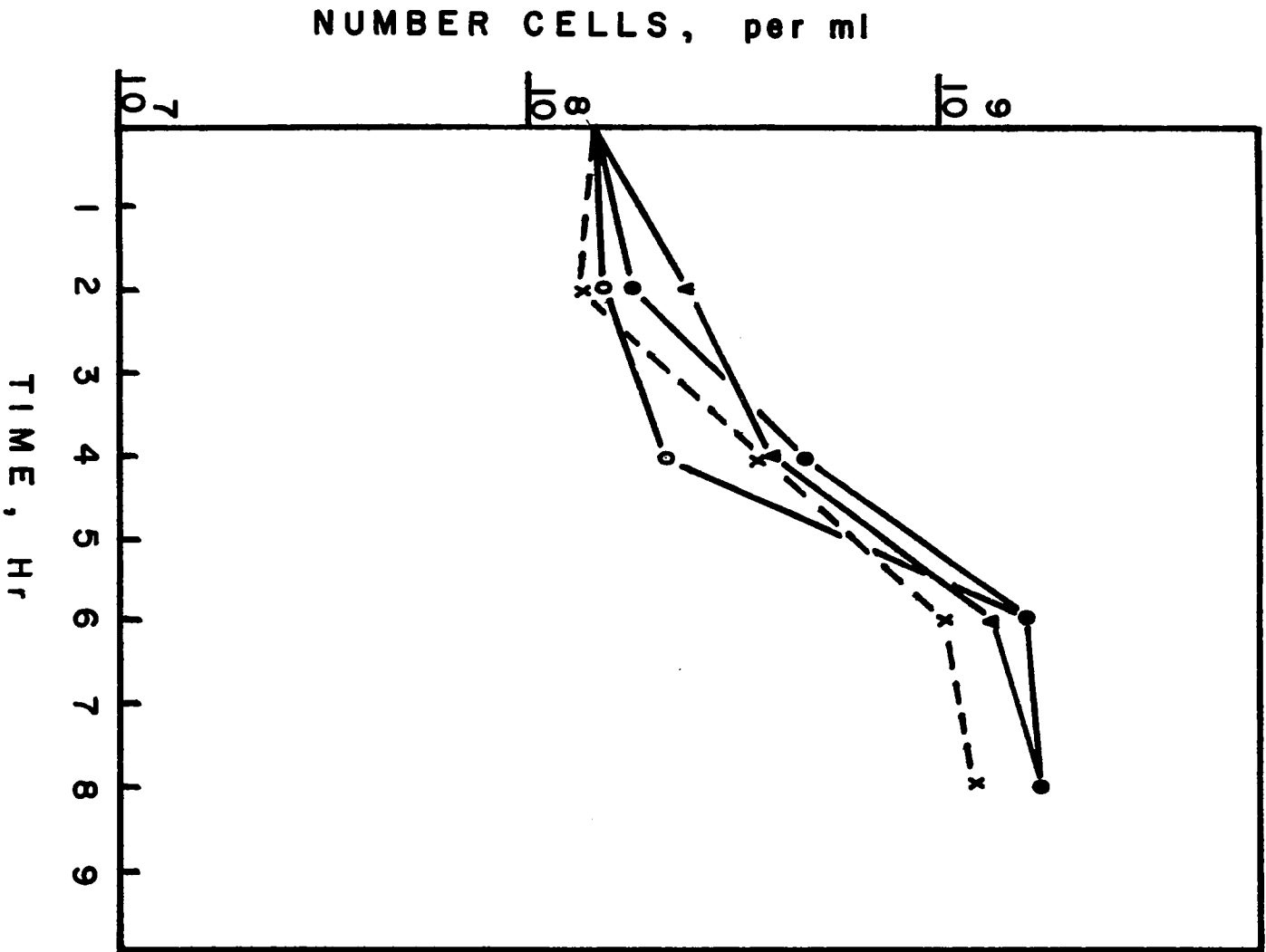


Fig. 10. Effect of starvation of E. coli B27T⁻ prior to resuspension in minimal broth lacking thymine. Two 100 ml cultures of E. coli B27T⁻ were grown to early logarithmic phase in minimal broth containing thymine. These cultures were pooled, centrifuged, washed, and resuspended in saline. The suspension was then divided into four 50 ml cultures. The cultures were shaken in a 37 C waterbath for 0, 30, 60, and 120 min, centrifuged, and resuspended in 50 ml of minimal broth lacking thymine. These cultures were then shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Cultures starved for 30 (○), 60 (●), 120 (▼) min (————). Unstarved control (x----x).



growth occurred in minimal broth lacking thymine from an initial inoculum as low as 10^3 cells/ml (Fig. 11).

Growth of E. coli B27T⁻ in minimal medium lacking thymine from an initial inoculum as low as 10^4 cells/ml suggested that cross-feeding was not responsible for growth. Evidence for excretion of protective nutrients into the medium was sought by examining the effect of E. coli B27T⁻, supernatant minimal broth lacking thymine on the growth of E. coli Y melT⁻ and E. coli 15T⁻, strains that were susceptible to thymineless death.

d. Effect of E. coli B27T⁻ Supernatant Minimal Broth Lacking Thymine, Supplemented with Inorganic Salts, Dextrose, Casamino Acids, and Tryptophan on the Growth of E. coli Y melT⁻, E. coli 15T⁻, and E. coli B27T⁻

The possibility that E. coli B27T⁻ excreted nutrient or protective substances into the medium during thymine starvation was investigated. E. coli Y melT⁻ (or 15T⁻) was resuspended in B27T⁻ supernatant minimal medium lacking thymine that had been supplemented with fresh nutrients in order to determine if the supernatant medium could protect E. coli Y melT⁻ (or E. coli 15T⁻) from thymineless death. The growth in mid-logarithmic phase (5 hr) B27T⁻ supernatant broth was compared to growth in minimal broth lacking thymine. The supplemented, supernatant medium from E. coli B27T⁻ failed to prevent loss of colony-forming ability by either E. coli Y melT⁻ (Fig. 12) or E. coli 15T⁻ (Fig. 13), although the onset of the lethal event was delayed about 60 min. Similar results (not shown) were obtained for thymine-requiring

Fig. 11. Effect of initial cell concentration on the growth of E. coli B27 in minimal broth containing thymine. An early logarithmic phase culture of E. coli B27 grown in minimal broth containing thymine was centrifuged, washed, and resuspended in minimal broth lacking thymine at a concentration of 10^8 cells/ml (curve a). At the same time, serial ten-fold dilutions were prepared in minimal broth lacking thymine to yield cultures containing 10^4 and 10^3 cells/ml (curves b and c). Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).

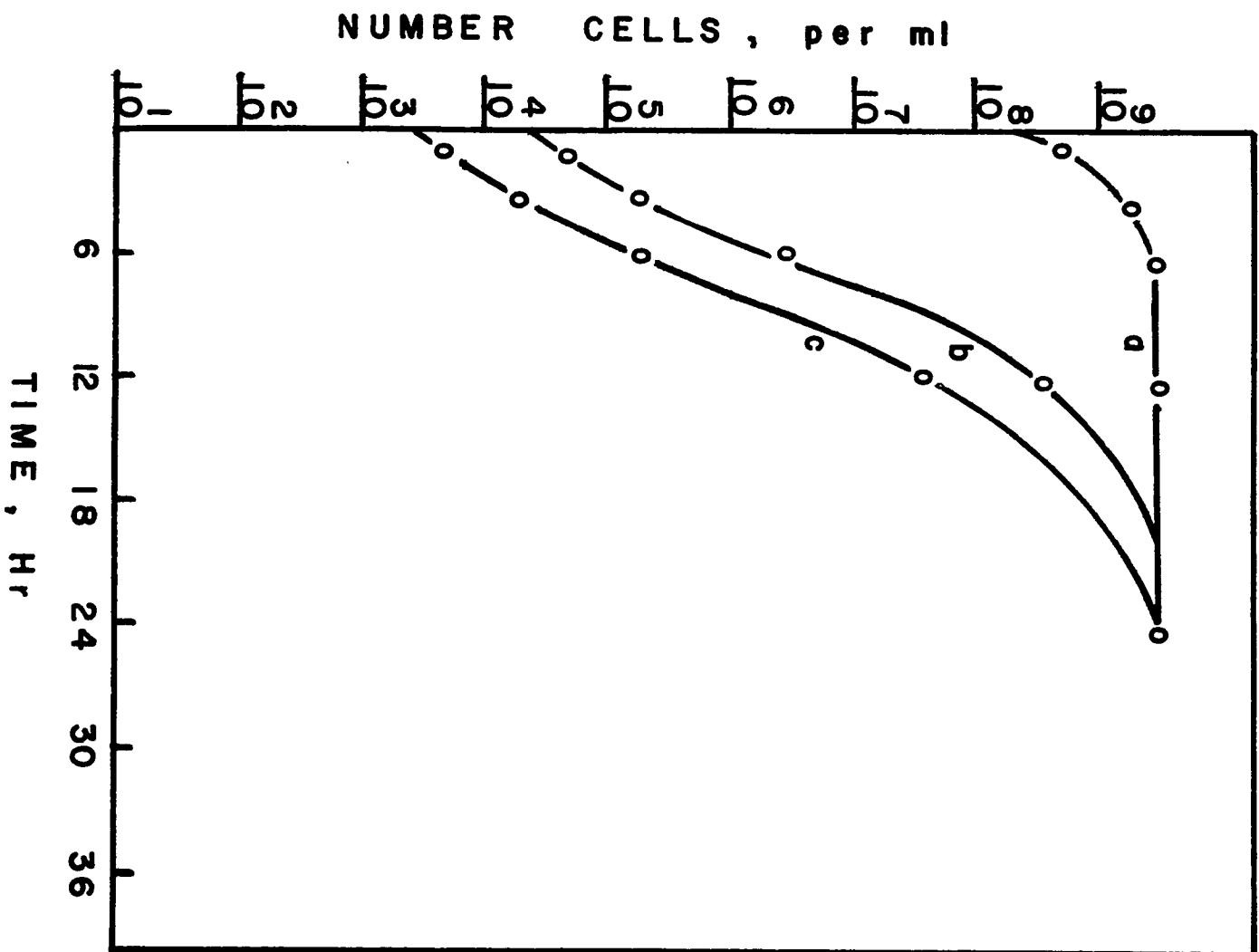


Fig. 12. Effect of mid-logarithmic phase E. coli B27T⁻ supernatant medium on the growth of E. coli Y melT⁻. An early logarithmic phase culture of E. coli Y melT⁻ grown in minimal broth lacking thymine was centrifuged, washed, and resuspended in either minimal broth lacking thymine or mid-logarithmic phase (5 hr) E. coli B27T⁻ supernatant minimal broth lacking thymine supplemented with fresh inorganic salts, dextrose, casamino acids, and tryptophan. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Minimal broth lacking thymine, X; E. coli B27T⁻ supplemented, supernatant broth, O; E. coli B27T⁻ supernatant broth without supplemented inorganic salts, ▼; E. coli B27T⁻ supplemented, supernatant broth containing 5 μg of thymine/ml, ●. (---) represents thy⁺ revertants obtained by plating a portion of the culture on minimal agar plates lacking thymine.

Inset: Turbidity of E. coli Y melT⁻ growing in minimal broth lacking thymine, X, and E. coli B27T⁻ supplemented, supernatant broth, O.

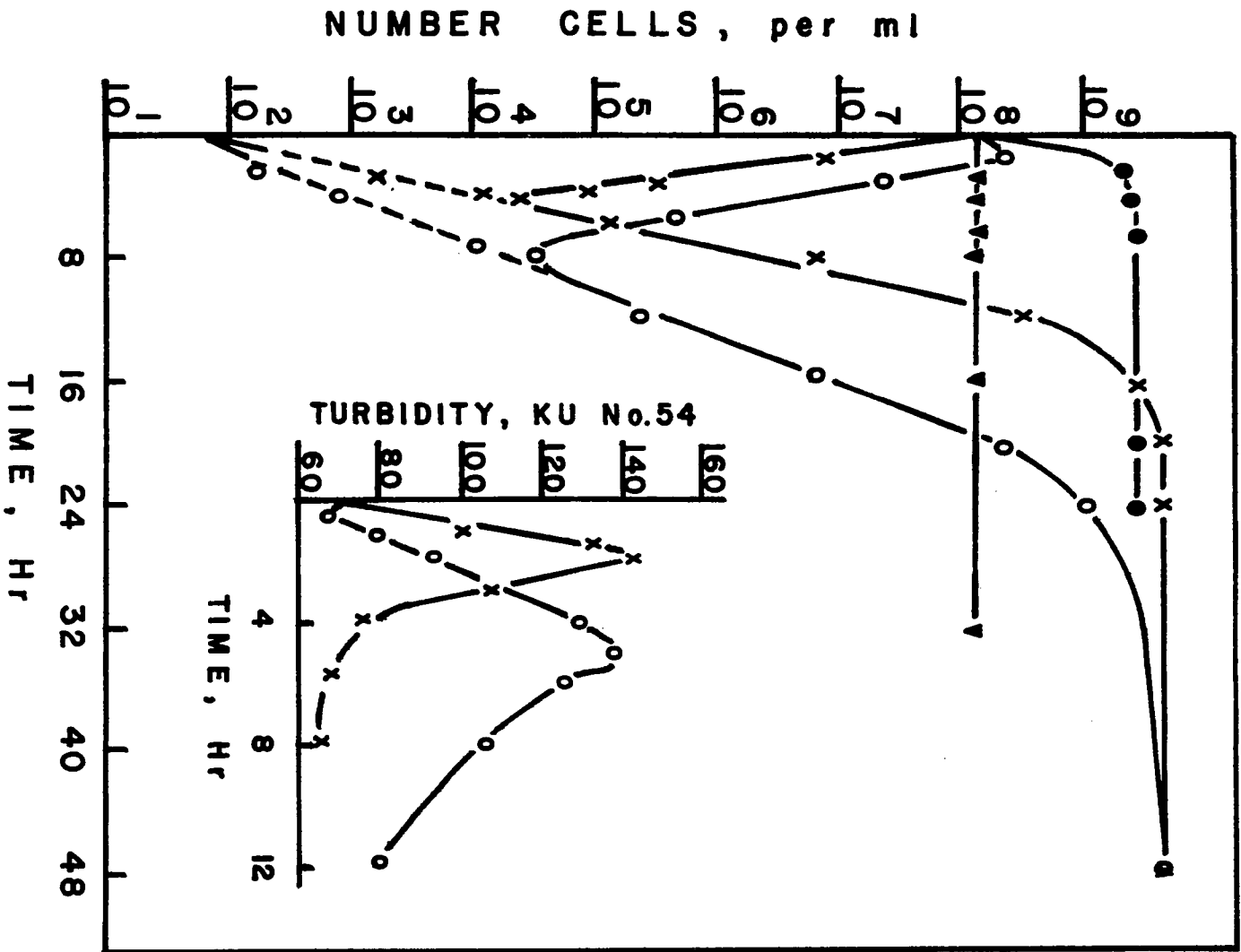
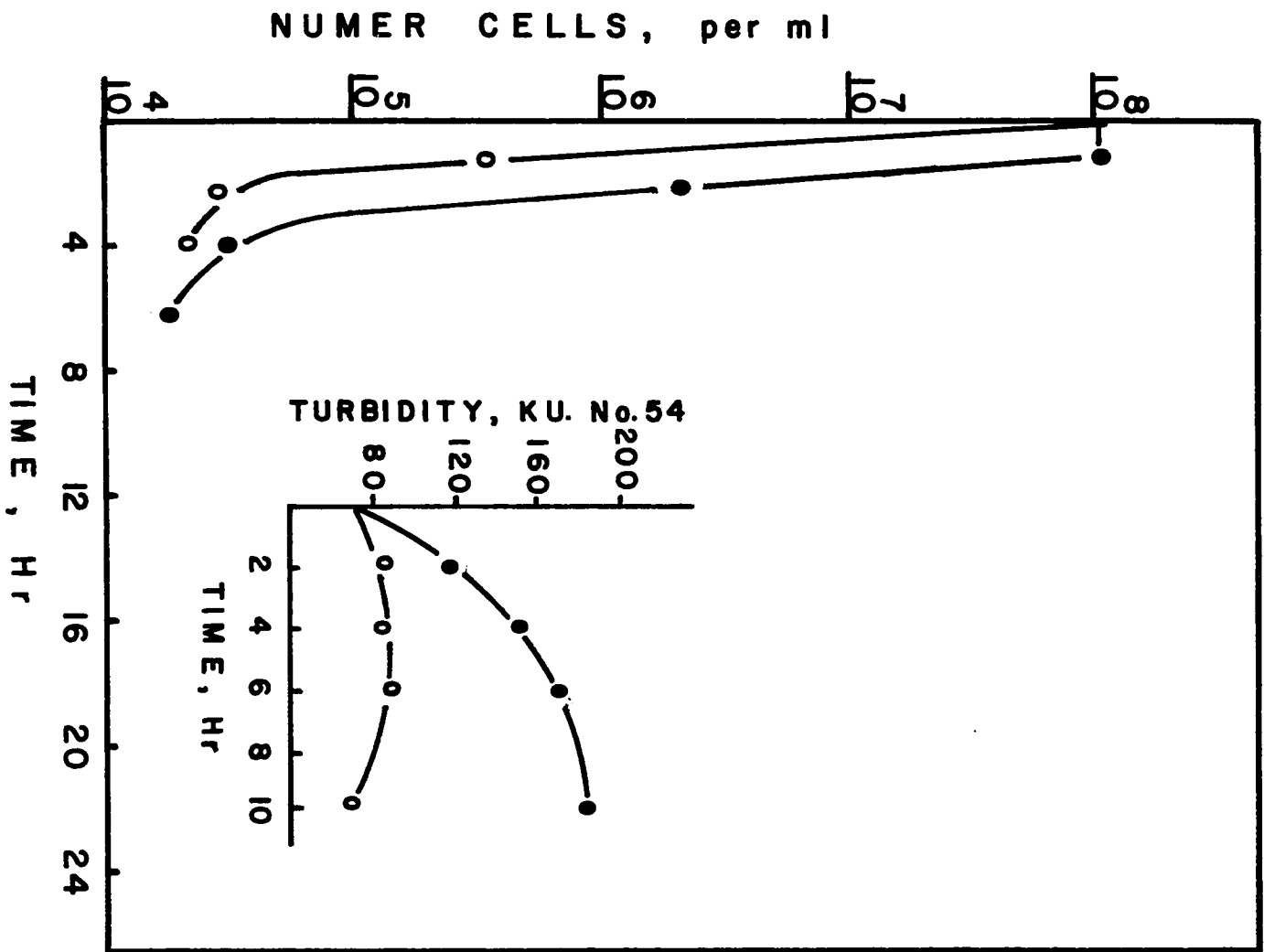


Fig. 13. Effect of mid-stationary phase E. coli B27T⁻ supernatant medium on the growth of E. coli B15T⁻. An early logarithmic phase culture of E. coli 15T⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in either minimal broth lacking thymine or in mid-stationary phase (24 hr) E. coli B27T⁻ supernatant minimal broth lacking thymine supplemented with fresh inorganic salts, dextrose, casamino acids, and tryptophan. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Minimal broth lacking thymine, 0; E. coli B27T⁻ supplemented, supernatant broth, ●.

Inset: Turbidity of E. coli 15T⁻ growing in minimal broth lacking thymine, 0, and E. coli B27T⁻ supplemented, supernatant broth, ●.



mutant, E. coli 307c. Elimination of the inorganic salt supplement from the E. coli B27T⁻ supernatant, minimal broth lacking thymine (Fig. 12) did not permit growth of E. coli Y melT⁻ but did prevent thymineless death. Since the addition of 5 μ g of thymine/ml to the E. coli B27T⁻ supplemented, supernatant medium (Fig. 12) permitted growth of E. coli Y melT⁻, thymineless death of this strain in the E. coli B27T⁻ supplemented, supernatant medium was not the result of an inhibitory substance excreted by E. coli B27T⁻. Measurements of turbidity (Fig. 12) showed that the cellular mass of E. coli Y melT⁻ increased during the period of thymineless death. A marked decrease in the turbidity of this strain occurred near the time of maximum loss of colony-forming ability in both minimal broth lacking thymine and E. coli B27T⁻ supplemented, supernatant broth. However, the decrease was less severe in E. coli B27T⁻ supplemented, supernatant broth.

E. coli B27T⁻ supplemented, supernatant medium obtained from a stationary phase culture (24 hr) of E. coli B27T⁻ also did not protect E. coli Y melT⁻ from thymineless death (Fig. 14). Thus, no substance which protected E. coli Y melT⁻ (or E. coli 15T⁻) from thymineless death was present in logarithmic or stationary phase E. coli B27T⁻ supplemented, supernatant broth.

The effect of E. coli B27T⁻ supplemented, supernatant medium on E. coli B27T⁻ was also investigated. Where the cold method of washing cells (Method I) was applied (Fig. 15), stationary phase (24 hr) E. coli B27T⁻ supplemented, supernatant

Fig. 14. Effect of mid-stationary phase E. coli B27T⁻ supernatant medium on the growth of E. coli Y melT⁻. An early logarithmic phase culture of E. coli Y melT⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in either mid-stationary phase (24 hr) E. coli B27T⁻ supernatant minimal broth lacking thymine supplemented with fresh inorganic salts, dextrose, casamino acids, and tryptophan (O) or in mid-stationary phase E. coli B27T⁻ supplemented, supernatant medium containing 5 μ g of thymine/ml (●).

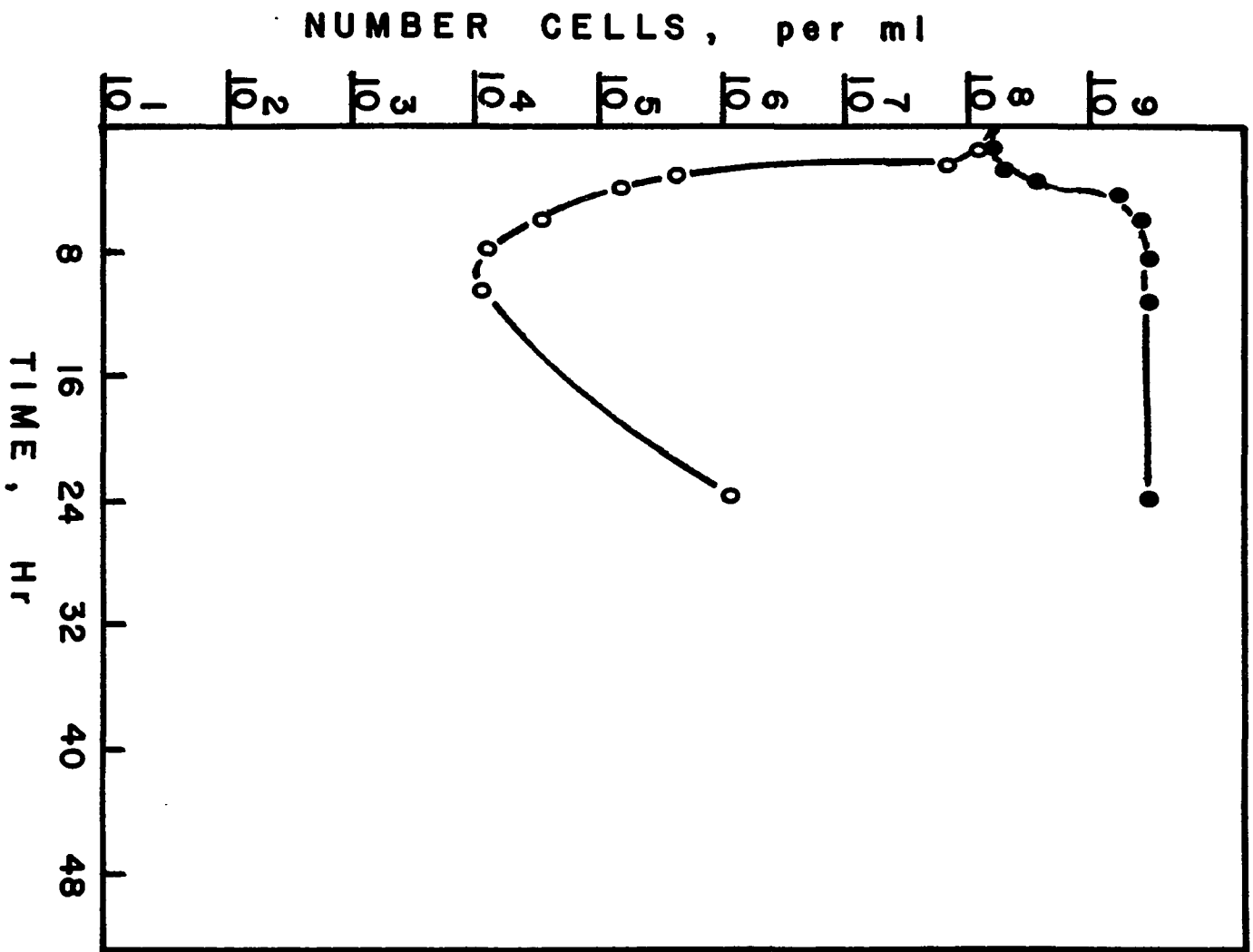
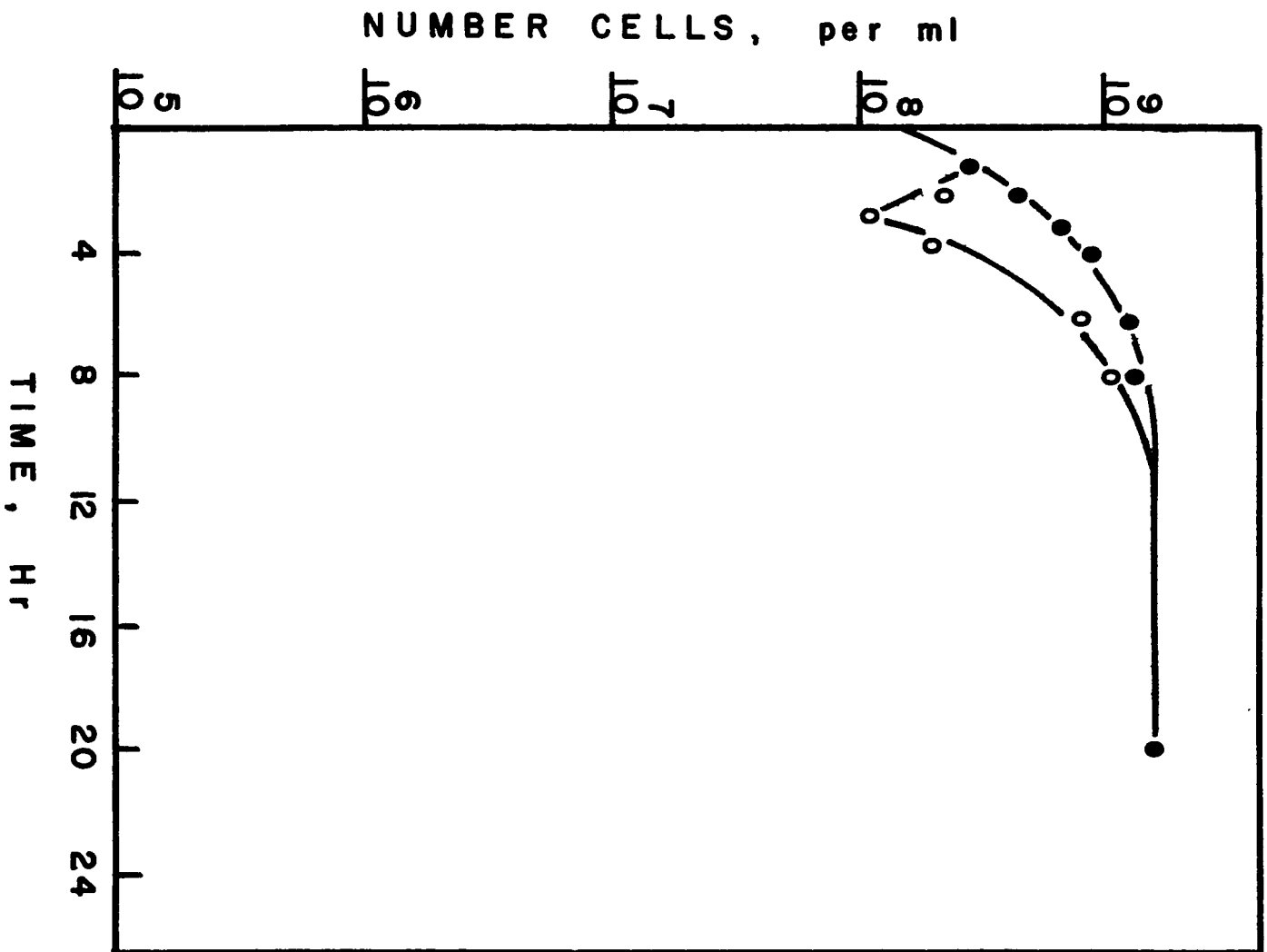


Fig. 15. Effect of mid-stationary phase E. coli B27T⁻ supernatant medium on the growth of E. coli B27T⁻. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed by the cold method (Method I), and resuspended in either minimal broth lacking thymine, 0, or in mid-stationary phase (24 hr) E. coli B27T⁻ supernatant minimal broth lacking thymine supplemented with fresh inorganic salts, dextrose, casamino acids, and tryptophan, ●. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).



broth protected E. coli B27T⁻ from the brief initial loss of colony-forming ability found with a comparable culture grown in minimal broth lacking thymine. Where cells were washed at room temperature (Fig. 16), cells grown in minimal broth lacking thymine and mid-logarithmic phase (5 hr) E. coli B27T⁻ supplemented, supernatant broth had similar initial growth rates. However, cells grown in E. coli B27T⁻ supplemented, supernatant broth had a lower terminal cell concentration compared with cells grown in minimal broth lacking thymine. In another experiment (Fig. 17) E. coli B27T⁻ cells were resuspended in E. coli B27T⁻ supplemented, supernatant broth (24 hr) at an initial cell concentration of 10^3 cells/ml. The data from this experiment showed that E. coli B27T⁻ supplemented, supernatant broth permitted only an initial phase of growth that resulted in an increase in number of viable cells equivalent to about two generations. This was followed by a slow loss of colony-forming ability. The rapid loss of colony-forming ability associated with growth of E. coli B27T⁻ from 10^3 cells/ml in minimal broth lacking thymine (Fig. 9) was not observed. On the other hand, when E. coli B27T⁻ supplemented, supernatant medium was supplemented with $5\mu\text{g}$ of thymine/ml (Fig. 17), the culture attained a terminal concentration of 1.7×10^9 cells/ml after 24 hr. Thus, E. coli B27T⁻ supplemented, supernatant broth protected E. coli B27T⁻ from thymineless death, but a significant increase in viable cell count was obtained only upon the addition of thymine.

Fig. 16. Effect of mid-logarithmic phase E. coli B27T⁻ supernatant medium on the growth of E. coli B27T⁻. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed at room temperature (Method II), and resuspended in either minimal broth lacking thymine, ●, or in mid-logarithmic phase (5 hr) E. coli B27T⁻ supernatant minimal broth lacking thymine supplemented with fresh inorganic salts, dextrose, casamino acids, and tryptophan, ○. Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).

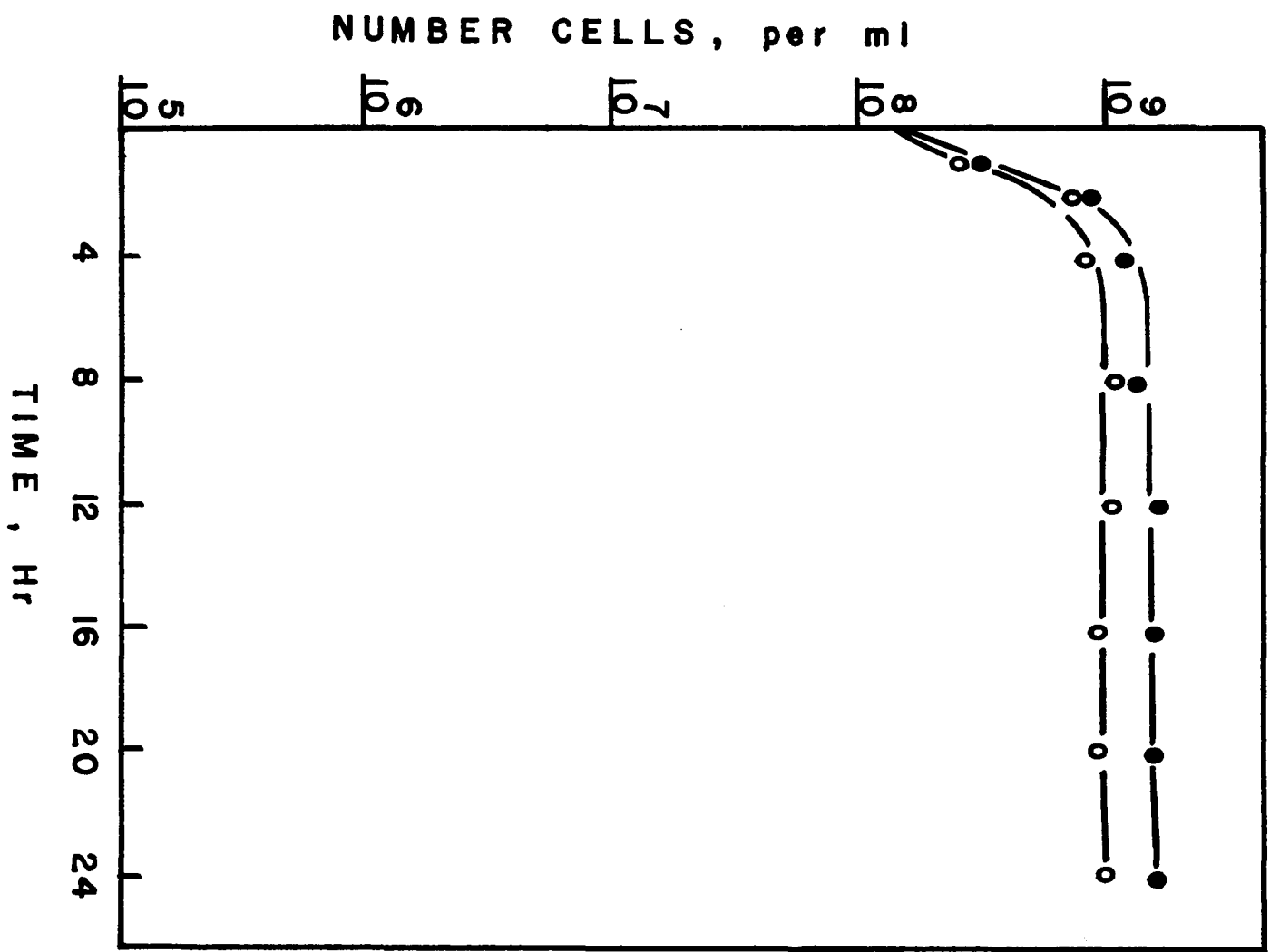
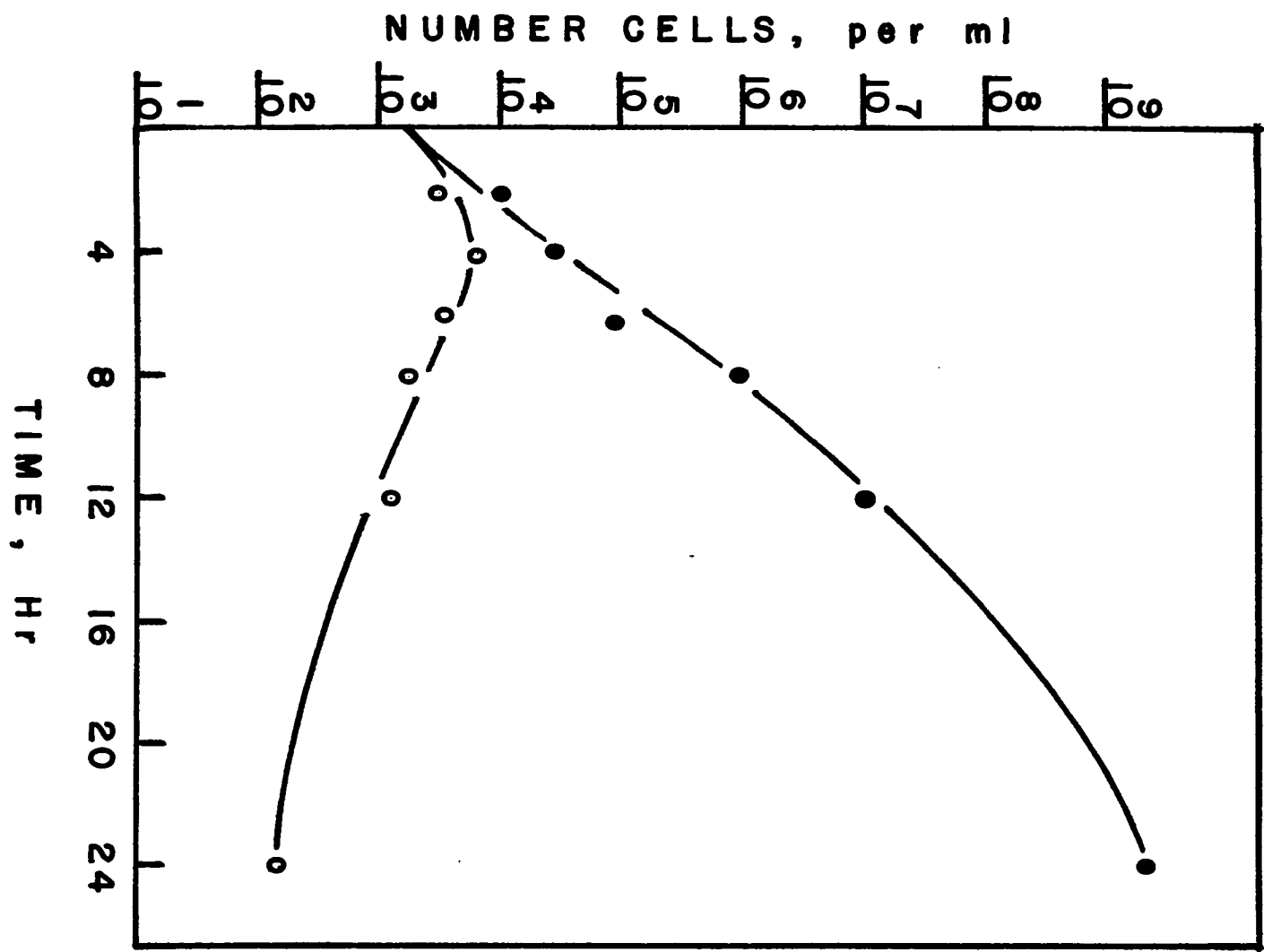


Fig. 17. Protection of E. coli B27T⁻ at a low cell concentration from thymineless death by mid-stationary phase E. coli B27T⁻ supernatant medium. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in minimal broth lacking thymine at a concentration of 2×10^8 cells/ml. The culture was diluted to a concentration of 2×10^3 cells/ml in either mid-stationary phase (24 hr) E. coli B27T⁻ supernatant minimal broth lacking thymine supplemented with fresh inorganic salts, dextrose, casamino acids, and tryptophan (O) or in mid-stationary phase E. coli B27T⁻ supplemented, supernatant broth containing $5 \mu\text{g}$ of thymine/ml (●). Cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).



e. Effect of the F'araB24 Plasmid on the Growth of E. coli B27T⁻ in Minimal Broth Lacking Thymine

The cistrons of the deo operon, which specify the enzymes involved in the catabolism of nucleosides and deoxynucleosides, are clustered between the hsp (host specificity) and ser B (serine biosynthesis) loci on the chromosome map of Escherichia coli (Ahmad and Pritchard, 1969) (Appendix 1). These enzymes confer on the cell the ability to utilize nucleosides or deoxynucleosides (e.g. thymidine) as a sole source of carbon (Dale and Greenberg, 1967). Double mutants, which are defective in the deo B or deo C regions and also in the structural gene for thymidylate synthetase (thy A), require low concentrations of thymine for growth (Lomax and Greenberg, 1968).

The F'araB24 plasmid is an extrachromosomal element which carries the markers of the deo region and the threonine region but possesses a mutation in the marker specifying arabinose utilization. Merodiploids of the genotype thy A⁻ deo B⁻/F'araB24 or thy A⁻ deo C⁻/F'araB24 require high concentrations (> 20 μ g/ml) of thymine for growth (Lomax and Greenberg, 1968), indicating that prototrophs of the deo B and deo C cistrons contain dominant alleles. The F'araB24 plasmid was employed to determine if the deo region of the chromosome had an effect upon the growth of E. coli B27T⁻ in minimal medium lacking thymine. It was anticipated that the merodiploid, E. coli B27T⁻/F'araB24, would require a high concentration of thymine and would utilize thymidine as a sole source of carbon.

E. coli B27T⁻ thr⁻/F'araB24 was isolated following the mating of E. coli B27T⁻thr⁻ with E. coli araB24/F'araB24. From this cross, in which approximately one in 10⁵ recipients was thr⁺, none of the thr⁺ isolates were found to utilize thymidine as a sole source of carbon. The F'araB24 plasmid was shown to exist in the episomal state in the isolated colonies of E. coli B27T⁻thr⁻/F'araB24 by mating such isolates with E. coli AB1157 (thr⁻) and selecting against the donor on medium lacking threonine, tryptophan and thymine. Threonine-independent recipients represented 85% of the recipient population (Table 8), indicating that the plasmid harbored by E. coli B27T⁻thr⁻/F'araB24 possessed the determinants for the transfer of the plasmid in addition to the threonine marker.

The effect of the F'araB24 plasmid on the growth of E. coli B27T⁻ was then investigated (Fig. 18, 19). The merodiploid, E. coli B27T⁻thr⁻/F'araB24, grew like the parent strain in minimal broth containing thymine. The parent, E. coli B27T⁻thr⁻, was also capable of growing in minimal broth lacking thymine to a concentration of about 1 x 10⁹ cells/ml. In contrast, merodiploid, E. coli B27T⁻thr⁻/F'araB24, lost colony-forming ability when resuspended in minimal broth lacking thymine, and at six hr the initial population had been reduced by approximately 90%. After six hr an increase in viable cells was apparent. An investigation of the surviving colonies showed the delayed growth phase was a result of the loss of the plasmid from E. coli B27T⁻thr⁻/F'araB24 (Table 9). Colonies from ENB plates were transferred by replica plating

Table 8. Transfer of the thr marker of E. coli B27T⁻thr⁻/F'araB24 to E. coli AB1157

<u>E. coli</u> Strain	Number Cells/ml		<u>thr</u> ⁺ Recipients, %
	<u>thr</u> ⁺	<u>thr</u> ⁻	
AB1157	1.1 x 10 ⁹	280	
B27T ⁻ thr ⁻ / <u>F'araB24</u>	0	0	
B27T ⁻ thr ⁻ / <u>F'araB24</u> X (AB1157)	6.7 x 10 ⁸	5.7 x 10 ⁸	85.1

E. coli B27T⁻thr⁻/F'araB24, thr⁺ and E. coli AB1157 (thr⁻) (1:3) were mated statically for 4 hr at 37 C in penassay broth. Thr⁺ recipient cells were selected on Vogel-Bonner glucose-salts medium containing leucine, proline, histidine, and arginine (20 μg/ml ea.) and B₁ (5 μg/ml). Threonine (20 μg/ml) was added to obtain total counts. Controls consisted of each strain cultured separately.

Fig. 18. Effect of the F'araB24 plasmid on colony-forming ability of E. coli B27T⁻thr⁻. Early logarithmic phase cultures of E. coli B27T⁻thr⁻/F'araB24 and E. coli B27T⁻thr⁻ grown separately in minimal broth containing thymine were centrifuged, washed, and resuspended in minimal broth lacking thymine. The cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). E. coli B27T⁻thr⁻ in minimal broth lacking thymine, X; E. coli B27T⁻thr⁻/F'araB24 in minimal broth lacking thymine, O; E. coli B27T⁻thr⁻/F'araB24 in minimal broth containing thymine, ●.

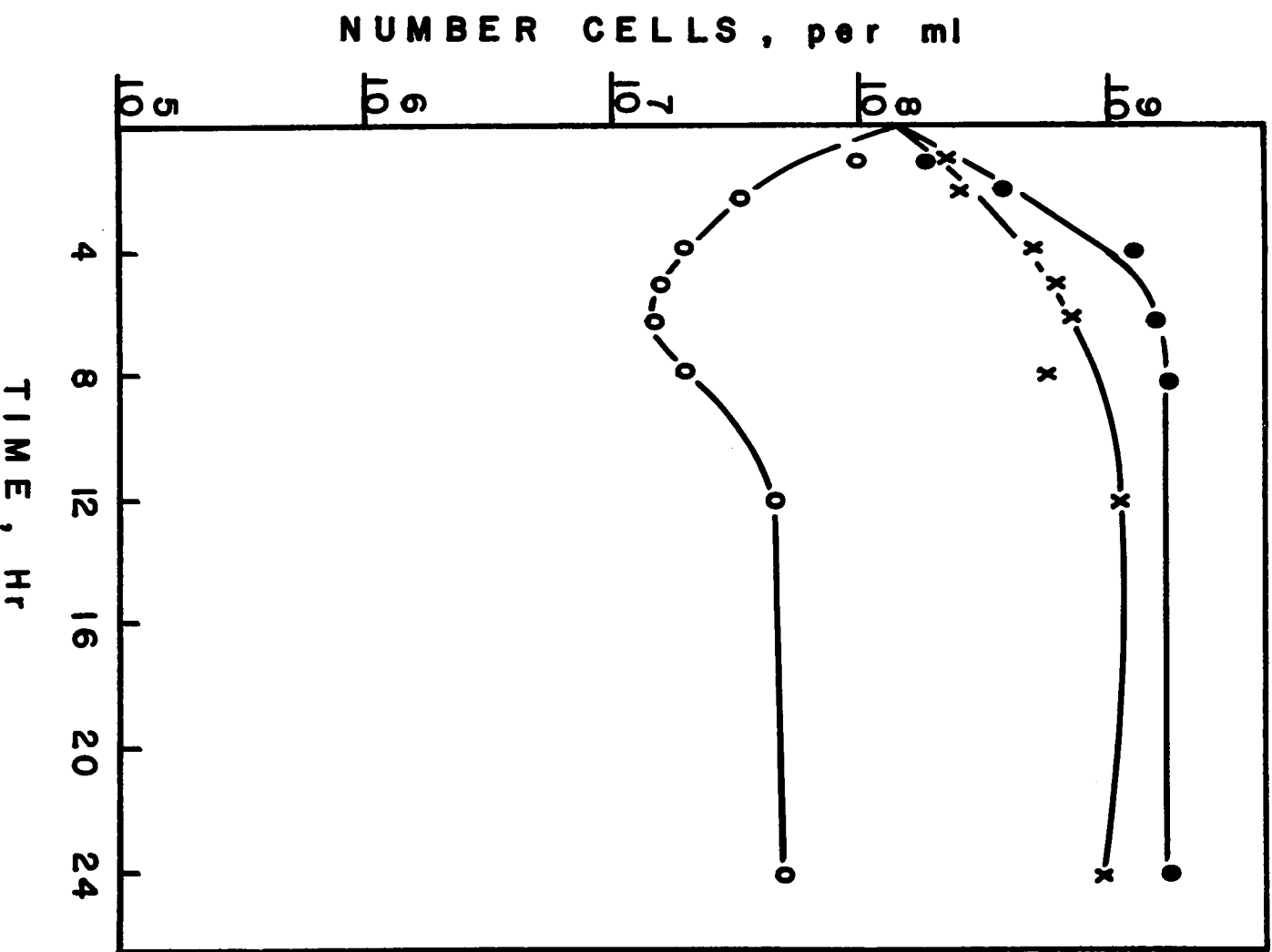


Fig. 19. Effect of the F'araB24 plasmid on the turbidity measurements of E. coli B27T⁻thr⁻. Early logarithmic phase cultures of E. coli B27T⁻thr⁻/F'araB24 and E. coli B27T⁻thr⁻ grown separately in minimal broth containing thymine were centrifuged, washed, and resuspended in minimal broth lacking thymine. The cultures were shaken in a 37 C waterbath, and turbidity measurements were obtained using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter. E. coli B27T⁻thr⁻ in minimal broth lacking thymine or E. coli B27T⁻thr⁻/F'araB24 in minimal broth containing thymine, ●; E. coli B27T⁻thr⁻/F'araB24 in minimal broth lacking thymine, ○.

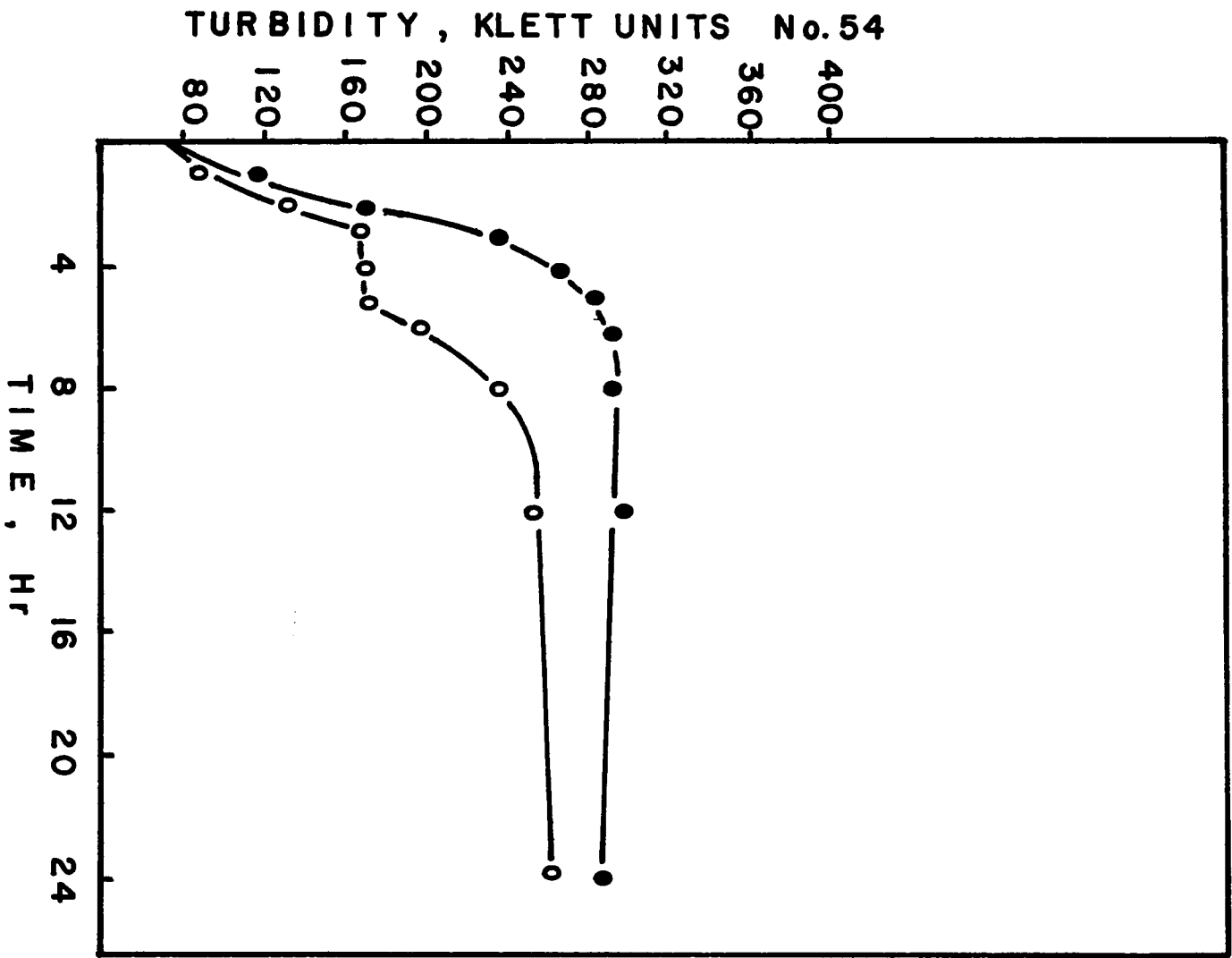


Table 9. Genotype of survivors of E. coli B27T⁻thr⁻/F'araB24 after exposure to thymine starvation conditions

Exposure to Thymine Starvation (hr)	Number Colonies Examined	Percentage	
		<u>thr</u> ⁻	<u>thr</u> ⁺
0	231	0.9	98.8
2	84	0	94.2
6	246	34.1	54.9
12	90	63.4	34.5

ENB plates containing surviving colonies of E. coli B27T⁻thr⁻/F'araB24 grown in minimal broth lacking thymine were replica plated to minimal agar containing thymine but lacking threonine and to the same medium containing threonine. The ENB plates were then further replicated on minimal medium lacking threonine, thymine, and tryptophan but previously spread with a lawn of E. coli AB1157 (thr⁻) cells (10⁸ cells/plate) to test for the ability of the survivors to transfer the plasmid (thr⁺). The plates were incubated 48 hr at 37 C, and the colonies were counted.

to minimal plates supplemented with threonine, to plates lacking threonine, and to plates lacking threonine, tryptophan, and thymine which had previously been spread with a lawn of E. coli AB1157(thr⁻) cells. The data show that an increasing proportion of the cells growing in minimal broth lacking thymine lost the ability to complement the threonine deficiency of E. coli AB1157. This suggested that E. coli B27T⁻thr⁻/F'araB24 had lost the F'araB24 plasmid and reverted to the threonine-requiring mutant, E. coli B27T⁻thr⁻, during prolonged exposure to thymine starvation. These experiments, however, showed that the introduction of the F'araB24 plasmid into E. coli B27T⁻thr⁻ resulted in an initial loss of colony-forming ability during thymine starvation in a manner similar to classical thymineless death though less severe. The recovery of colony-forming ability, after thymineless death (12 hr), suggested that a growth factor such as threonine was exhausted. In addition, E. coli B27T⁻thr⁻/F'araB24 may harbor a F'araB24 plasmid defective in one or more of the deo cistrons since it was unable to utilize thymidine as a sole source of carbon. Therefore, a mutant was sought which harbored the F'araB24 plasmid and was capable of utilizing thymidine.

An E. coli B27T⁻/F'araB24 strain was isolated following mating of the E. coli B27T⁻(sm^F) with E. coli araB24/F'araB24 (sm^S) and subsequent selection on minimal agar (lacking casamine acids and dextrose) supplemented with tryptophan (20 μ g/ml) and streptomycin (250 μ g/ml) and in which thymidine (500 μ g/ml) as the sole source of carbon.

An average of one thymidine-positive colony per 10^8 recipient colonies was isolated. Initially, E. coli B27T⁻/F'araB24 strains grew poorly on minimal agar plates supplemented with 5 μ g of thymine/ml, although satisfactory growth was obtained in the presence of 50 μ g of thymine/ml. However, after several transfers on ENB stock agar slants, E. coli B27T⁻/F'araB24 grew equally as well on minimal agar supplemented with 5 μ g or 50 μ g of thymine/ml.

Further information regarding the thymine requirement of E. coli B27T⁻/F'araB24 was obtained from thymine limitation experiments. Terminal turbidity measurements were obtained for cultures of E. coli B27T⁻ and E. coli B27T⁻/F'araB24 previously grown in minimal broth containing 50 μ g of thymine/ml. Media containing various concentrations of thymine (0, 0.5, 1.0, 2.0, 5.0, 10, 20, and 50 μ g/ml) was seeded with washed cells at a concentration of either 10^3 or 10^6 cells/ml. The experiment (Table 10) employing a low (10^3) cells concentration, previously shown (Fig. 8) to induce thymineless death in E. coli B27T⁻, indicated that under these conditions E. coli B27T⁻/F'araB24 required less thymine (about 5 μ g/ml) to obtain maximum growth than did E. coli B27T⁻ (about 20 μ g/ml). The presence of the F'araB24 plasmid in E. coli B27T⁻/F'araB24 was demonstrated through a complementation test using E. coli AB1157 (thr⁻). Growth occurred when the two strains were streaked together on minimal medium lacking threonine, tryptophan, and thymine on which neither strain could grow alone. In a second experiment employing a high (10^6) initial cell concentration (Table 11), E. coli B27T⁻ was shown to require

Table 10. Limiting concentration of thymine necessary for growth of E. coli B27T⁻ and E. coli B27T⁻/F'araB24 at low cell concentration

Concentration ^a	Turbidity ^b	
	<u>E. coli</u> B27T ⁻	<u>E. coli</u> B27T ⁻ / <u>F'araB24</u>
0	0	0
0.5	0	0
1	0	0
2	2	5
5	139	251
10	229	257
20	244	257
50	243	255

Cultures were grown to 2×10^8 cells/ml in minimal broth containing $50 \mu\text{g}$ of thymine/ml, centrifuged, washed, and diluted to a concentration of 10^5 cells/ml in minimal broth lacking dextrose and thymine. One-tenth of a ml of diluted cells (10^4 cells) was added to duplicate tubes containing 10 ml of minimal broth containing various concentrations of thymine. Cultures were shaken in tubes at 37 C for 24 hr.

- a. Concentration of thymine in $\mu\text{g}/\text{ml}$ added to minimal broth.
- b. Mean of duplicate turbidity measurements obtained after 24 hr using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter.

Table 11. Limiting concentration of thymine necessary for growth of E. coli B27T⁻ and E. coli B27T⁻/F'araB24 at high cell concentration

Concentration ^a	Turbidity ^b	
	<u>E. coli</u> B27T ⁻	<u>E. coli</u> B27T ⁻ /F'araB24
0	260	120
0.5	260	127
1	264	163
2	265	219
5	262	271
10	267	277
20	254	273
50	247	255

Cultures were grown to 2×10^8 cells/ml in minimal broth containing $50 \mu\text{g/ml}$ of thymine, centrifuged, washed, and resuspended in an equal volume of minimal broth lacking dextrose and thymine. One-tenth of a ml of cell suspension (10^7 cells) was added to 10 ml of minimal broth containing various concentrations of thymine. Cultures were shaken in tubes at 37 C for 24 hr.

- a. Concentration of thymine in $\mu\text{g/ml}$ added to minimal broth.
 b. Mean of duplicate turbidity measurements obtained after 24 hr using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter.

only 2 μg of thymine/ml to obtain maximum turbidity, while approximately 5 μg of thymine/ml were required for E. coli B27T⁻/F'araB24. Thus, it appeared that the thymine requirements for E. coli B27T⁻ and E. coli B27T⁻/F'araB24 were dependent on the cell concentration used to inoculate the tubes. Under conditions similar to those employed in growth experiments where high cell concentrations were employed, E. coli B27T⁻/F'araB24 required higher concentrations of thymine for maximum growth than did E. coli B27T⁻.

The effect of thymine starvation on E. coli B27T⁻/F'araB24 was investigated next. E. coli B27T⁻/F'araB24 was grown in minimal broth supplemented with 50 μg thymine/ml, centrifuged, washed, and resuspended in minimal broth lacking thymine. The growth of this strain was then followed during incubation in minimal broth lacking thymine (Fig. 20). E. coli B27T⁻/F'araB24 cells rapidly lost colony-forming ability following a brief lag phase, and after five hr incubation, the viable count was reduced by 99%. From six hr on, the number of surviving colony-forming units remained constant. Fig. 21 shows turbidity measurements obtained for E. coli B27T⁻/F'araB24 grown in minimal broth lacking thymine. The increase in turbidity observed during the initial three hr incubation in this medium was equivalent to 1.2 mass doublings. Following the initial growth phase, a marked decrease in turbidity was observed resulting in a terminal turbidity equivalent to the initial turbidity measurement. On the other hand, growth of E. coli B27T⁻/F'araB24 incubated in minimal broth containing thymine resulted in a terminal cell

Fig. 20. Effect of the F'araB24 plasmid on colony-forming ability of E. coli B27T⁻. An early logarithmic phase culture of E. coli B27T⁻/F'araB24 grown in minimal broth containing 50 μ g of thymine/ml was centrifuged, washed, and resuspended in either minimal broth lacking thymine, ●, or in minimal broth containing 5 μ g of thymine/ml, ○. The cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).

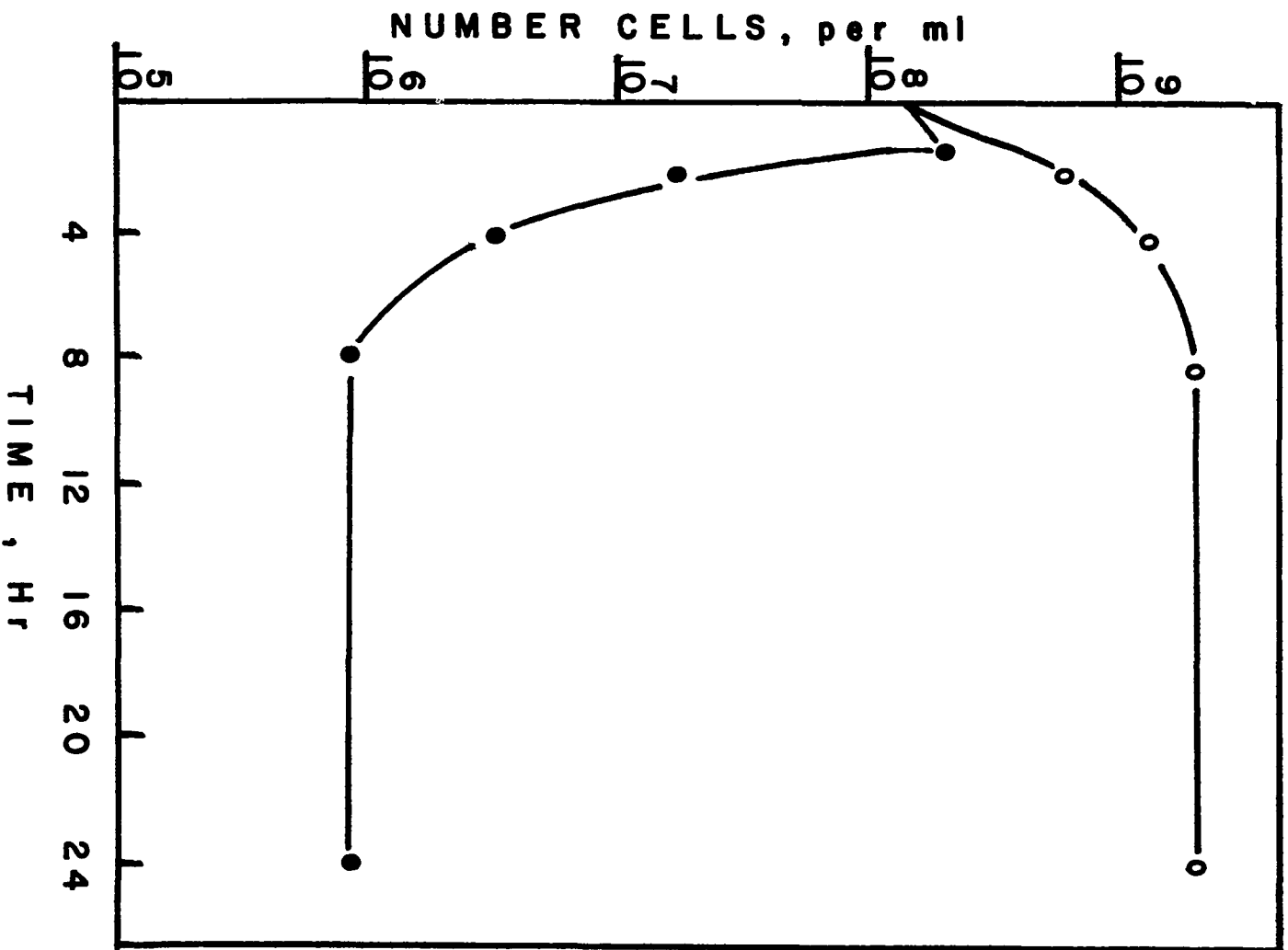
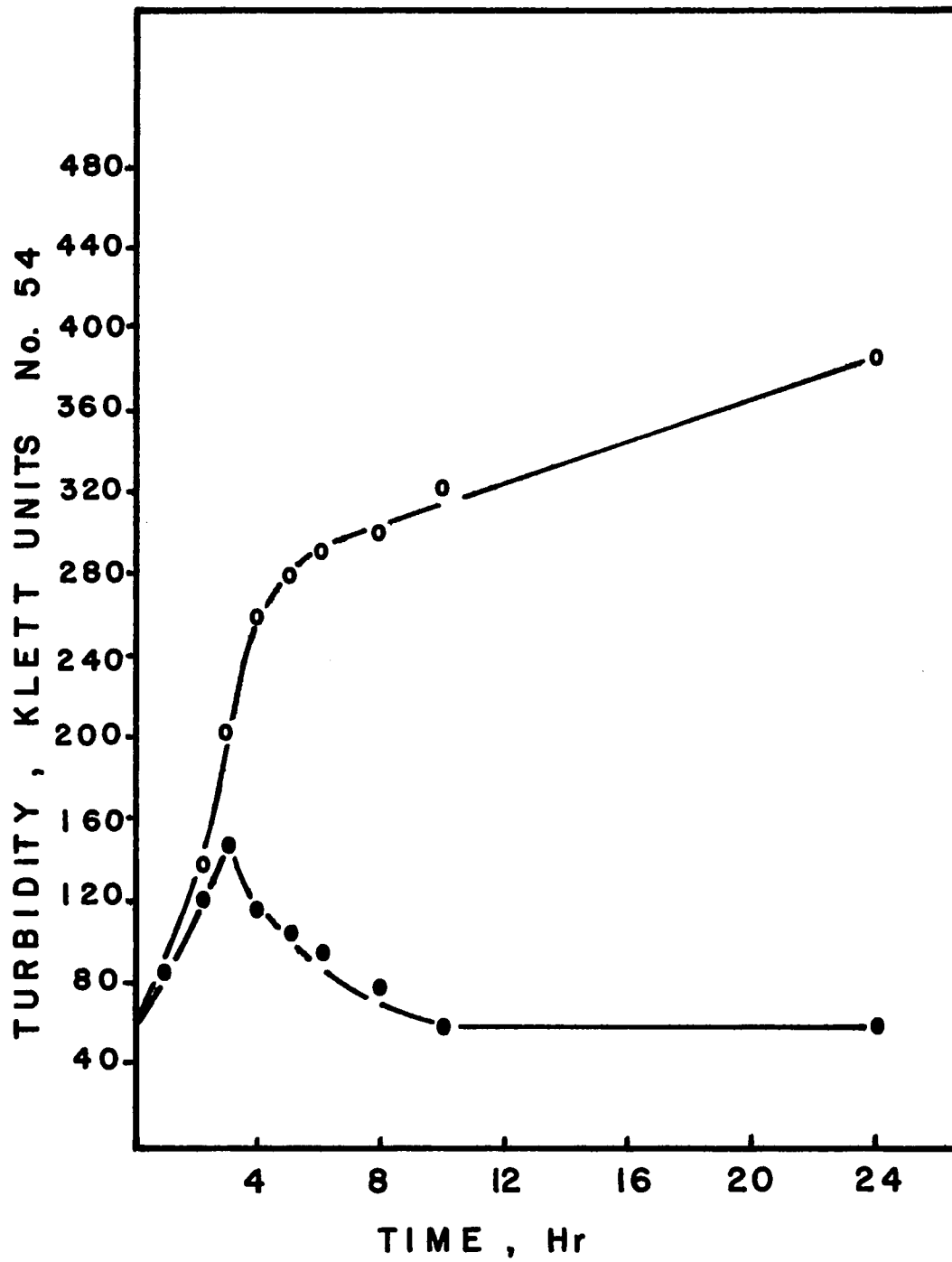


Fig. 21. Effect of the F'araB24 plasmid on the turbidity measurements of E. coli B27T⁻. An early logarithmic phase culture of E. coli B27T⁻/F'araB24 grown in minimal broth containing 50 μ g/ml of thymine was centrifuged, washed, and resuspended in either minimal broth lacking thymine, ●, or in minimal broth containing 5 μ g/ml of thymine, ○. The cultures were shaken in a 37 C waterbath, and turbidity measurements were obtained using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter.



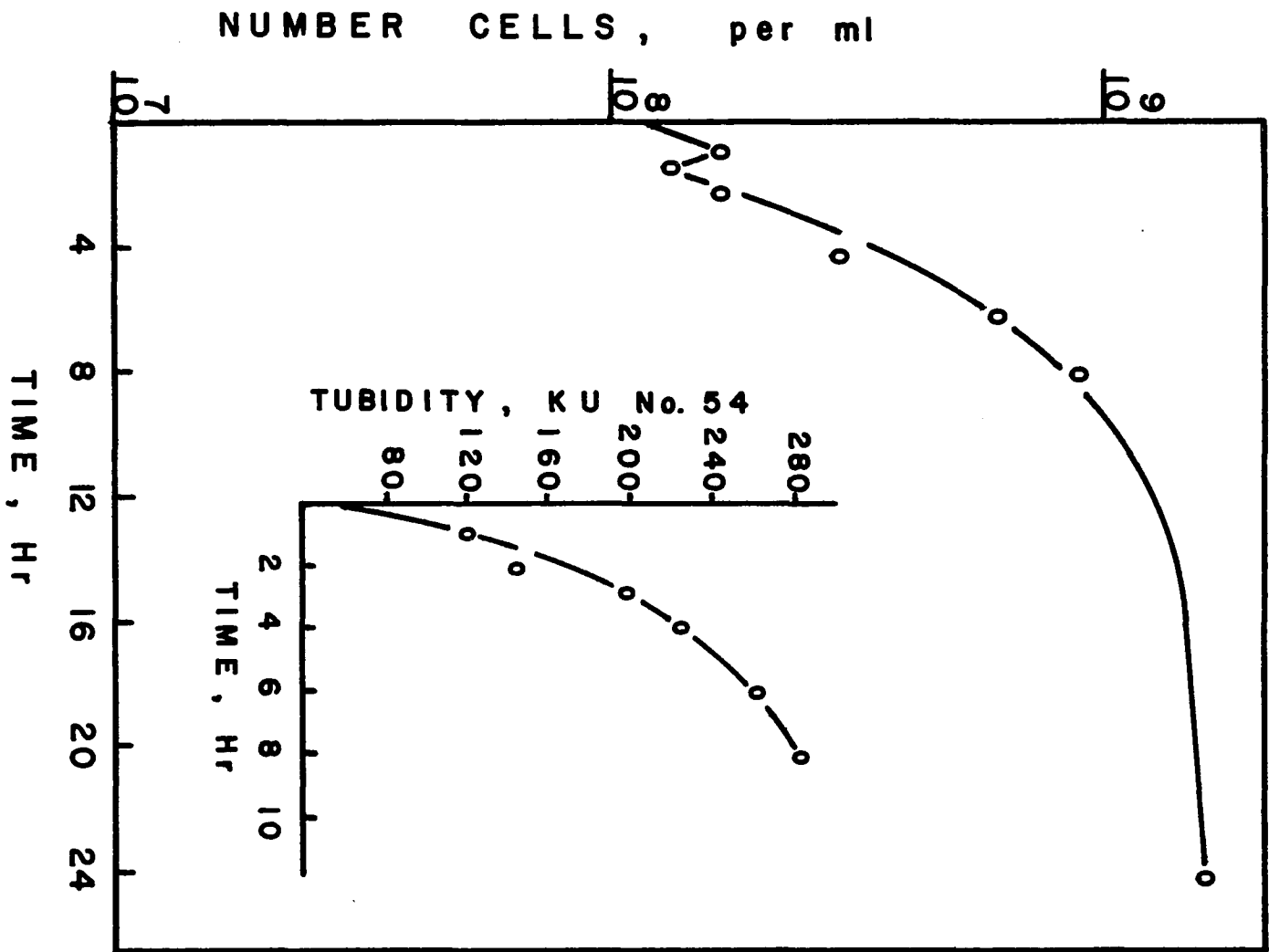
concentration of 2×10^9 cells/ml after 8 hr (Fig. 20). In conclusion, the presence of the F'araB24 plasmid in E. coli B27T⁻ caused a loss of colony-forming ability similar to thymineless death while in the absence of the plasmid and under similar conditions, this strain was capable of growth.

f. Effect of the Tryptophan Deletion on the Growth of E. coli B27T⁻ in Broth Lacking Thymine

Escherichia coli B27T⁻ contains a deletion in the tryptophan operon which covers part of the trp B and trp C cistrons (Fig. 1). Experiments were performed to determine what effect, if any, the deletion had on the growth of E. coli B27T⁻ in minimal broth lacking thymine. E. coli B27T⁻ was transduced with the generalized transducing phage Plkc which had previously been grown on E. coli Y mel, a strain prototrophic for the tryptophan operon. Three tryptophan-independent, thymine-requiring transductants were selected, and the growth response of these trp⁺ revertants in minimal broth lacking thymine was determined. Fig. 22 illustrates the growth of one of the E. coli B27T⁻trp⁺ isolates when resuspended in minimal broth lacking thymine. The data showed that the trp⁺ revertant grew to a terminal concentration of 3×10^9 cells/ml from an initial inoculum of 10^8 cells/ml. Growth in terms of cellular mass, as measured by the increase in turbidity of the culture, was also similar to the parent strain. Other trp⁺ isolates showed similar growth patterns (data not shown). These data indicated that the mutation in the tryptophan operon was not responsible for the growth of E. coli B27T⁻ in minimal broth lacking thymine.

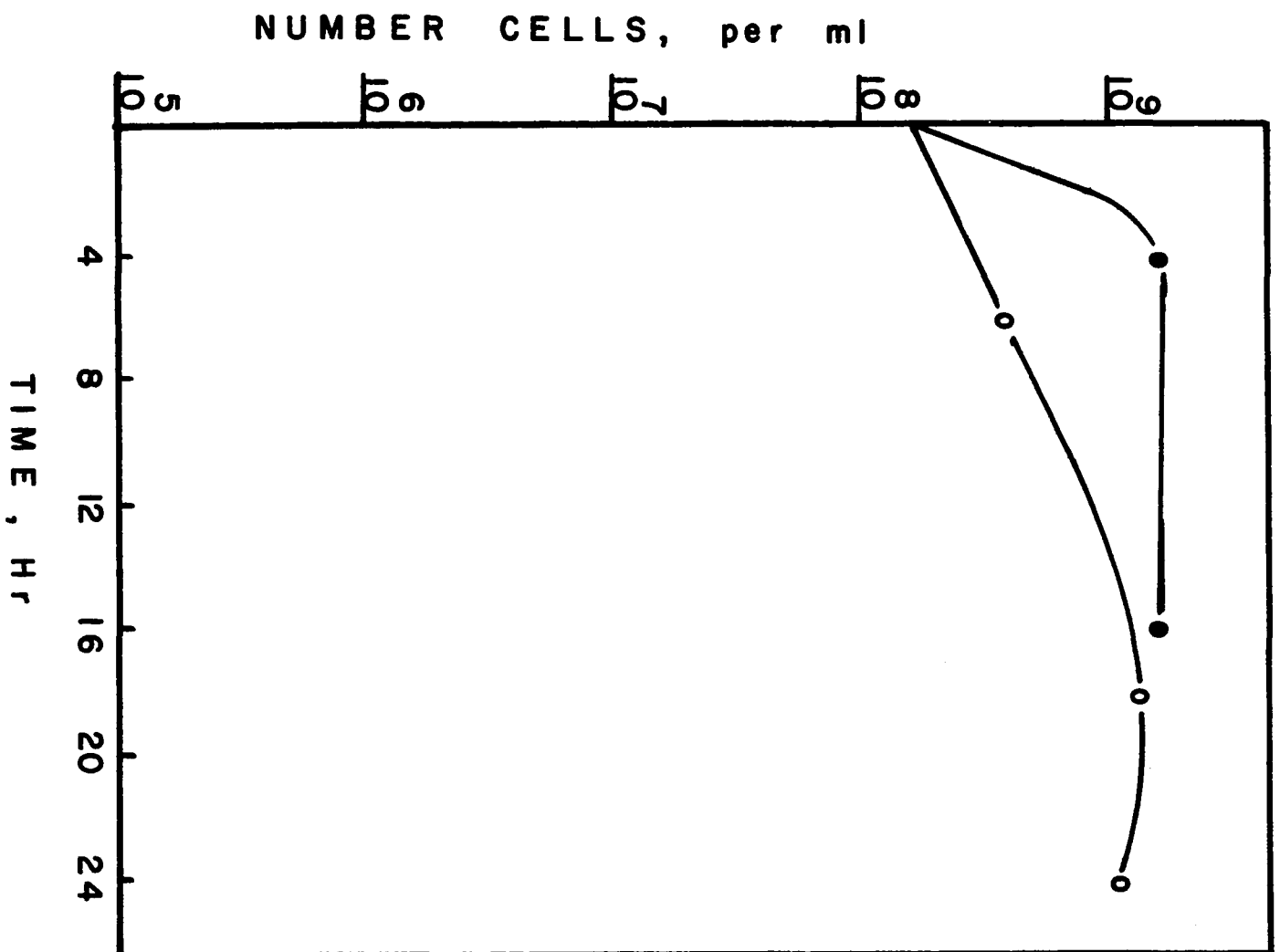
Fig. 22. Effect of the tryptophan deletion on the colony-forming ability of E. coli B27T⁻. A trp⁺ revertant of E. coli B27T⁻ was grown to early logarithmic phase in minimal broth containing thymine, centrifuged, washed, and resuspended in minimal broth lacking thymine. The culture was shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8).

Inset: Turbidity measurements of E. coli B27T⁻trp⁺ in minimal broth lacking thymine obtained using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter.



Supporting evidence to the effect, that the tryptophan operon was not responsible for the protection of E. coli B27T⁻ from thymine starvation, was obtained from experiments employing a merodiploid strain, E. coli B27T⁻/F'ColVBM-K260, trp, which harbors a plasmid containing the entire tryptophan operon. This strain was isolated following conjugation of E. coli B27T⁻ (sm^r recipient) with E. coli JC411T⁻/F'ColVBM-K260, trp (sm^s donor) on Vogel-Bonner minimal medium without casamino acids supplemented only with 5 μ g/ml of thymine and 250 μ g/ml of streptomycin. Merodiploid strains thus isolated, which were colicinogenic, thy⁻, and trp⁺, were capable of growing in minimal broth lacking thymine to about 2×10^9 cells/ml (Fig. 23). Omission of both casamino acids and tryptophan from the minimal broth lacking thymine resulted in more rapid growth of E. coli B27T⁻/F'ColVBM-K260, trp than that observed in minimal broth lacking thymine. Evidence that the F'ColVBM-K260, trp plasmid, itself, was not responsible for protection against thymine starvation was obtained from growth experiments where the thymine-requiring mutant, E. coli JC411T⁻/F'ColVBM-K260, trp was resuspended in minimal broth lacking thymine (Fig. 23). These experiments indicated that an intact tryptophan operon was not essential for thymineless death and that protection of E. coli B27T⁻ from thymine starvation did not result from a product of the mutant tryptophan operon nor a conformational effect in the bacterial DNA due to the deletion.

Fig. 23. Effect of the F'ColVBM-K260, trp plasmid on the colony-forming ability of E. coli B27T⁻. E. coli B27T⁻/F'ColVBM-K260, trp was grown to early logarithmic phase in minimal broth containing thymine, centrifuged, washed by the cold method (Method I), and resuspended in minimal broth containing thymine. The cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Minimal broth lacking thymine, O, and minimal broth lacking casamino acids, tryptophan and thymine, ●.



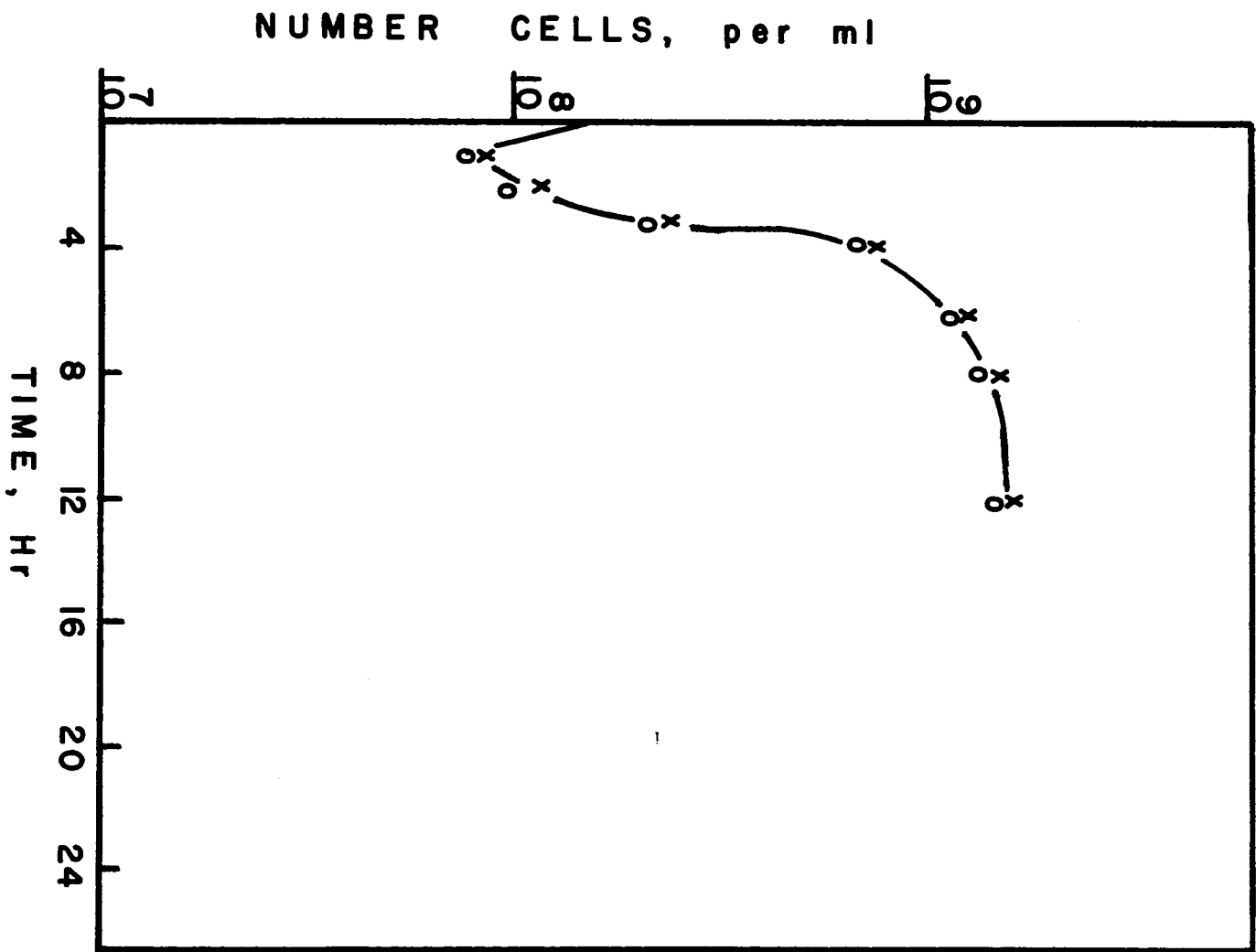
g. Effect of Uridine in Minimal Broth Lacking Thymine on the Colony-Forming Ability of E. coli B27T⁻

Freifelder (1965) showed that the addition to the growth medium of 50 μ g/ml of any riboside (e.g. uridine) antagonized the protection of thymine-requiring E. coli B/r against thymineless death provided by low concentrations of thymine. In the conditions employed by Freifelder, a concentration of 0.1 μ g of thymine/ml stabilized E. coli B/r(T⁻) for three hr against loss of colony-forming ability. It was hypothesized that E. coli B27T⁻ might synthesize enough thymine at a low rate under thymine starvation to support growth in minimal broth lacking thymine. If this were the case, the addition of uridine to the growth medium might inhibit growth and result in thymineless death. Resuspension of a culture of E. coli B27T⁻ in minimal broth lacking thymine but supplemented with uridine (50 μ g/ml) or uridine (50 μ g/ml) and thymine (0.1 μ g/ml) resulted in growth of both strains (Fig. 24). The initial loss of colony-forming ability in this experiment was attributable to the experimental technique employed since the cultures were washed at 4 C (Method I) prior to resuspension, a technique which was shown to sensitize the culture. The results indicated that thymineless death could not be induced by this method.

h. Effect of Pantoyl Lactone in Minimal Broth Lacking Thymine on the Colony-Forming Ability of E. coli B27 and E. coli B27T⁻

Escherichia coli B27T⁻ cells growing in minimal broth lacking thymine were observed by light microscopy and were

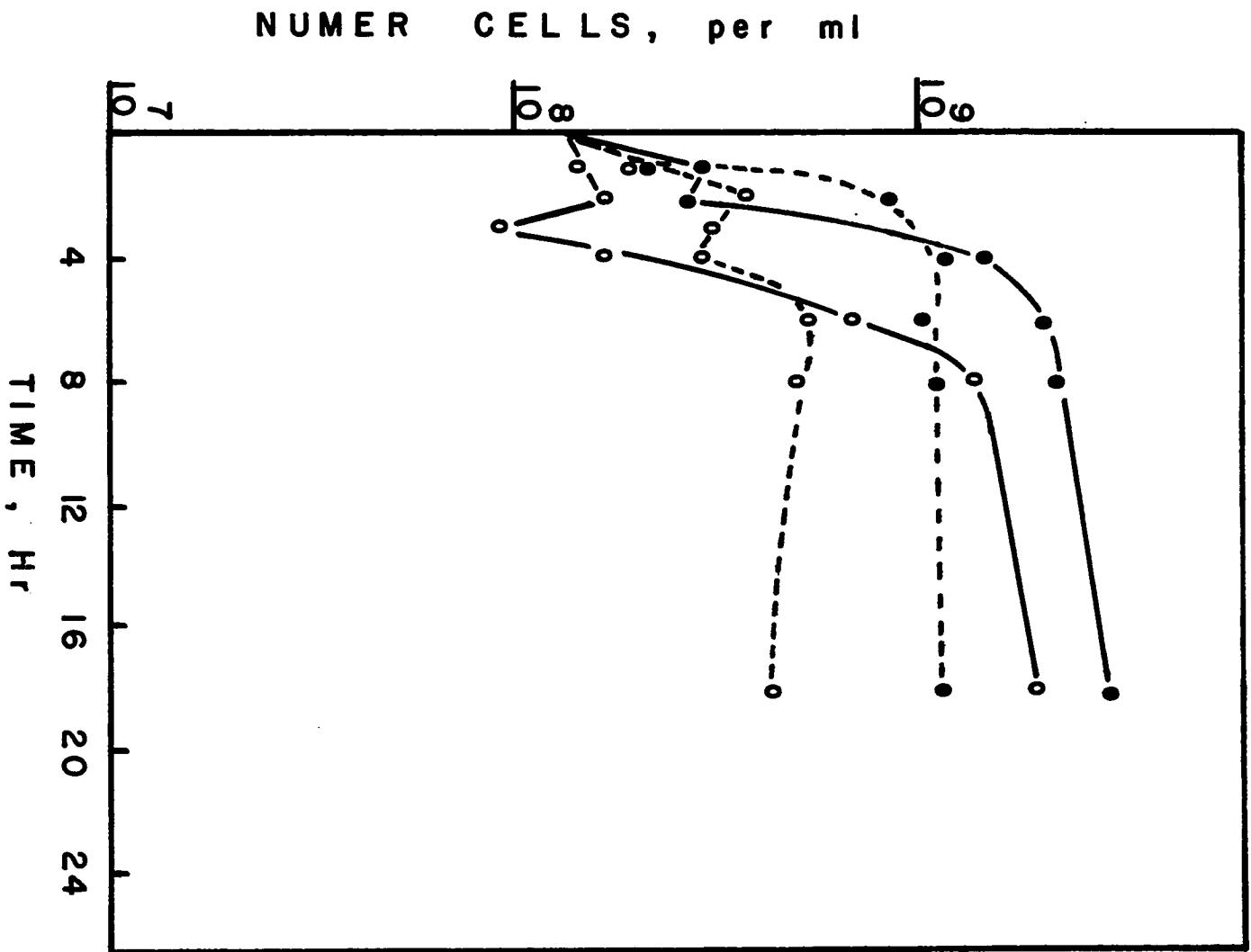
Fig. 24. Effect of uridine in minimal broth lacking thymine on the colony-forming ability of E. coli B27T⁻. A culture of E. coli B27T⁻ grown to early logarithmic phase in minimal broth containing thymine was centrifuged, washed by the cold method (Method I), and resuspended in minimal broth. The cultures were shaken in a 37 C waterbath, and samples were removed, diluted, and plated on ENB agar (Methods section 8). Minimal broth lacking thymine supplemented with uridine (50 μ g/ml), O; minimal broth containing thymine (0.1 μ g/ml) and uridine (50 μ g/ml), X.



found to form long rod-shaped cells as compared to the short rods formed by the same culture grown in the presence of thymine. E. coli B27 though did not form the elongated cells during thymine starvation. Grula and Grula (1962) found that division inhibition caused by D-serine, penicillin, or ultraviolet irradiation could be reversed in a species of Erwinia by the addition of divalent cations or pantooyl lactone, a precursor of pantothenic acid. Adler and Hardigree (1964) observed that DL-pantooyl lactone (0.08M) allowed filamentous cells of E. coli, which formed after exposure to ultraviolet irradiation, to divide normally. Cummings and Mondale (1967) found that pantooyl lactone rescued cells of E. coli B which had previously been exposed either to thymine starvation or ultraviolet irradiation. These workers suggested that the mechanism of thymineless death and filament formation might be related. Thus, experiments were performed to study the effect of DL-pantooyl lactone on cell elongation in E. coli B27T⁻.

Several experiments were conducted in which DL-pantooyl lactone (0.08M) was incorporated in minimal broth lacking thymine. In a preliminary experiment (Fig. 25), in which E. coli B27T⁻ and E. coli B27 cells were washed by the cold (Method I) procedure prior to resuspension in minimal broth lacking thymine and in the same medium supplemented with pantooyl lactone, the presence of the pantooyl lactone had two effects. Initially, the loss of colony-forming ability of E. coli B27T⁻ was retarded. Then after 4 to 6 hr incubation, pantooyl lactone inhibited colony-formation for both strains.

Fig. 25. Effect of growth in pantoyl lactone, minimal broth lacking thymine on the colony-forming ability of E. coli B27 and E. coli B27T⁻. Cultures of E. coli B27 and E. coli B27T⁻ grown to early logarithmic phase in minimal broth containing thymine were centrifuged, washed by the cold method (Method I), and resuspended in either minimal broth lacking thymine (——) or in minimal broth lacking thymine but supplemented with 0.08M DL-pantoyl lactone (---). E. coli B27, ●; E. coli B27T⁻, ○.



Growth of E. coli B27 during the initial four hr incubation appeared to be unaffected by the presence of pantooyl lactone.

When E. coli B27T⁻ cells were washed by the warm procedure (Method II), pantooyl lactone added at the beginning prevented the initial increase in colony-forming units typical of E. coli B27T⁻ grown in minimal broth lacking thymine (Fig. 26). However, when pantooyl lactone was added to a culture of E. coli B27T⁻ growing in minimal broth lacking thymine after one hr of incubation, the shape of the growth curve was similar to a comparable culture incubated in minimal broth lacking thymine. Thus, pantooyl lactone did not have a marked effect on the ability of E. coli B27T⁻ to form colonies after growth in minimal broth lacking thymine.

Observations by light microscopy of cultures growing in minimal broth lacking thymine and in minimal broth supplemented with either thymine (5 μ g/ml) or pantooyl lactone (0.08M) indicated that E. coli B27T⁻ formed elongated cells only in minimal broth lacking thymine after two hr incubation. E. coli B27 did not form elongated cells under thymine starvation nor did cultures of E. coli Y mel or E. coli B9. Thymine-requiring mutants, E. coli Y melT⁻, E. coli B9T⁻, and E. coli 15T⁻ also were observed to form elongated cells when grown in minimal broth lacking thymine.

Photomicrographs were obtained of E. coli B27T⁻ growing in minimal broth lacking thymine and the same medium supplemented with 0.08M pantooyl lactone. The photomicrographs (Fig. 27-31) illustrate the growth of E. coli B27T⁻ in minimal broth lacking thymine initially, and after 1, 2, 4, and 8 hr.

Fig. 26. Effect on colony-forming ability of E. coli B27T⁻ if pantoyl lactone is added one hr after E. coli B27T is inoculated into minimal broth lacking thymine. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in either minimal broth lacking thymine (——) or in minimal broth lacking thymine but supplemented with 0.08M DL-pantoyl lactone (- - -). E. coli B27T⁻ in minimal broth lacking thymine, O; lactone added initially, ●; lactone added at one hr, ▼.

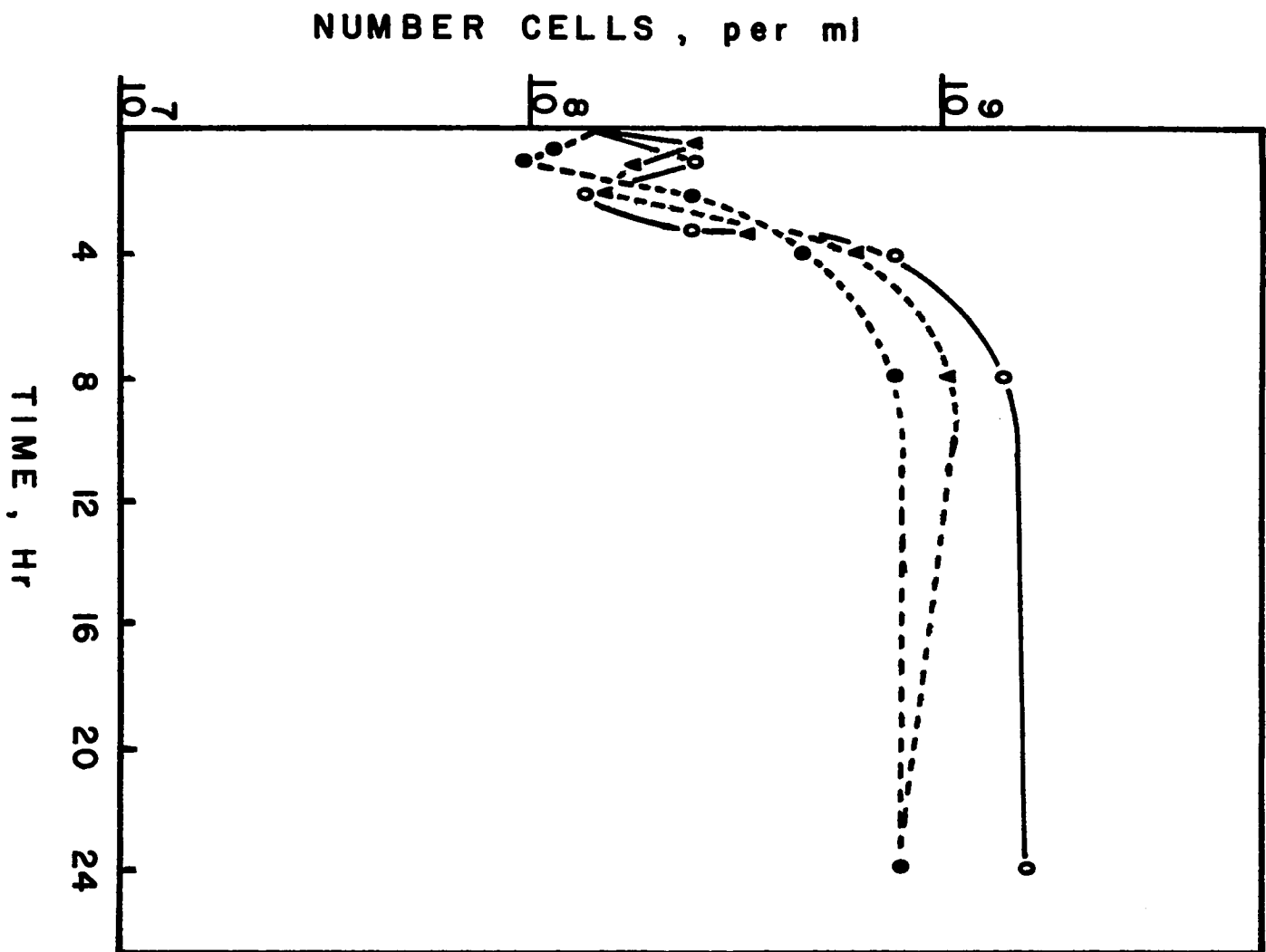
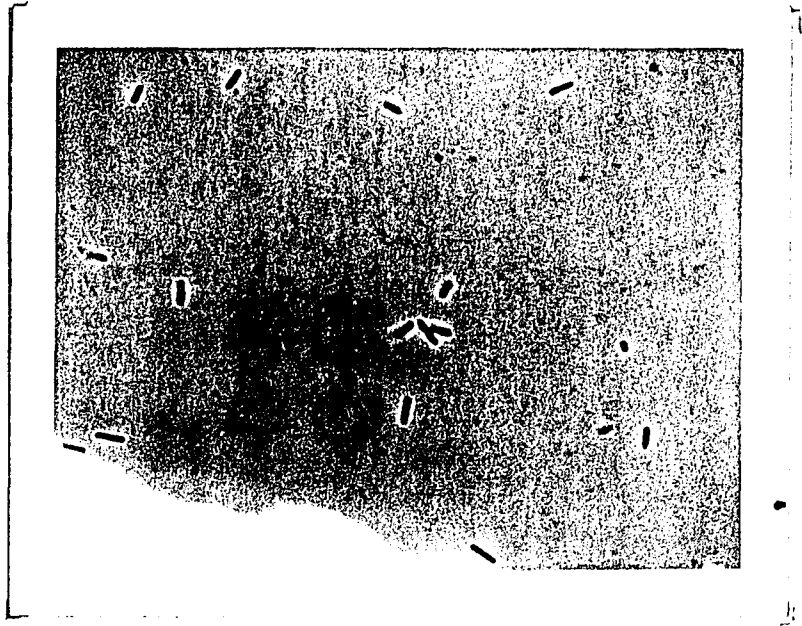


Fig. 27-31. Photomicrographs of E. coli B27T⁻ cells growing in minimal broth lacking thymine. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in minimal broth lacking thymine. The culture was shaken in a 37 C waterbath, and samples were removed. Photomicrographs were obtained for the incubation times indicated using a Zeiss WL Research microscope equipped with a Polaroid Land Instrument Camera (Model ED-10) on Polaroid black and white film (#107). Photographic magnification was 800x.

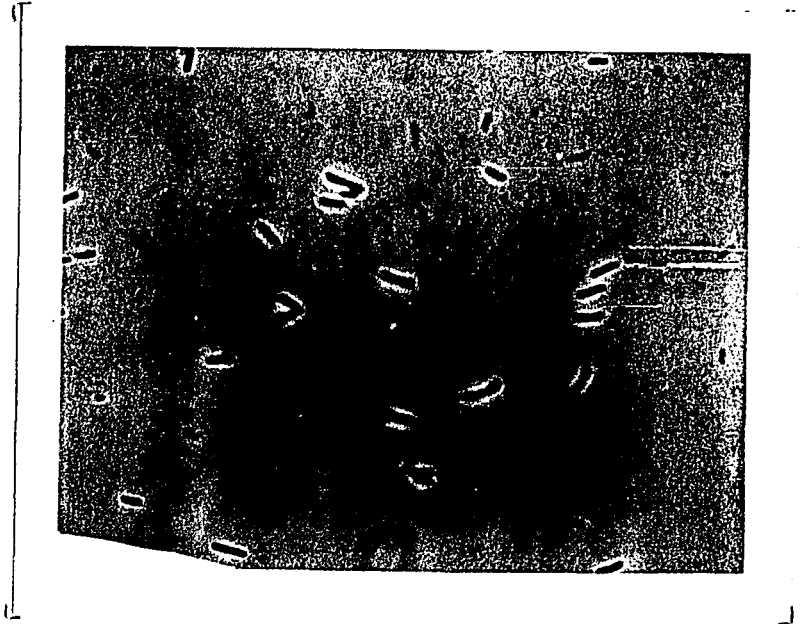
Fig. 27



E. coli B27T⁻ Cells in Minimal Broth Lacking Thymine Just After Inoculation



Fig. 28



E. coli B27T⁻ Cells in Minimal Broth
Lacking Thymine After 1 Hour

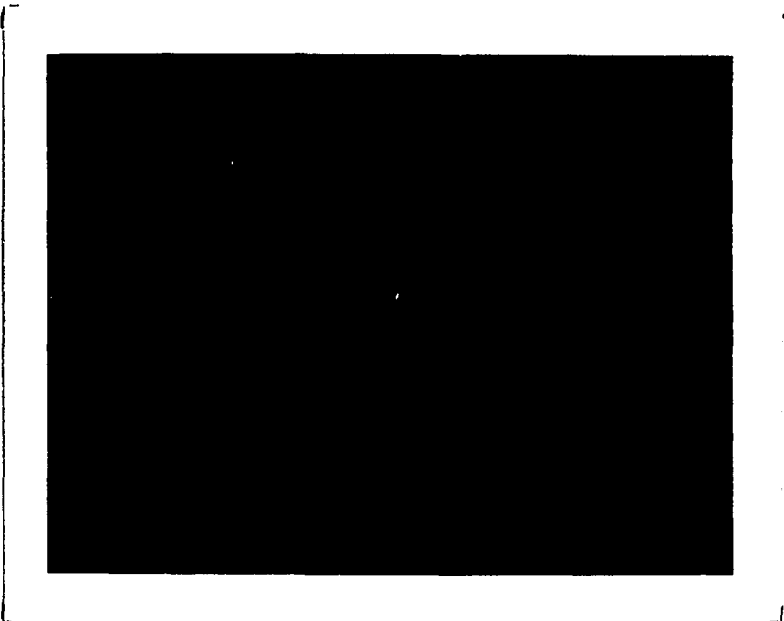
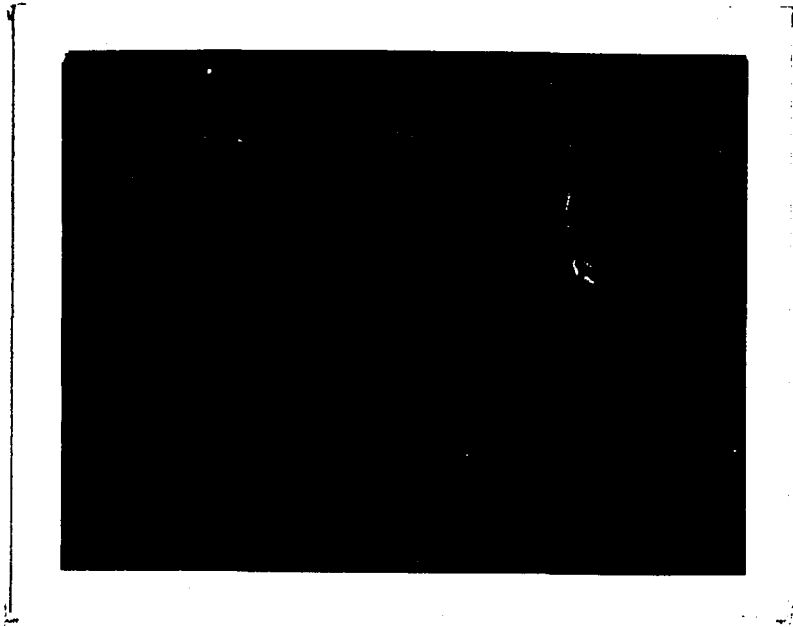


Fig. 29



E. coli B27T⁻ Cells in Minimal Broth
Lacking Thymine After 2 Hours

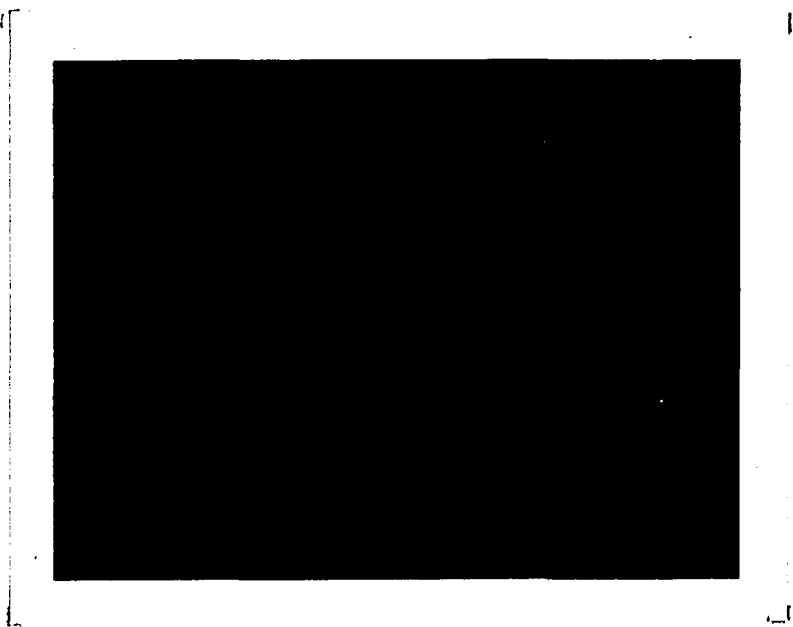
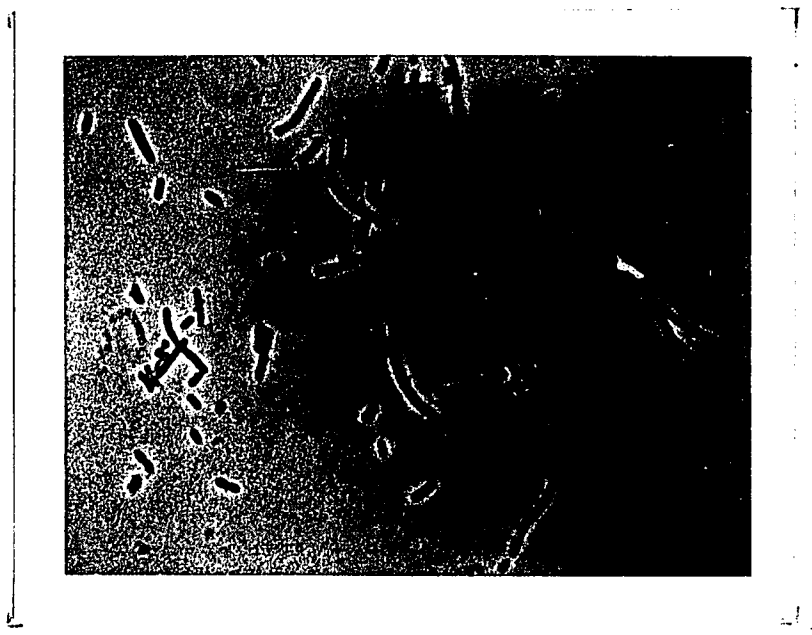


Fig. 30



E. coli B27T⁻ Cells in Minimal Broth Lacking Thymine After 4 Hours

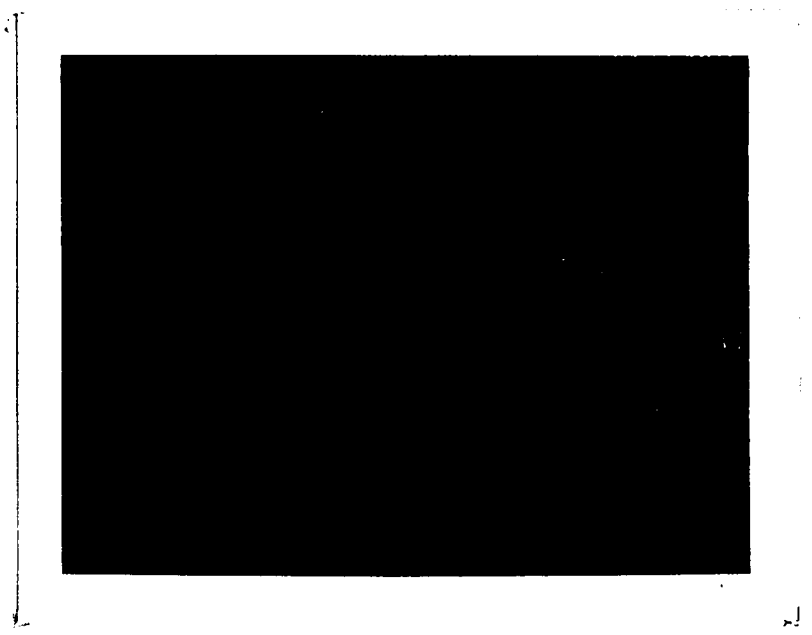
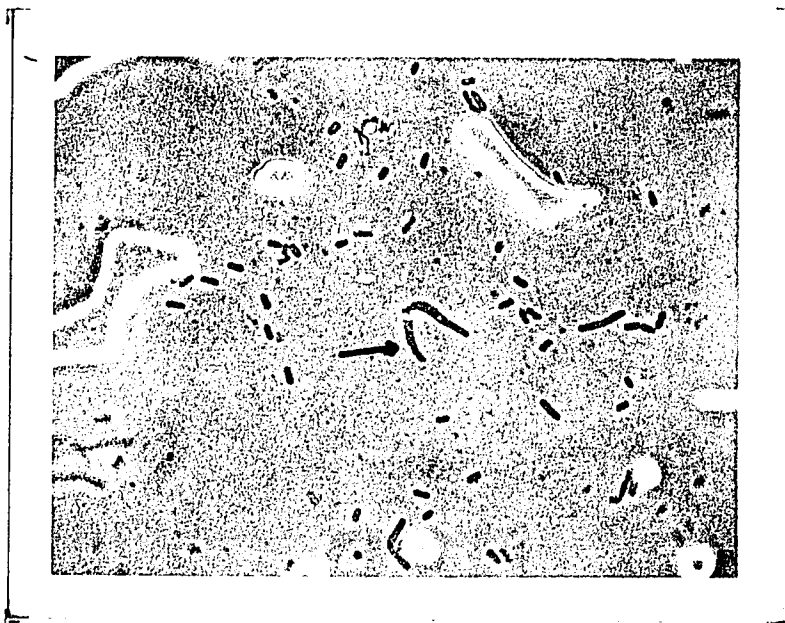
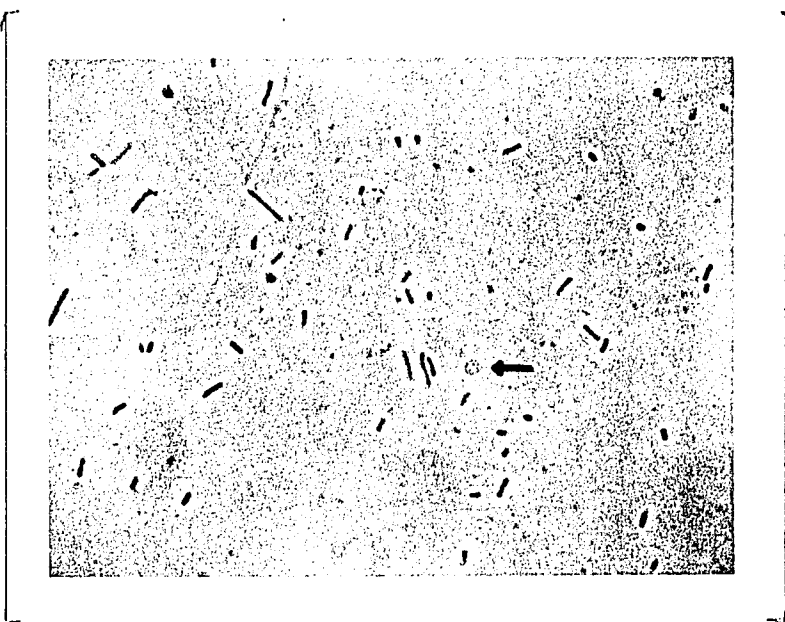


Fig. 31



E. coli B27T⁻ Cells in Minimal Broth
Lacking Thymine After 8 Hours

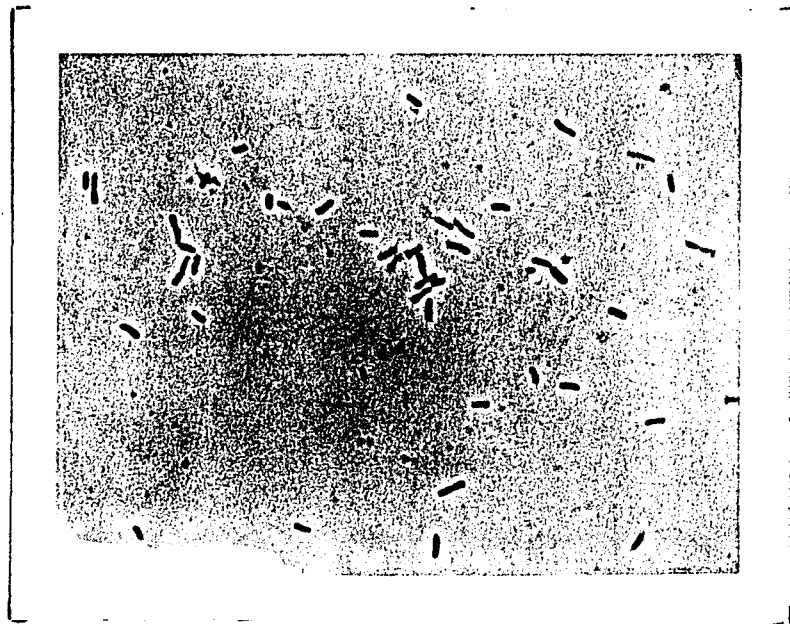


Photomicrographs (Fig. 32-36) depict growth of E. coli B27T⁻ in minimal broth lacking thymine containing 0.08M pantoyl lactone. The mean cell length for all samples was estimated from measurements of 20-30 cells/sample (Table 12). The photomicrographs and measurements of the mean cell length show that E. coli B27T⁻ formed elongated cells while growing in minimal broth lacking thymine, and that very little change in the length of these cells was observed in the presence of 0.08M pantoyl lactone. It was of interest to note that, at four hr (Fig. 30, Table 12) of growth in minimal broth lacking thymine, E. coli B27T⁻ cells were of two distinct sizes, 3.2 and 13.9 μm , while after 8 hr exposure only the smaller form was found. Fig. 26 had revealed that the number of viable E. coli B27T⁻ cells in minimal medium lacking thymine increased from 8.4×10^8 to 1.3×10^9 cells/ml during the 4 to 8 hr period of incubation. Thus, it appeared that the number of colony-forming units did not increase greatly during the period when the longer cell form was disappearing. This phenomenon may be explained by the division of the elongated cells during growth, forming both viable and non-viable daughter cells (the latter may be DNA-less minicells). Alternatively, prolonged thymine starvation may result in the lysis of the elongated cells which would leave only the smaller form.

Other abnormal variations in cellular morphology were also observed to occur in a minor fraction of the population of E. coli B27T⁻ cells. Branched cells were found in minimal broth lacking thymine (photomicrograph not shown) as well as in the presence of pantoyl lactone (arrow Fig. 33, 34), as

Fig. 32-36. Photomicrographs of E. coli B27T⁻ cells growing in minimal broth lacking thymine but supplemented with 0.08M DL-pantoyl lactone. An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in minimal broth lacking thymine but supplemented with 0.08M DL-pantoyl lactone. The culture was shaken in a 37 C waterbath, and samples were removed. Photomicrographs were obtained for the incubation times indicated using a Zeiss WL Research microscope equipped with a Polaroid Land Instrument Camera (Model ED-10) on Polaroid black and white film (#107). Photographic magnification was 800x.

Fig. 32



E. coli B27T⁻ Cells in Lactone Minimal
Broth Just After Inoculation

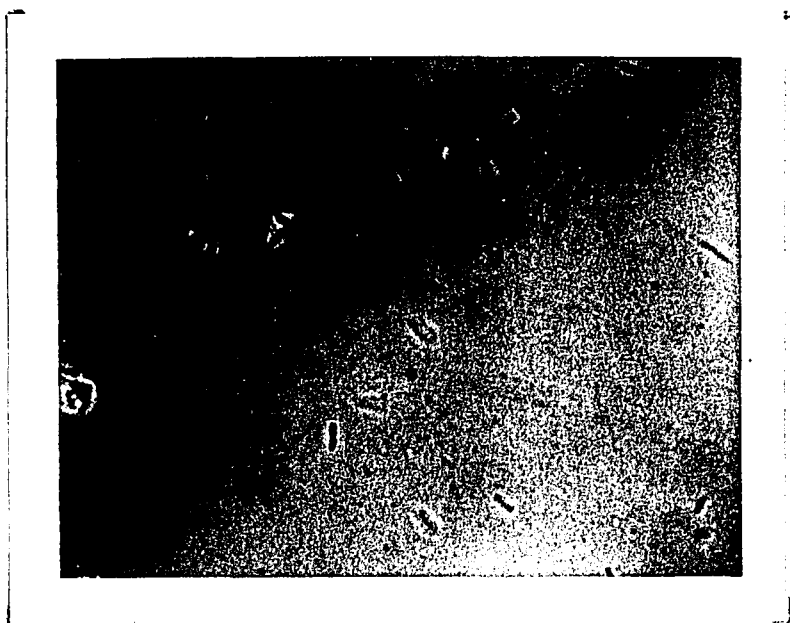
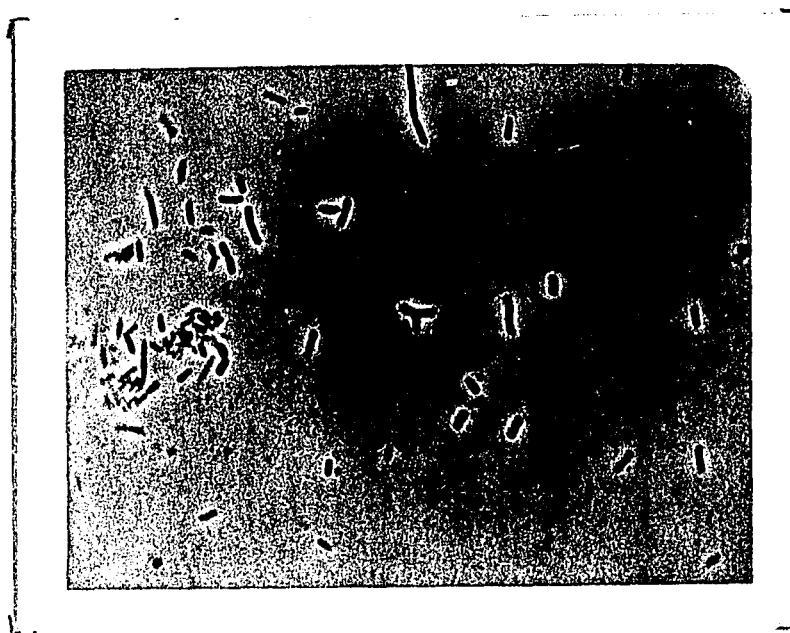


Fig. 33



E. coli B27T⁻ Cells in Lactone
Minimal Broth After 1 Hour

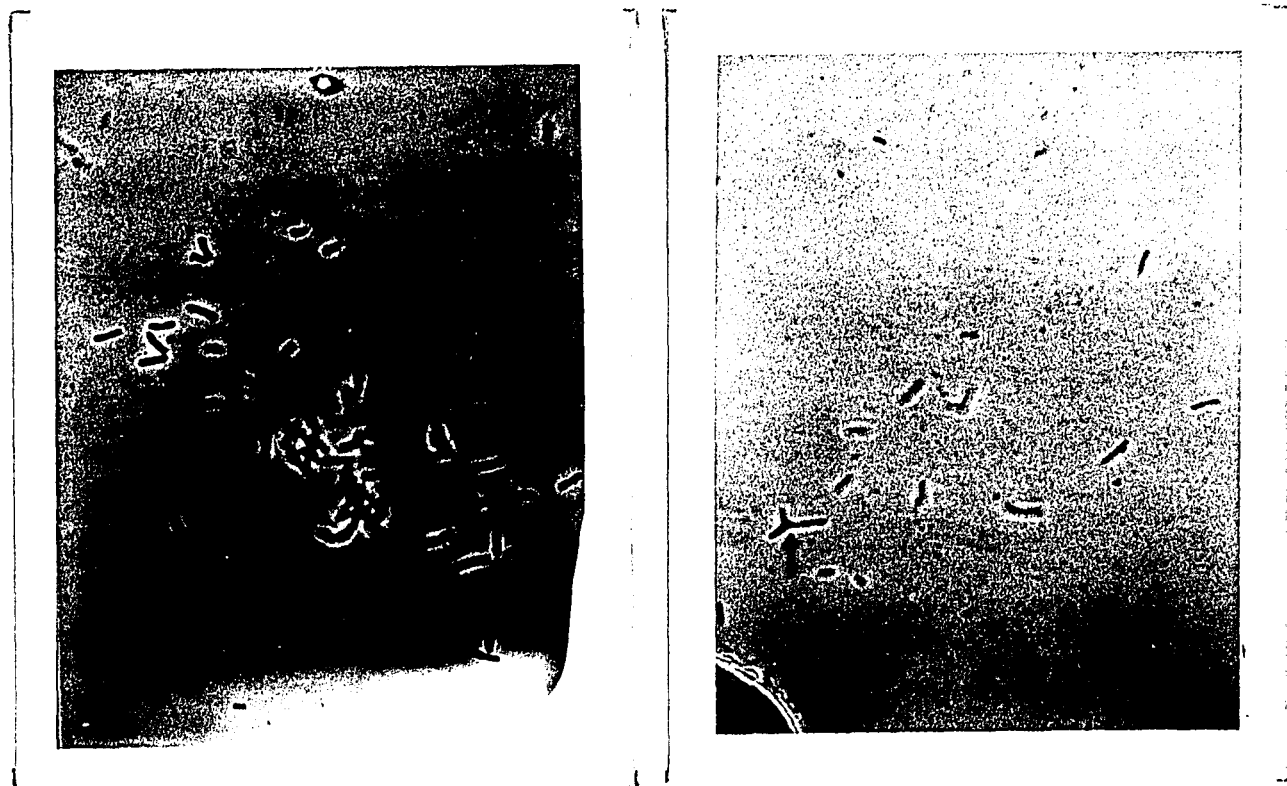


Fig. 34



E. coli B27T⁻ Cells in Lactone Minimal
Broth After 2 Hours

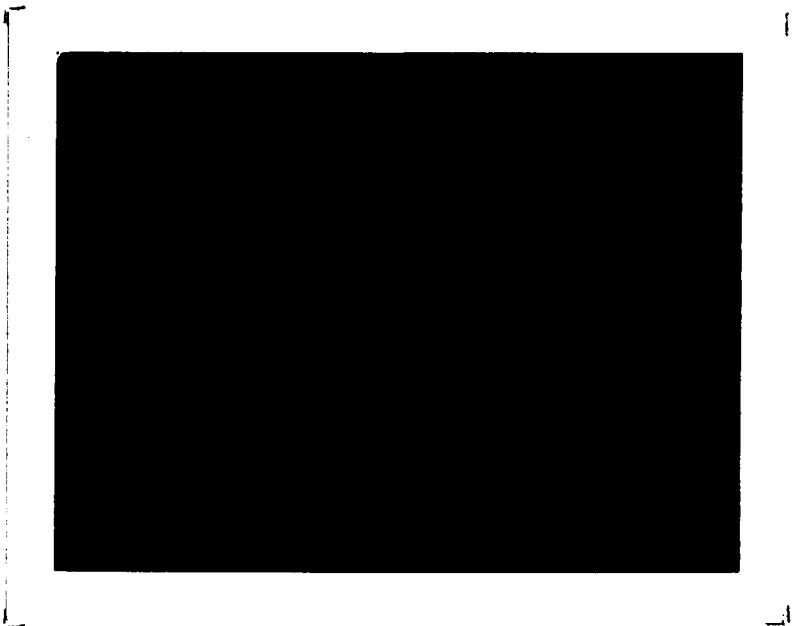
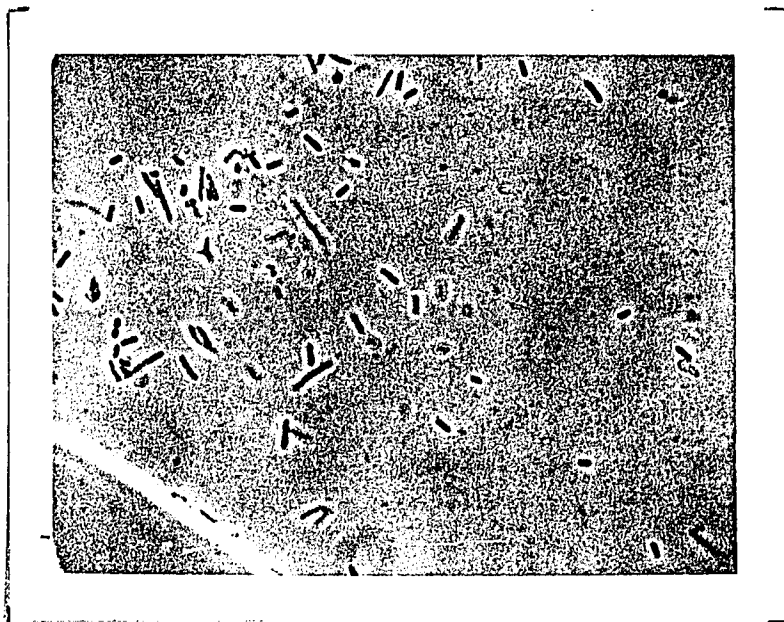
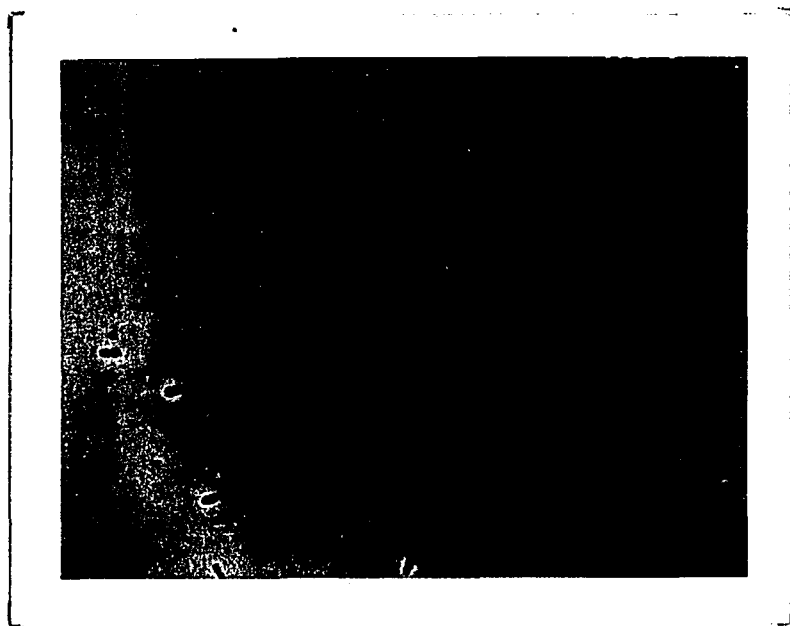


Fig. 35



E. coli B27T⁻ Cells in Lactone Minimal
Broth After 4 Hours

Fig. 36



E. coli B27T⁻ Cells in Lactone Minimal
Broth After 8 Hours

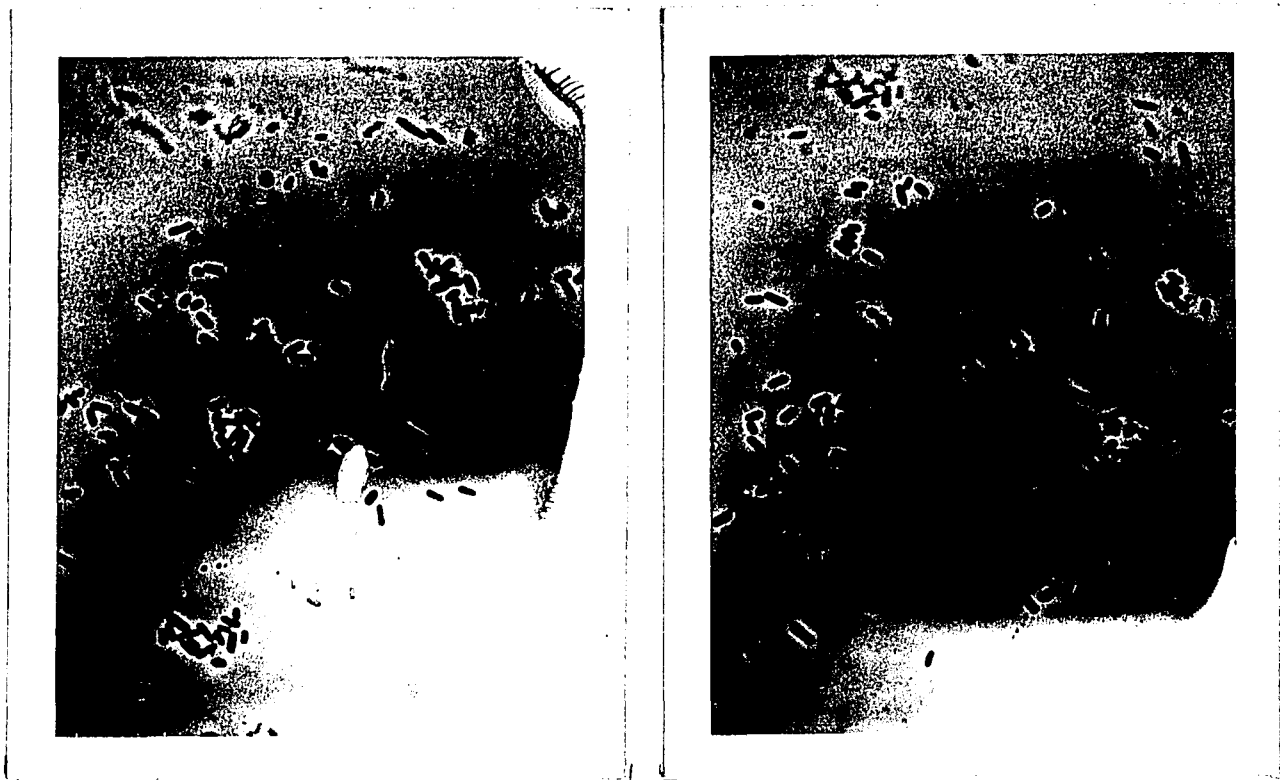


Table 12. Cell sizes of E. coli B27T⁻ exposed to pantoyl lactone

Time of Growth (Hr)	Mean Length of <u>E. coli</u> B27T ⁻ Cell in: ^a	
	A	B
0	4.1	3.6
1	4.6	3.6
2	8.8	4.4
4	13.2 and 3.2	3.7
8	3.5	3.5

An early logarithmic phase culture of E. coli B27T⁻ grown in minimal broth containing thymine was centrifuged, washed, and resuspended in either (A) minimal broth lacking thymine or in (B) minimal broth lacking thymine but supplemented with 0.08M DL-pantoyl lactone. The cultures were shaken in a 37 C waterbath. Samples were removed at the times indicated and then examined as wet mounts by light microscopy.

- a. The mean cell length expressed as micrometers (20-30 cells) was obtained using an ocular micrometer.

well as swollen, bent (arrow Fig. 31) or club-shaped cells. Spheroplast-like cells were also found in cultures of E. coli B27T⁻ growing in minimal broth lacking thymine (Fig. 30, 31, 2.8 μ m diameter) and in the presence of pantoyl lactone (Fig. 36, 2.5 μ m diameter) after 4 hr. Thus, during thymine starvation, E. coli B27T⁻ formed elongated cells which attained a mean length of about three times the normal length for these cells. Pantoyl lactone prevented cellular elongation, but did not greatly alter colony-forming ability. Other morphological abnormalities were observed in the form of swollen and branched cells, suggesting a possible defect in wall formation and division.

3. Thymidylate Synthetase Activity in Crude Cell Extracts of E. coli Y mel, E. coli B27, and E. coli B27T⁻.

The enzyme thymidylate synthetase, which is specified by the thy A locus on the E. coli chromosome, catalyzes the conversion of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP) in the presence of Mg⁺⁺ and N⁵, N¹⁰-methylene-tetrahydrofolate. In the thymidylate synthetase reaction, the change from tetrahydrofolate to dihydrofolate can be measured as an increase in absorbance at 340 nm. This spectrophotometric method, described by Freidkin (1963), was used to compare the thymidylate synthetase activity found in crude extracts from cultures of E. coli Y mel, E. coli B27, and E. coli B27T⁻ grown in minimal broth lacking thymine. Minimal medium was chosen as the growth medium so as to determine if growth under conditions of thymine starvation resulted because of de novo synthesis of thymine using the thymidylate synthetase pathway.

The data (Fig. 37; Table 13) showed an increase in absorbance at 340 nm for crude extracts of E. coli Y mel and E. coli B27. Extracts of E. coli B27T⁻ prepared from either logarithmic or early stationary phase cultures exhibited no increase in absorbance. Thymidylate synthetase activity associated with the crude extract obtained from E. coli B27 exhibited slightly higher activity than the corresponding activity found for the crude extract from E. coli Y mel.

When the crude extract from E. coli B27T⁻ was mixed with the crude extract from either E. coli Y mel or E. coli B27, the initial rate of the reaction and the terminal absorbance for the thymidylate synthetase assay were reduced (Fig. 37; Table 14). A mixture of crude extracts from E. coli Y mel and E. coli B27 resulted in the same initial rate of reaction as that observed for each extract separately, but a lower terminal absorbance.

The data from the assay of crude cell extracts for thymidylate synthetase activity, indicated that E. coli B27T⁻ lacked detectable levels of enzyme activity. Also, the extracts obtained from E. coli B27T⁻ inhibited the thymidylate synthetase activity of extracts of E. coli Y mel and E. coli B27.

4. Study of Bacterial DNA

a. DNA Base Composition

Escherichia coli B27T⁻ was capable of growing in minimal broth lacking thymine from an initial cell concentration of as low as 10^4 to 10^8 cells/ml. Thus, this mutant was

Fig. 37. Thymidylate synthetase activity in crude cell extracts of E. coli Y mel, E. coli B27, and E. coli B27T⁻ and mixed extracts of these strains. Cell-free extracts were prepared by the method described by Breitman and Bradford (1967) from cultures grown to early logarithmic phase in minimal broth lacking thymine (see Methods section 23). Thymidylate synthetase activity was estimated by following the spectral change which occurs during the oxidation of tetrahydrofolate to dihydrofolate at 340 nm using a Bausch and Lomb 600 spectrophotometer. For the reaction mixture see Methods section 24.

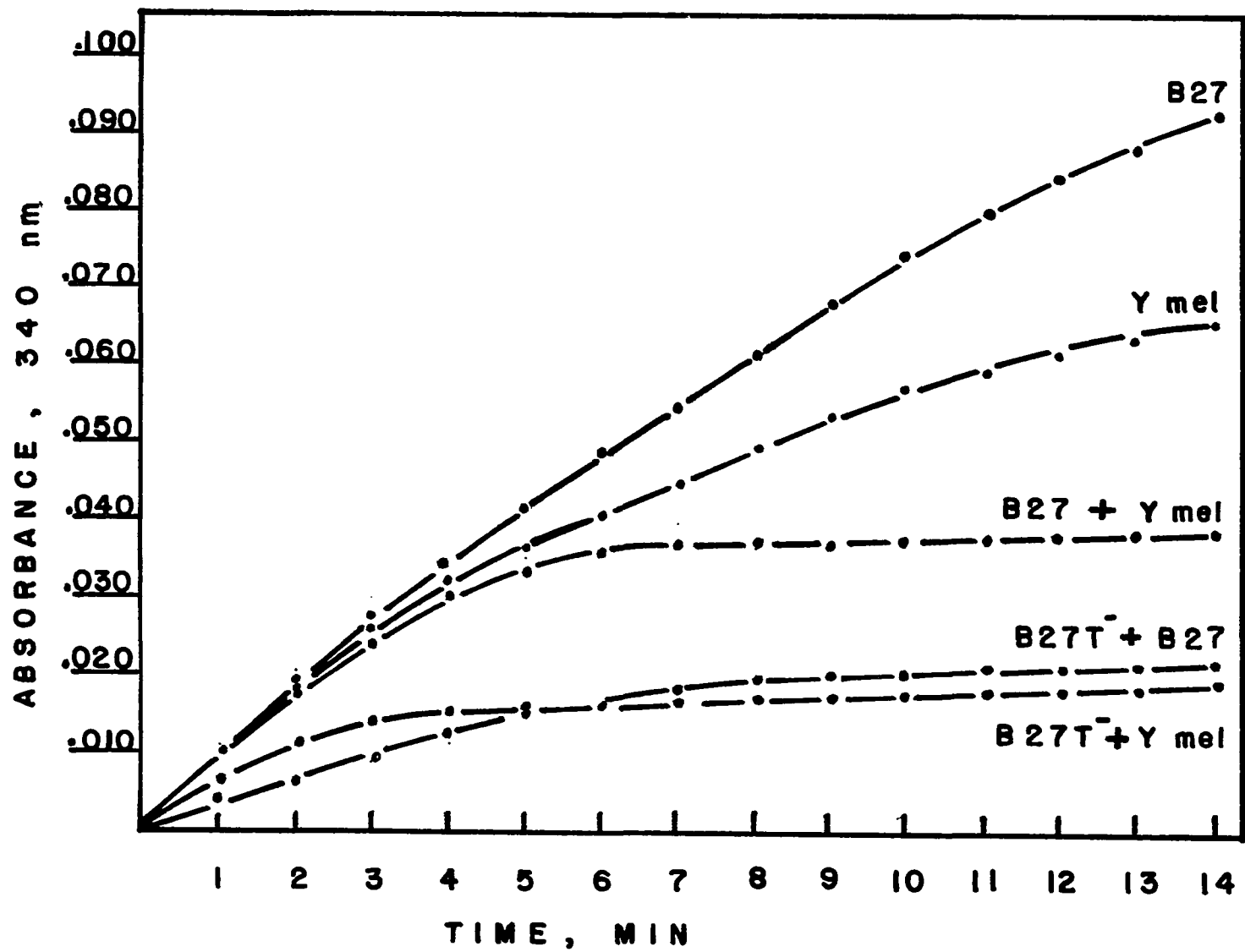


Table 13. Thymidylate synthetase activity of crude extracts of E. coli Y mel, E. coli B27, and E. coli B27T⁻^a

Crude Extract from	Number Cells/ml	Turbidity ^b	Lowry Protein (mg/ml)	Absorbance ^c	Protein in Reaction Mixture ^d	Activity Units ^e
Y mel	1.4 x 10 ⁹	217	4.6	0.073	2.07	3.5
B27	1.7 x 10 ⁹	214	4.2	0.083	1.89	4.4
B27T ⁻	5.6 x 10 ⁸	215	5.6	0	2.52	0
	1.2 x 10 ⁹	280	5.6	0	2.52	0

- a. Crude extracts were prepared as described in Methods section 23, and thymidylate synthetase was assayed as described in Methods section 24.
- b. Turbidity measurements obtained using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter.
- c. Absorbance at 340 nm was determined by extrapolating the initial rate of reaction to 10 min.
- d. Protein in the reaction mixture in milligrams. The reaction mixture contained 0.9 ml from a dilution containing 1.0 ml of enzyme and 1.0 ml of buffer (Methods section 24).
- e. 1 unit = 0.001 O.D. unit/mg protein/min.

Table 14. Thymidylate synthetase activity of mixed crude extracts from E. coli Y mel, E. coli B27, and E. coli B27T⁻ ^a

Crude Extract Mixture from	Absorbance ^b	Protein in Reaction Mixture ^c	Activity Units ^d
B27T ⁻ and Y mel	0.041	4.59	0.89
B27T ⁻ and B27	0.031	4.41	0.70
Y mel and B27	0.085	3.96	2.1

- a. Crude extracts were prepared as described in Methods section 23, and thymidylate synthetase was assayed as described in Methods section 24.
- b. Absorbance at 340 nm was determined by extrapolating the initial rate of reaction to 10 min.
- c. Protein in the reaction mixture in milligrams. The reaction mixture contained 0.9 ml from a dilution of 1.0 ml of extract from each organism in the mixture (Methods section 24).
- d. 1 unit = 0.001 O.D unit/mg protein/min.

undergoing division and cytoplasmic growth for approximately 16 generations in the absence of exogenous thymine. The possibility that E. coli B27T⁻ might possess the biosynthetic ability to form an analogue of thymine such as 5-hydroxymethyluracil was tested.

The base composition of deoxyribonucleic acid from E. coli B27T⁻ was determined following the extraction and purification of the DNA (Marmur, 1961) and subsequent hydrolysis and separation of the nucleic acid bases by paper chromatography (Lin and Maes, 1963). Nucleic acid bases from the chromatogram were identified on the basis of mobility (R_f) and absorption spectra. The characteristics of E. coli B27T⁻ DNA and isolated DNA bases were compared with similar determinations obtained for DNA extracted from E. coli B, E. coli Y mel, and E. coli B27 as well as commercial preparations of salmon sperm DNA, E. coli DNA, and a mixture of free bases.

DNA was extracted and purified from a liter culture of early stationary phase cells grown in minimal broth lacking thymine (Table 15). The concentration of purified DNA preparations was determined by the diphenylamine reaction (Burton, 1968) and the absorbance at 260 nm. Purity was determined empirically from the absorption spectra for each sample. The data (Table 16) showed that all of the DNA preparations were of reasonable purity, since the maximum absorbance for all preparations occurred at 258 nm and the ratios of 260:280 extinctions were equal to or greater than 1.9:1.

Table 15. DNA obtained from liter cultures of various E. coli strains^a

<u>E. coli</u> Strain	Turbidity ^b	Cell Pack Wet Weight (gm)	Total Viable Count (cells/ml)	Purified DNA Obtained (mg)
B	300	6.1	3.0×10^9	9.5
Y mel	300	6.7	3.0×10^9	5.4
B27	310	6.4	3.1×10^9	9.7
B27T ⁻	310	8.9	1.7×10^9	9.5

- a. DNA was extracted and purified (Marmur, 1961) from early stationary phase cultures grown in minimal broth lacking thymine (Methods section 25).
- b. Terminal turbidity measurements were obtained using a Klett-Sommerson colorimeter equipped with a No. 54 (green) filter.

Table 16. Concentration and absorption properties of DNA preparations from E. coli Y mel, E. coli B27, and E. coli B27T⁻ ^a

Type of DNA	Estimated Concentration ($\mu\text{g/ml}$)		Absorption Properties ^b			
	Diphenylamine	Absorbance ^{b,c} 260 nm	Maximum			
			nm	O.D.	$\frac{260}{280}$	$\frac{260}{230}$
<u>E. coli</u> B	950	816	258	.83	1.93	2.28
<u>E. coli</u> (Worthington)	500	460	258	.47	1.90	2.24
Salmon sperm	380	345	258	.70	1.96	2.34
<u>E. coli</u> Y mel	540	540	258	.55	1.93	2.30
<u>E. coli</u> B27	970	960	258	.97	1.92	2.26
<u>E. coli</u> B27T ⁻	950	828	258	.84	1.94	2.30

- a. DNA was extracted and purified (Marmur, 1961) from early stationary phase cultures grown in minimal broth lacking thymine (Methods section 25).
- b. Absorbance was determined in a Bausch and Lomb 600 spectrophotometer using 1.0 ml samples.
- c. Concentration was estimated from the relation that 20 $\mu\text{g/ml}$ of pure DNA was equivalent to an absorbance at 260 nm of 0.04. Thus: E. coli B DNA diluted 1:20, 260 O.D. = 0.815 $\frac{20}{0.4} \times 20 = 816 \mu\text{g/ml}$.

The mobility of hydrolyzed DNA was determined using paper chromatography, and the R_f values for isolated ultraviolet absorbing spots are presented in Table 17. Four spots were readily discernible following chromatography with a solvent system consisting of isopropanol, hydrochloric acid and water. However, guanine trailed and cytosine and thymine were somewhat labile during hydrolysis. R_f values obtained indicated that the resolved bases consisted of guanine, adenine, cytosine and thymine. Other ultraviolet absorbing spots were not observed. Additional evidence that thymine was present in E. coli B27T⁻ DNA was obtained using a second solvent system, aqueous n-butanol. However, in this solvent system, guanine and cytosine were not completely resolved. On the other hand, thymine had a greater mobility than the other nucleic acid bases and was readily resolved (Table 17). Thus, E. coli B27T⁻ DNA appeared to contain thymine. This result was confirmed by a study of the absorption spectra of eluted spots.

Ultraviolet absorption spectra were obtained for each eluate, and the maximum absorbance and the ratio of extinctions was determined (Tables 18, 19, 20, and 21). The values obtained for the adenine, guanine and cytosine spots from the chromatogram where isopropanol was the solvent showed that these bases in the various DNA preparations were consistent with the values reported by Bendich (1957) and thus confirmed the identity of these compounds. However, the ratios of extinctions obtained for the ultraviolet absorbing compounds tentatively identified as thymine were inconsistent (Table 21).

Table 17. Mobility of hydrolyzed DNA preparations on paper chromatographs

Spot #	Base Mixture ^c	Rf of DNA bases in Isopropanol: HCl:H ₂ O ^a						Probable Identity
		<u>E. coli</u> B DNA	<u>E. coli</u> DNA (Worthington)	Salmon Sperm DNA	<u>E. coli</u> Y mel DNA	<u>E. coli</u> B27 DNA	<u>E. coli</u> B27T DNA	
1	0.24	0.26	0.29	0.27	0.25	0.26	0.26	Guanine
2	0.32	0.34	0.36	0.35	0.34	0.35	0.35	Adenine
3	0.48	0.50	0.51	0.50	0.50	0.51	0.50	Cytosine
4	0.73	0.74	0.78	0.76	0.73	0.76	0.74	Thymine
Rf in aq. n-butanol ^b								
4	0.44	0.44	N.D.	N.D.	0.45	0.44	0.43	Thymine

Purified DNA (Methods section 25) was hydrolyzed and chromatographed as described in Methods section 26.

- a. Isopropanol (redistilled) 325 ml. concd. HCl 83.5 ml, water to make 500 ml; Wyatt, Biochem. J. 48:584 (1951).
 - b. 86% (vol./vol.) aq. n-butanol; Markham and Smith, Biochem. J. 45:294 (1949).
 - c. Base mixture = adenine sulfate, guanine hydrochloride, cytosine, and thymine (80 μ g/ml ea.) dissolved in 5 mM NaOH.
- N.D. = not determined.

Table 18. Ultraviolet absorption properties of eluted DNA bases: adenine from a isopropanol-HCl solvent chromatogram

Ultraviolet absorbing spots from a isopropanol-HCl solvent chromatogram were eluted in 0.1 N HCl (5 ml) for 2 hr at room temperature. The absorbance spectra were determined in a Bausch and Lomb 600 Spectrophotometer.

a. The concentration was determined from the absorption maxima according to Bendich (1957). The absorbance at the maximum was multiplied by the volume of eluate (5 ml) and the number of micromoles of base/O.D. unit (0.0794, 0.100, 0.0901, and 0.126 for adenine, cytosine, guanine, and thymine, respectively). Thus the absorbance of adenine in the mixture of bases at the maximum (260 nm) = 0.58, and $0.58 \times 5 \text{ ml} \times 0.0794 = 0.23 \mu\text{mole}$.

b. Base mixture = adenine sulfate, guanine hydrochloride, cytosine, and thymine (80 $\mu\text{g/ml}$ ea.) dissolved in 5 mM NaOH.

DNA from Which Bases Were Obtained	Wavelength at Maximum (nm)	Ratio of Extinctions			Concentration ^a (μ moles)
		$\frac{250}{260}$	$\frac{280}{260}$	$\frac{290}{260}$	
Base Mixture ^b	260	0.81	0.33	0.026	0.23
<u>E. coli</u> B	265	0.69	0.43	0.043	0.20
<u>E. coli</u> (Worthington)	265	0.65	0.47	0.044	0.12
Salmon Sperm	267	0.63	0.47	0.042	0.19
<u>E. coli</u> Y mel	262	0.76	0.37	0.039	0.28
<u>E. coli</u> B27	265	0.74	0.42	0.047	0.41
<u>E. coli</u> B27T ⁻	265	0.67	0.44	0.042	0.24

Table 19. Ultraviolet absorption properties of eluted DNA bases: cytosine from a isopropanol-HCl solvent chromatogram

DNA from Which Bases Were Obtained	Wavelength at Maximum (nm)	Ratio of Extinctions			Concentration ^a (μ moles)
		$\frac{250}{260}$	$\frac{280}{260}$	$\frac{290}{260}$	
Base Mixture ^b	275	0.57	1.30	0.62	0.55
<u>E. coli</u> B	275	0.24	1.90	0.92	0.27
<u>E. coli</u> (Worthington)	275	0.32	1.77	0.88	0.16
Salmon Sperm	275	—	0.27	1.35	0.17
<u>E. coli</u> Y mel	275	0.43	1.50	0.73	0.33
<u>E. coli</u> B27	275	0.42	1.51	0.73	0.51
<u>E. coli</u> B27T ⁻	275	0.23	1.87	0.91	0.31

a, b, see footnotes, Table 18.

Table 20. Ultraviolet absorption properties of eluted DNA bases: guanine from a isopropanol-HCl solvent chromatogram

DNA from Which Bases Were Obtained	Wavelength at Maximum (nm)	Ratio of Extinctions			Concentration ^a (μ moles)
		$\frac{250}{260}$	$\frac{280}{260}$	$\frac{290}{260}$	
Base Mixture ^b	250	1.25	0.70	0.38	0.20
<u>E. coli</u> B	246	1.39	1.06	0.61	0.15
<u>E. coli</u> (Worthington)	247	1.38	1.04	0.62	0.12
Salmon Sperm	245	1.39	1.15	0.66	0.12
<u>E. coli</u> Y mel	249	1.35	0.87	0.50	0.21
<u>E. coli</u> B27	246	1.35	1.06	0.59	0.16
<u>E. coli</u> B27T ⁻	247	1.41	1.15	0.66	0.15

a,b, see footnotes, Table 18.

Table 21. Ultraviolet absorption properties of eluted DNA bases: thymine from a isopropanol-HCl solvent chromatogram

DNA from Which Bases Were Obtained	Wavelength at Maximum (nm)	Ratio of Extinctions			Concentration ^a (μ moles)
		$\frac{250}{260}$	$\frac{280}{260}$	$\frac{290}{260}$	
Base Mixture ^b	265	0.70	0.56	0.16	0.43
<u>E. coli</u> B	268	0.61	1.15	0.81	0.19
<u>E. coli</u> (Worthington)	270	0.52	1.76	1.43	0.15
Salmon Sperm	269	0.53	1.43	1.07	0.17
<u>E. coli</u> Y mel	267	0.33	0.81	0.10	0.26
<u>E. coli</u> B27	268	0.07	1.11	0.21	0.35
<u>E. coli</u> B27T ⁻	269	0.40	1.53	1.09	0.18

a,b, see footnotes, Table 18.

The reason for these discrepancies was not clear. However, the identity of thymine was confirmed from the data for the ratios of extinctions obtained for the spots tentatively identified as thymine that were eluted from the chromatogram developed with aqueous n-butanol (Table 22). These values are in good agreement with those reported by Bendich (1957) for thymine.

b. Incorporation of Thymidine-methyl-C¹⁴ Into the DNA of E. coli Y mel and E. coli B27T⁻.

Since Escherichia coli B27T⁻ DNA was found to contain thymine in addition to adenine, cytosine and guanine, it was of interest to determine if E. coli B27T⁻ could incorporate exogenous thymidine. Boyce and Setlow (1962) described a method for increasing the incorporation of exogenous labeled thymidine into E. coli B DNA by supplementing the growth medium with a deoxyriboside.

E. coli Y mel and E. coli B27T⁻ were grown separately in the presence of thymidine-methyl-C¹⁴ and deoxyadenosine for about two generations, centrifuged, washed, and resuspended in minimal broth lacking thymine (Table 23). The cultures were then grown to 10⁹ cells/ml in the minimal broth lacking thymine, DNA extracted, purified, and chromatographed. Table 24 shows the data of the concentration and absorption properties of the purified labeled DNA. The results in Table 23 show that E. coli Y mel contained a greater amount of labeled thymidine/mg of DNA than E. coli B27T⁻. Nevertheless, E. coli B27T⁻ incorporated a significant amount of the label, and the percentage of counts/minute of the DNA applied to the

Table 22. Ultraviolet absorption properties of eluted DNA bases: thymine from a n-butanol solvent chromatogram

DNA From Which Bases Were Obtained	Wave length at maximum (nm)	Ratio of Extinctions		
		$\frac{250}{260}$	$\frac{280}{260}$	$\frac{290}{260}$
Base mixture ^a	265	0.69	0.53	0.097
Y me1	265	0.69	0.54	0.103
B	265	0.71	0.56	0.148
B27	265	0.68	0.55	0.117
B27T ⁻	265	0.68	0.53	0.094

Ultraviolet absorbing spot for thymine was cut from the n-butanol chromatogram and eluted in 5.0 ml of 0.1N HCl. Absorbance spectra were obtained using a Bausch and Lomb 600 Spectrophotometer.

a. Base mixture = adenine sulfate, guanine hydrochloride, cytosine, and thymine (80 μ g/ml ea.) dissolved in 5 mM NaOH.

Table 23. Distribution of thymidine-methyl-C¹⁴ label in E. coli Y mel and E. coli B27T⁻. Cultures (500 ml) of E. coli Y mel and E. coli B27T⁻ were grown in minimal broth containing 0.375 g of deoxyadenosine, 1500 μg of thymidine, and 25 μCi of thymidine-methyl-C¹⁴ (Specific activity, 54.7 m Ci/mM, New England Nuclear) until a cell concentration of about 10⁸ cells/ml was attained. The cells were centrifuged, washed, and resuspended in 1 liter of minimal broth lacking thymine. Ten ml of cell suspension were filtered for the initial isotope count; one ml of filtrate was also counted. Cultures were shaken at 37 C until the population reached early stationary phase. Ten ml of cell suspension were filtered for the terminal isotope count; one ml of filtrate was also counted. DNA was extracted, purified and dissolved in 10 ml of 5 mM NaOH. One ml of purified DNA was hydrolyzed and chromatographed.

Condition	<u>E. coli</u> Y mel	<u>E. coli</u> B27T ⁻
Uninoculated thymidine-methyl-C ¹⁴ broth (CPM/ml)	79,430	90,408
Initial inoculum into thymidine- methyl-C ¹⁴ broth (cells/ml)	2.6 x 10 ⁷	2.7 x 10 ⁷
Cell count after growth in thymidine-methyl-C ¹⁴ broth (cells/ml)	8.5 x 10 ⁷	1.2 x 10 ⁸
No. generations growth in thymidine- methyl-C ¹⁴ broth	1.7	2.1
Supernatant thymidine-methyl-C ¹⁴ broth at end of cell growth (CPM/ml)	61,511	50,781
Initial cell count after resuspension in minimal broth lacking thymine (cells/ml)	8.5 x 10 ⁷	1.2 x 10 ⁸
Initial isotope count for minimal broth lacking thymine (CPM/ml)	23	35
Initial isotope count for cells from minimal broth lacking thymine (CPM/ml)	3609	3505

Table 23., continued. Distribution of thymidine-methyl-C¹⁴ label in E. coli Y mel and E. coli B27T⁻

Condition	<u>E. coli</u> Y mel	<u>E. coli</u> B27T ⁻
Terminal cell count in minimal broth lacking thymine (cells/ml)	3.8 x 10 ⁹	1.2 x 10 ⁹
Turbidity (Klett units)	325	315
No. generations growth in minimal broth lacking thymine	5.5	3.3
Terminal isotope count for supernatant minimal broth lacking thymine (CPM/ml)	285	735
Terminal isotope count for cells from minimal broth lacking thymine (CPM/ml)	3247	2486
Cell pack wet weight (g)	6.1	7.1
Purified DNA obtained (mg)	7.7	5.4
Total isotope count of purified DNA (CPM/ml)	50,330	26,379
(CPM/mg DNA)	(65,300)	(48,900)

Table continued next page.

Table 23., continued. Distribution of thymidine-methyl-C¹⁴ label in E. coli Y mel and E. coli B27T⁻

Condition	<u>E. coli</u> Y mel	<u>E. coli</u> B27T ⁻
Isotope count for thymine from isolated DNA (1.0 ml) after chromatography (CPM)	21,509	11,103
Isotope count recovered as thymine, %	43	42

Table 24. Concentration and absorption properties of labeled DNA preparations

DNA isolated from	Concentration Estimated by ($\mu\text{g/ml}$)		Absorption Properties		
	Diphenylamine	Absorbance at 260 nm	Maximum nm	$\frac{260}{280}$	$\frac{260}{230}$
Thymidine-methyl- C^{14} labeled <u>E. coli</u> Y mel	770	544	258	1.91	2.25
Thymidine-methyl- C^{14} labeled <u>E. coli</u> B27T	540	424	258	1.91	2.28

See Methods section 27 for isolation procedure.

chromatogram and recovered as thymine was similar for E. coli Y mel (43%) and E. coli B27T⁻ (42%). It was also noted that the supernatant minimal broth lacking thymine, from which E. coli B27T⁻ cells were removed, contained higher counts/minute than the corresponding medium for E. coli Y mel, suggesting that E. coli B27T⁻ might leak thymidine (or thymine) into the medium during growth in minimal broth lacking thymine. Thus, E. coli B27T⁻ was capable of incorporating thymidine-methyl-C¹⁴ into its DNA and retaining the label through several generations of growth in minimal broth lacking thymine.

5. Transduction of the Thymine Requirement from E. coli B27T⁻ to E. coli RG274.

Arginine-positive transductants were selected following the infection of E. coli RG274 (argA⁻, thy A⁺) with a phage Plkc lysate previously grown on E. coli B27T⁻ (argA⁺, thyA⁻). A similar lysate prepared from E. coli B27 (argA⁺, thyA⁺) was used to infect a second culture. This served as a control. Arg⁺ transductants were found infrequently (51 arg⁺/8 x 10⁶ cells) when E. coli RG274 was infected with the phage lysate from E. coli B27T⁻ cells (Table 25). However, nine of 51 arg⁺ isolates were also thy⁻ (18%), indicating that the thymine requirement of E. coli B27T⁻ was cotransducible with arg A. In addition, all arg⁺thy⁻ isolates grew on minimal agar plates lacking thymine in the presence of 5% carbon dioxide. Thymine-requiring arg⁺ transductants were not isolated from E. coli RG274 following infection with the phage lysate from E. coli B27. Thus, E. coli B27T⁻ appeared to contain a mutation, which was responsible for the thymine requirement as

Table 25. Transduction of the thymine requirement from E. coli B27T⁻ to E. coli RG274

<u>E. coli</u> Donor	Number <u>Arg</u> ⁺ <u>E. coli</u> RG274 Recipients		
	Isolated	# <u>Arg</u> ⁺ <u>Thy</u> ⁺	# <u>Arg</u> ⁺ <u>Thy</u> ⁻
B27	200	200	0
B27T ⁻	51	42	9

Phage Plkc lysates were prepared following infection of E. coli B27 and E. coli B27T⁻ at a ratio of 20 plaque-forming units/cell (Methods section 22). Lysates were then employed to infect E. coli RG274 (arg⁻,thy⁺), and arg⁺ transductants were selected on minimal agar containing thymine (50 μg/ml) but lacking arginine. The thymine requirement was tested by streaking arg⁺ transductants on minimal agar lacking thymine or on minimal agar containing thymine (50 μg/ml).

well as the sparing effect exhibited by carbon dioxide, in or closely linked with the thy A region which, in turn, is closely linked to Arg A.

CHAPTER V

DISCUSSION

Thymine-requiring mutants, E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ were isolated following treatment with trimethopterin (Stacey and Simson, 1965). Successive streaking of isolated colonies on decreasing concentrations of thymine resulted in the isolation of low thymine-requiring strains. Unusual difficulty was encountered when thymine-requiring mutants of E. coli B27 and E. coli B9 were sought following treatment of these strains with trimethopterin. Penicillin counter-selection, normally, cannot be used to isolate thymine-requiring mutants since incubation in the absence of thymine leads to thymineless death (Cohen and Barner, 1954). When trimethopterin selection failed to yield thymine-requiring mutants of E. coli B27 and E. coli B9, penicillin selection was applied after trimethopterin treatment, and mutants E. coli B27T⁻ and E. coli B9T⁻ were isolated. The following properties (Table 1) of these mutants were observed: (1) Mutants E. coli Y melT⁻, E. coli B9T⁻, and E. coli B27T⁻ were incapable of utilizing thymidine as a sole source of carbon - a characteristic of mutants defective in pyrimidine deoxyribonucleoside catabolism (Lomax and Greenberg, 1968); (2) low concentrations (2-5 μ g/ml) of thymine were required by these strains for aerobic growth on minimal agar plates - a characteristic of strains containing mutations in both the thy A and deo regions (Breitman and Bradford, 1964; Harrison, 1965); (3) growth of E. coli B9T⁻ was inhibited

(sensitive) by high concentrations of thymidine (tds), a characteristic of strains defective in the synthesis of deoxy-riboaldolase (dra⁻, Ahmad and Pritchard, 1969; or deo C⁻, Lomax and Greenberg, 1968); (4) E. coli Y melT⁻ and E. coli B27T⁻ were not inhibited (resistant) by high concentrations of thymidine.

In addition, carbon dioxide (5%) permitted colony development of E. coli Y melT⁻ and E. coli B27T⁻ when these mutants were plated on minimal agar lacking thymine, while thymine-requiring mutants E. coli 15T⁻, E. coli B9T⁻, E. coli 307c, E. coli 840c, and E. coli JC411T⁻/F'ColVBM-K260, trp failed to develop colonies on the same agar (Tables 5, 6). Increased carbon dioxide tension, possibly associated with broth cultures containing high cell densities ($> 10^4$ cells/ml), did not solely account for the ability of E. coli B27T⁻ to grow in minimal broth lacking thymine, since E. coli Y melT⁻ exhibited thymineless death under similar conditions. The effect of carbon dioxide on the growth of E. coli Y melT⁻ and E. coli B27T⁻ in the absence of exogenous thymine remains unexplained. This sparing effect was also found cotransducible along with the thymine requirement of E. coli B27T⁻ which indicated that the carbon dioxide sensitive locus was closely linked to or identical with the thy A locus.

Thymine-requiring mutant, E. coli B27T⁻, was capable of growing without exogenous thymine for many generations under certain conditions. These included initial cell concentrations of greater than 10^4 cells/ml in minimal broth lacking thymine (Fig. 9) or in the presence of increased carbon dioxide

tension on minimal agar plates lacking thymine (Tables 5, 6). Thymineless death of E. coli B27T⁻ resulted when minimal broth lacking thymine was inoculated with 10³ cells/ml or less (Fig. 9), or by the formation of the merodiploid, E. coli B27T⁻/F'araB24, by the introduction of the F'araB24 plasmid, which carries the genetic region involved in the catabolism of deoxyribonucleosides (Lomax and Greenberg, 1968), into E. coli B27T⁻ (Fig. 20).

Survival of E. coli B27T⁻ cells during thymine starvation may, in part, result from cross-feeding and/or unusual intracellular pools of thymidine (or a derivative). Experiments designed to examine the ability of the supplemented, supernatant broth from a culture of E. coli B27T⁻ grown in minimal broth lacking thymine to protect other thymine-requiring mutants from thymineless death indicated that protective substances were not in sufficient concentration to prevent thymineless death of E. coli Y melT⁻ (Fig. 12) or E. coli 15T⁻ (Fig. 13), although the onset of lethality was delayed in both strains. In contrast, E. coli B27T⁻ supplemented, supernatant broth protected E. coli B27T⁻ from thymineless death when a low (10³ cells/ml) initial concentration of cells of E. coli B27T⁻ was employed as an inoculum (Fig. 17). Barner and Cohen (1954) also found that supernatant broth from a thymine starved culture of E. coli 15T⁻ protected cells of this strain from thymineless death even though it did not support growth. These investigators postulated the existence of a compound, other than thymine, present in the supernatant broth which was essential for the maintenance of viability.

They further suggested that thymine may be involved in the synthesis of essential metabolites other than intermediates in the synthesis of DNA. Cohen and Barner (1954) isolated several ultraviolet absorbing compounds from the supernatant broth of a thymine starved culture of E. coli 15T⁻ including uracil, orotic acid, and hypoxanthine. Data from an experiment on the incorporation of thymidine-methyl-C¹⁴ into DNA (Table 23) suggested that E. coli B27T⁻ excreted more labeled thymidine into the supernatant medium than the parent strain, E. coli Y mel. Cross-feeding was also observed to occur in a culture of E. coli 15T⁻ infected with phage T2r⁺ in minimal broth lacking thymine and yielded a culture containing 10⁹ cells/ml (Barner and Cohen, 1954). The infected cells synthesized thymidine via phage-mediated thymidylate synthetase and released sufficient thymine into the medium to support growth of cells resistant to phage infection but which retained the thymine requirement. The presence of a phage-mediated cross-feeding effect in E. coli B27T⁻ was unlikely; since the supernatant broth from this mutant failed to support growth of any strain examined which was susceptible to thymineless death. Mathews (1965) found that the yield of thymidylate synthetase defective phage, td 8, when mixedly infected in E. coli B with parental phage, td⁺, was reduced in cultures containing low cell concentrations (10⁴ cells/ml) as compared with the yield of phage from cultures containing high cell concentrations (10⁸ cells/ml). At high cell concentrations, lysis was proposed to cause the liberation of cellular components including thymine, thymidine, and thymine-nucleotides

into the medium in sufficient concentrations to support the multiplication of phage $\text{td } 8$.

Experiments on the effect of the initial cell concentration of E. coli B27T⁻ suggested that growth of this mutant without exogenous thymine did not result from the utilization of unusual intracellular pools of thymidine (or a derivative) (Fig. 9). Starvation of E. coli B27T⁻ cells in saline prior to their resuspension in minimal broth lacking thymine supported this conclusion (Fig. 10). Freifelder (1965) demonstrated that low ($0.1 \mu\text{g/ml}$) levels of thymine protected a thymine-requiring strain of E. coli B/r from thymineless death, but that the addition of $50 \mu\text{g/ml}$ of any riboside (e.g. uridine) to this medium resulted in thymineless death. These ribosides appeared to prevent the utilization of thymine pools. The mechanism was postulated to involve the conversion of thymine to ribosyl thymine by transribosylation (Mantsavinos and Zamenhof, 1961). When experiments similar to those described by Freifelder (1965) were performed, the addition of uridine to a culture of E. coli B27T⁻ in minimal broth lacking thymine did not prevent growth (Fig. 24) indicating that this mutant was not a leaky strain, producing low levels of thymidylate synthetase. O'Donovan *et. al.* (1971) reported that a mutant of E. coli, which contained abnormal nucleoside triphosphate pools, was unable to synthesize thymidine nucleotides. The mutation involved the synthesis of deoxycytidine triphosphate deaminase which is needed for the synthesis of deoxyuridine-5'-monophosphate (dUMP). The mutation in E. coli B27T⁻ was not similar to that reported by O'Donovan *et. al.*

(1971); since this strain cannot utilize deoxyuridine-5'-monophosphate for the synthesis of thymidylate.

Introduction of the F'araB24 plasmid, which carries the genetic region involved in the catabolism of deoxyribonucleosides (Lomax and Greenberg, 1968), into E. coli B27T⁻ resulted in a merodiploid strain, E. coli B27T⁻/F'araB24, which was susceptible to thymineless death (Fig. 20). Merodiploids of the thy A⁻drm⁻ (or dra⁻)/F'araB24 genotype can utilize thymidine as a sole source of carbon and require high (> 20 µg/ml) concentrations of thymine for growth (Lomax and Greenberg, 1968). Even though E. coli B27T⁻/F'araB24 grew on thymidine as a sole source of carbon, an elevated requirement for thymine was not detectable when the aerobic growth of this strain on minimal agar containing various concentrations of thymine was compared with E. coli B27T⁻. However, experiments, where a high concentration (10⁶ cells/ml) of cells was added to tubes of minimal broth supplemented with various concentrations of thymine (Table 11), showed that E. coli B27T⁻/F'araB24 required a higher concentration of thymine than that required by E. coli B27T⁻ to attain maximum turbidity. Thymineless death of E. coli B27T⁻/F'araB24 may thus result from the catabolism of the synthesized thymidine by enzymes specified by the deo region of the F'araB24 plasmid. Alternatively, the F'araB24 plasmid may specify the synthesis of a product lacking in E. coli B27T⁻ that is responsible for the thymineless death of E. coli B27T⁻/F'araB24. Two types of proteins have been postulated to be involved in the lethal event of thymineless death - a structural and an initiator

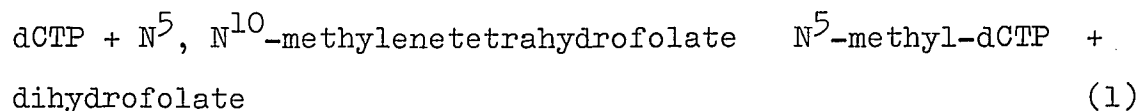
protein (Lark and Lark, 1964; Lark and Lark, 1966). Synthesis of the structural protein was postulated to be controlled by the completion of a round of DNA replication. Therefore, the structural protein was not produced during thymine starvation. Pritchard and Lark (1964) found that premature initiation of replication occurred at the origin of the chromosome during thymine starvation. In addition, Wilkins, Gallant, and Harada (1971) showed that the presence in E. coli B of a plasmid containing the tsx-pur E region of the E. coli chromosome (Appendix 1) caused increased sensitivity to thymine deprivation.

Analogues of nucleic acid bases have been detected in the DNA of several systems. Wyatt and Cohen (1952) showed that the DNA of T-even bacteriophage contained 5-hydroxymethyl cytosine in place of cytosine. DNA of several Bacillus subtilis phage including SP8 (Kallen et. al., 1962), SPO-1 (Okubo et. al., 1964), SP5C (Aposhian, 1965), ϕ e (Roscoe and Tucker, 1966), and ϕ 25 (Liljemark and Anderson, 1970) contained 5-hydroxymethyl uracil in place of thymine. Zamenhof, Giovanni, and Rich (1956) demonstrated that E. coli may incorporate 5-bromouracil into its DNA without an apparent change in phenotype, although modifications, such as elongated cells, were found. Under certain conditions of thymine deprivation, E. coli B27T⁻ may synthesize thymidine or an analogue of thymidine. For example, purified DNA preparations, obtained from cultures of E. coli B27T⁻ grown in the absence of exogenous thymine, contained, following hydrolysis and paper chromatography, four ultraviolet absorbing spots. Mobility (R_f)

of the ultraviolet absorbing spots and subsequent absorption spectra obtained for eluates of these spots indicated the presence of adenine, guanine, cytosine, and thymine in the DNA (Tables 17-22). Other ultraviolet absorbing spots were not detected.

Evidence that E. coli B27T⁻ DNA contained thymine suggested the possibility of an alternate pathway, independent of thymidylate synthetase, for the biosynthesis of thymidine. Evidence has been obtained for the presence of two pathways for the biosynthesis of 2'-deoxythymidine-5'-triphosphate in Bacillus subtilis (O'Donovan and Neuhard, 1970). The thymine requirement in B. subtilis consisted of two unlinked loci, thy A and thy B. Mutation of both loci was required to make thymine-requiring strains (Wilson, Farmer, and Rothman, 1966). Mutants of the thy A⁻thy B⁺ genotype were resistant to aminopterin while mutants of the thy A⁺thy B⁻ genotype were sensitive to the drug. Thymidylate synthetase activity was associated with the thy A region. Wilson, Farmer, and Rothman (1966) suggested that the thy B region coded for an alternate pathway which was not dependent on tetrahydrofolate but required a closely related coenzyme. Genetic mapping of thymine-requiring mutants of E. coli (Alikhanian *et. al.*, 1966) and Salmonella typhimurium (Eisenstark, Eisenstark, and Cunningham, 1968) indicated that the thy⁻ mutations occurred within a narrow region of the chromosome near arg A. The possibility of two closely linked loci, both of which must be mutated to produce thymine-requiring strains, has not been eliminated. Evidence supporting the hypothesis for two

thymine loci in E. coli was obtained by Eisenstark, Eisenstark, and Cunningham (1968) who found that cotransduction of the thy marker with a closely linked marker such as arg A or lys A was less frequent than was the cotransduction of the prototrophic, thy⁺, phenotype with the same loci. In addition, some thymine-independent revertants of these mutants retained resistance to aminopterin and were, therefore, phenotypically similar to the thy A⁻ thy B⁺ mutants found in B. subtilis. Alikhanian et. al. (1966) reported that 62 of 134 thymine-requiring mutants of E. coli mapped at a single locus and all were temperature sensitive. The remaining strains were located in 17 sites to one side of the temperature sensitive region. An alternate pathway was described for thymine-requiring mutants of E. coli (Forster and Holldorf, Abstract, 2nd FEBS meeting, Vienna, p. 146, 1965; discussed in O'Donovan and Neuhard, 1970) where deoxycytidine triphosphate (dCTP) was converted directly to deoxythymidine triphosphate (dTTP) by the sequential methylation and deamination and employed N⁵, N¹⁰-methylenetetrahydrofolate as a cofactor.



Enzymes catalyzing the above reactions were N⁵-methyl-dCTP synthetase (reaction 1) and N⁵-methyl-dCTP deaminase (reaction 2). The methylating enzyme was separatable from thymidylate synthetase by chromatography on DEAE-cellulose. Using this

technique, neither enzyme was detectable in cell extracts of the thymine-requiring mutants. Two types of thymine-independent revertants were found. One contained thymidylate synthetase and lacked the methylating enzyme, while the other lacked thymidylate synthetase but contained the methylating enzyme. This supported the hypothesis that two mutations were necessary in order to obtain thymine-requiring mutants (Holldorf, A. W. thesis. 1967. Albert-Ludwigs - Universitat, Freiburg; discussed in O'Donovan and Neuhard, 1970). Direct evidence for the presence of an alternate pathway for thymidine biosynthesis in E. coli B27T⁻ was not obtained. However, thymidylate synthetase activity was not detected in extracts of E. coli B27T⁻, although the bacterial DNA apparently contained thymine. Growth curves (Fig. 3) of E. coli B27T⁻ in minimal broth lacking thymine exhibited a biphasic nature in terms of the increase in colony-forming units which suggested a possible metabolic shift following resuspension of this strain in minimal broth lacking thymine. The difficulty encountered during the isolation of E. coli B27T⁻ after trimethopterin selection suggested that more than a single-step mutation was necessary in order to obtain a thymine-requiring mutant. Treatment of E. coli B27 with penicillin in the absence of thymine following trimethoprim selection may have resulted in death of "normal" thymine-requiring mutants by thymineless death and the selection of strains which were defective in thymidylate synthetase but contained an enzyme associated with an alternate pathway for the synthesis of thymine.

Methylation of nucleic acid bases at the polynucleotide level has been described in several systems and may constitute the basis for an alternate pathway for the biosynthesis of thymine in bacteria. Mandel and Borek (1961) showed that the methyl group of thymine found in soluble RNA of a methionine-requiring mutant, E. coli K12W6, was donated by methionine, although the thymine of the DNA in this strain was formed by de novo synthesis of the methyl group. Methylation of DNA, on the other hand, was found to be an established phenomenon occurring in all organisms examined and involved the enzymatic transfer of the methyl group of S-adenosylmethionine to specific bases in DNA (Mandel and Borek, 1963; Srinivasan and Borek, 1964). Transfer of the methyl group, also, occurred at the polynucleotide level in bacteria at or near the point of replication (Gold and Hurwitz, 1964; Fleissner and Borek, 1962; Billon, 1968). Grippo et. al. (1970) found that the DNA of sea urchin embryos, cultured in the presence of tritiated methionine labeled in the methyl group, contained labeled thymine in a small fraction of the total thymine isolated from the DNA. The nonrandom distribution of the labeled thymine suggested that methylation occurred at the polynucleotide level and was followed by the enzymatic deamination of the 5-methylcytosine. Guanine-cytosine to adenine-thymine (GC to AT) base transitions were suggested to occur following the formation of the minor thymine component that resulted in alterations in the genetic code which affected chemical events associated with cellular differentiation.

Alternatively, E. coli B27T⁻ may possess a defective thymidylate synthetase protein which is active only under certain conditions (increased carbon dioxide tension). The thymidylate synthetase protein may undergo a conformational change during growth leading to an active protein. Evidence has been obtained which suggested that thymidylate synthetase of E. coli is a multimeric enzyme (S. T. Roodman, Ph.D. Dissertation, Univ. of Mich., 1968). Association of the subunits of thymidylate synthetase of E. coli B27T⁻ may not occur under growth conditions, which lead to death, due to an amino acid substitution in the polypeptide chain which causes only a slight change in conformation. Alternatively, an inhibitor of the enzyme may be present and may only be effective in vivo under certain conditions. Transduction experiments (Table 25) showed that the thymine requirement of E. coli B27T⁻ was cotransducible along with the arg A region, indicating that this mutant possessed a mutation in the region (or closely linked to it) which specifies the synthesis of thymidylate synthetase. The sparing effect exhibited by carbon dioxide was also cotransduced with arg A.

An example of a thymine-requiring mutant containing a defective thymidylate synthetase protein was the conditional mutant, E. coli thy 101, described by Roodman and Greenberg (1971). E. coli thy 101 was temperature sensitive and was postulated to be blocked at the level of the polyribosome complex through a conformational defect in the elongation of the polypeptide chain. A shift to the permissive temperature resulted in the normal configuration of the partially synthesized

protein, and synthesis of the active enzyme was completed by a process dependent on protein synthesis.

Thymidylate synthetase activity was not detected in crude cell extracts of E. coli B27T⁻ grown in minimal broth lacking thymine (Fig. 37; Table 13). However, such extracts inhibited the thymidylate synthetase activity of similar extracts prepared from E. coli Y mel and E. coli B27 (Fig. 37; Table 14). In addition, supplemented, supernatant broth, from a culture of E. coli B27T⁻ grown in minimal broth lacking thymine, slightly inhibited the growth of a thymine-independent strain, E. coli Y mel. A thymine-requiring mutant of E. coli B was found by Barner and Cohen (1959) to contain trace amounts of thymidylate synthetase activity which lead Barner and Cohen to postulate the presence of an inhibitor. However, such a substance was not detected since crude cell extracts of the mutant did not inhibit the phage-induced thymidylate synthetase from phage T2 (or T5)-infected cultures of the mutant. During the purification of thymidylate synthetase from E. coli B, a fluorescent material was isolated by Sephadex G-75 chromatography which strongly inhibited the enzyme (Friedkin *et. al.*, 1962a). The inhibitor appeared to be a mixture of several derivatives of pteroylglutamate which inhibited both E. coli thymidylate synthetase and dihydrofolate reductase of mouse leukemia cells (Friedkin *et. al.*, 1962b). The inhibitor of E. coli thymidylate synthetase was identified as 5-formyl-tetrahydropteroyl-polyglutamate, a compound one hundred times more effective than the monoglutamate derivative (Friedkin, Crawford, and Plante, 1971). These investigators found that the inhibitor

of E. coli thymidylate synthetase was converted to a cofactor of the enzyme when it was transformed to the methylene form. The component which inhibited dihydrofolate reductase of mouse leukemia cells was identified as 10-formyl-~~pteroyl~~-polyglutamate. It was a stronger inhibitor of the mouse leukemia reductase enzyme than was 10-formyl-pteroylmonoglutamate and was only weakly active against E. coli dihydrofolate reductase. The functions of the pteroylglutamate derivatives in vivo are not understood, although they may be involved in regulation of or occur as products of folic acid degradation (Friedkin et. al., 1962a). Crusberg, Leary, and Kisliuk (1970) found that tetrahydrofolate could be replaced by tetrahydrohomopteroyl-monoglutamate in the synthesis of thymidylate in Streptococcus faecium and Lactobacillus casei.

Under certain conditions E. coli B27T⁻ was capable of growing in the absence of exogenous thymine. If pteroylglutamate derivatives are present, a role in the regulation of the synthesis of deoxythymidine triphosphate may be postulated. Depending on the physiological conditions, the interconversion of various forms of pteroyl derivatives may result in compounds which either inhibit or act as cofactors for thymidylate synthetase. Since thymidylate synthetase activity was not detected in crude cell extracts of E. coli B27T⁻ (Fig. 37; Table 13), the inhibitor may be either inactive in vivo under permissive growth conditions or serve as a cofactor for an enzyme associated with an alternate pathway when this mutant is grown in the presence of increased carbon dioxide tension and in the absence of thymine.

E. coli B27T⁻ cells growing in minimal broth lacking thymine formed elongated cells during the initial 4 hr of incubation (Fig. 27-31; Table 12). Later only small, normal-sized cells were observed. Several elongated cells were observed under the light microscope to contain evidence of septum formation and or branching. Cohen and Barner (1954) also reported that filamentous cells of E. coli 15T⁻ were formed during thymine starvation but that multiple nuclear bodies were not observed. On the other hand, Deering (1958) found that exposure of E. coli W cells to ultraviolet light resulted in inhibition of cell division and the formation of elongated cells which consisted of single chains containing many nuclear bodies. Shaw (1968) found that E. coli ML30 formed filaments when exposed to temperatures near or below the minimum temperature required for growth. Crosswalls were not formed under these conditions, although synthesis of DNA, RNA, protein, and mucopeptide continued. The cells contained abundant nuclear material as well as areas of incomplete septum formation. Mutants of E. coli sensitive to ultraviolet irradiation and which form filaments were designated as lon⁻mutants (Adler and Hardigree, 1964). Such mutants were observed by Howard-Flanders, Simon, and Theriot (1964) to exhibit distinctive colonial morphology on minimal agar plates, including a glossy, mucoid-like appearance. Mucoid-like colonial morphology was not observed when E. coli B27T⁻ was plated on minimal agar plates. Cummings and Mondale (1967) found that during thymine starvation, filament formation of several strains of E. coli was a common feature with the

exception of E. coli K12 rec⁻21 which was sensitive to thymineless death but did not form filaments. Gherardi and Sicard (1970) conducted experiments on mutants of E. coli K12, which carried mutations controlling filament formation following exposure to ultraviolet irradiation, suggested that there was not necessarily a relationship between filament formation and thymineless death.

Donachie (1969) suggested that the ratio of DNA to cellular mass was a critical factor affecting the timing of division. When the mass necessary for the initiation of a new round of division was reached during a period of inhibition of DNA synthesis, a new round of DNA replication would be initiated immediately upon the resumption of DNA synthesis. The division of these cells was, however, delayed until the completion of the new round of replication. The polyamine content of the cell was postulated by Inouye and Pardee (1970) to play a role in the regulation of cell division. These investigators suggested that the ratio of putrescine to spermidine may be a controlling factor for division. E. coli B27T⁻ exhibited some difficulty completing division during thymine deprivation. Effects of polyamines on the growth of E. coli B27T⁻ in the absence of thymine were not investigated experimentally since this mutant does not require arginine and thus would be expected to synthesize putrescine. Spermidine could also be synthesized in E. coli from putrescine and ornithine.

Thymidine has been shown to be involved in metabolic processes other than DNA metabolism since thymine diphosphate

derivatives function in the conversion of glucose to rhamnose, a cell wall constituent (Cohen, 1971). Mutations affecting the synthesis of rhamnose led to defective cell wall synthesis that resulted in osmotically unstable cells. Thymidine diphosphate rhamnose was isolated from E. coli 15T⁻ and Lactobacillus acidophilus R-26 (Okazaki, 1959) and from E. coli B (Okazaki, Okazaki, and Kuriki, 1960). Okazaki (1960) suggested that thymidine diphosphate sugar compounds could serve as intermediates in both DNA and polysaccharide synthesis in Lactobacillus acidophilus. Similar compounds were isolated from Pseudomonas aeruginosa (Glaser and Kornfeld, 1961) and Streptomyces griseus (Baddiley and Blumson, 1960). E. coli B27T⁻, however, did not appear to be blocked in its ability to form precursors of wall synthesis since this mutant grew normally on exogenous thymine or thymidine.

E. coli Y mel and its derivatives, E. coli B9 and E. coli B27, are lysogenic for phage λ (I. P. Crawford personal communication, 1970, and Table 7). The lethal effect of thymineless death has been attributed to the induction of bacteriophage (Endo et. al., 1965; Medoff and Swartz, 1969). Recently, however, Medoff (1972) suggested that the mechanism of thymineless death was separable from phage induction since lysogenic strains of E. coli were sensitive to thymine starvation and lost viability more rapidly than nonlysogenic strains. These investigators postulated two mechanisms to explain lethality during thymine starvation. One type of death resulted from induction of the phage and subsequent lysis of

the host cell during thymine starvation. The second mechanism was independent of phage induction. Significant differences in the kinetics of induction of phage in E. coli were not observed following the exposure of lysogenic strains to Mitomycin-C, thymine starvation, or heat (Lieb, 1966). Cummings and Mondale (1967) examined the survival kinetics of λ lysogenic, thymine-requiring strains of E. coli K12 and non-lysogenic parent strains and found no differences in their response to thymine deprivation. If this is true, phage λ in a mutant, such as E. coli B27T⁻, would not be expected to influence the response of this strain to thymine starvation. The mutant, E. coli Y melT⁻, is lysogenic for phage λ and is susceptible to thymineless death during thymine deprivation, suggesting that the presence of phage λ in E. coli Y melT⁻ and in E. coli B27T⁻ does not affect their response to thymine deprivation.

Several possible explanations for the growth of E. coli B27T⁻ in the absence of exogenous thymine that are based both on experimental data and data reported by others are: (1) an alternate pathway mediated by tetrahydrofolate for the biosynthesis of thymidylate such as the sequential methylation and deamination of deoxycytidine-5'-triphosphate (dCTP) to yield deoxythymidine-5'-triphosphate (dTTP), (2) an alternate pathway catalyzed by a pteroylglutamate derivative which may also inhibit thymidylate synthetase, (3) the in vivo interconversion of pteroylglutamate derivatives to serve either as cofactors or inhibitors of thymidylate synthetase depending on the growth conditions, (4) the biosynthesis

of an analogue of thymine which does not affect the transcription of the genetic code and is unstable during the purification of the bacterial DNA, (5) methylation at the polynucleotide level of DNA, (6) a defective thymidylate synthetase protein which is subject to a conformational change or to the association of the proper subunits under permissive growth conditions, and (7) crossfeeding among the population at high cell density.

In conclusion, E. coli Y melT⁻ and E. coli B9T⁻, though not studied extensively, were thymine-requiring mutants similar to other mutants of the thy A and deoxyribonucleoside regions. E. coli Y melT⁻ was susceptible to thymineless death, while E. coli B9T⁻ was resistant to thymine starvation but showed a gradual loss of colony-forming ability following a prolonged lag phase. E. coli B9T⁻ was also sensitive to high concentrations of thymidine suggesting a mutation in the region which specifies deoxyriboaldolase (dra⁻).

In contrast, E. coli B27T⁻ was capable, under certain conditions, of growing extensively in the absence of exogenous thymine. Data from several experiments suggested that this mutant may synthesize thymidylate by an alternate pathway. Experiments on the growth of E. coli B27T⁻ in minimal broth lacking thymine suggested a metabolic shift may occur following the removal of thymine from the growth medium (Fig. 3). E. coli B27T⁻ contained mutations in the thy A and deoxyribonucleoside regions of the chromosome. The lack of detectable thymidylate synthetase activity in crude cell extracts (Fig. 37; Table 13) and the cotransduction of the thymine requirement

of E. coli B27T⁻ along with arg A (Table 25) indicated a mutation in the thy A region. An analogue of thymine probably was not synthesized since a base having the mobility and absorption spectra expected for thymine was isolated from E. coli B27T⁻ DNA (Tables 17, 22). The introduction of the F'araB24 plasmid, which carries the markers associated with deoxyribonucleoside catabolism, into E. coli B27T⁻ resulted in a merodiploid strain, E. coli B27T⁻/F'araB24, that was susceptible to thymineless death (Fig. 20). Loss of colony-forming ability by this strain in the absence of thymine suggested that thymidine was indeed synthesized by E. coli B27T⁻ during thymine starvation in sufficient concentration to permit growth. Thus, the introduction of the F'araB24 plasmid into E. coli B27T⁻ led to the catabolism of the synthesized thymidine which then gave rise to thymineless death. Morphological studies of E. coli B27T⁻ during thymine starvation showed that division was delayed (Fig. 27-31; Table 12), an observation consistent with the hypothesis that a metabolic shift must occur when this strain is resuspended in minimal broth lacking thymine. The role of the inhibitor was not examined.

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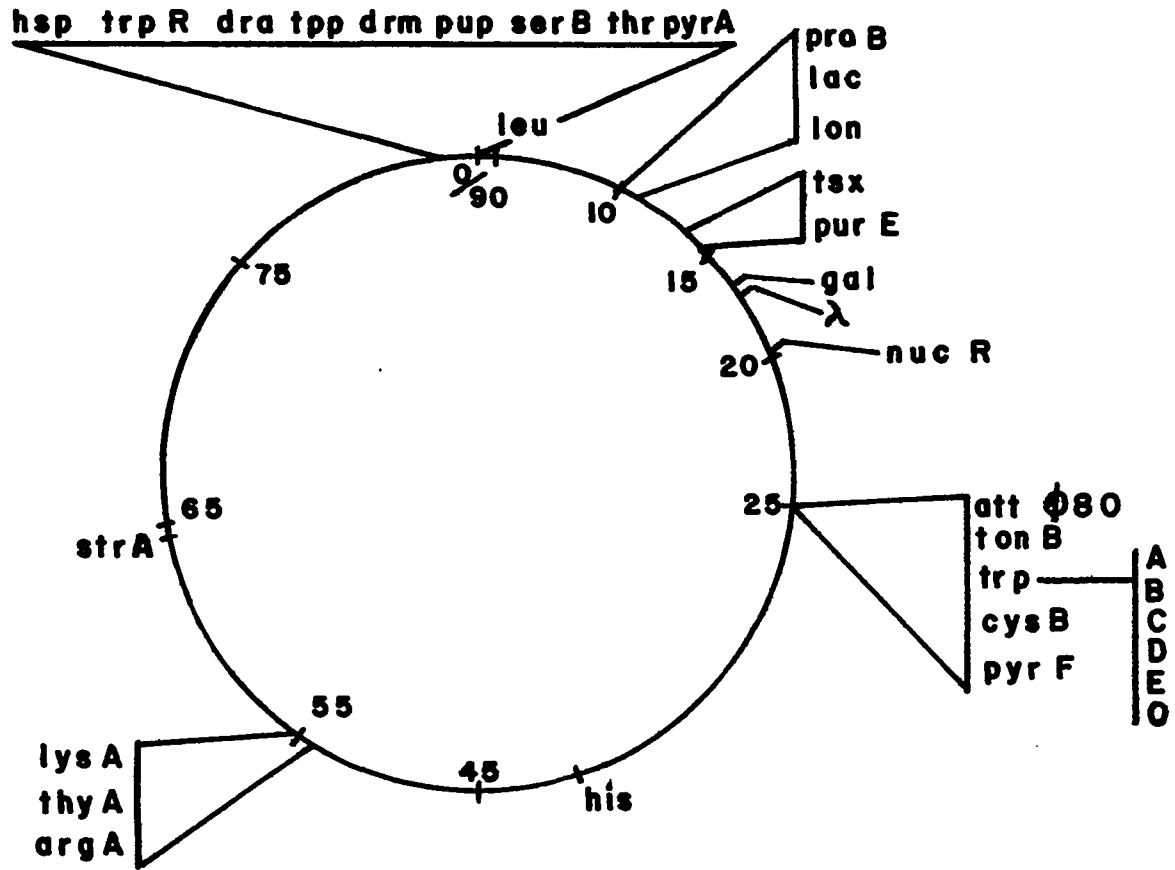
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APPENDIX

Escherichia coli Linkage Map



Appendix 1. Escherichia coli Linkage Map.^a

Symbol	Map Position (min.)	Enzyme or Function
arg A	54	arginine synthesis
att	17	attachment site for phage
att 80	24	attachment site for phage 80
cys _b B	25	cysteine synthesis
dra _b	89	deoxyribonucleoside catabolism; deoxyriboaldolase (<u>deoC</u>)
drm _b	89	deoxyribonucleoside catabolism; deoxyribomutase (<u>deoB</u>)
gal	17	galactose utilization
his	38	histidine synthesis
hsp	89	host specificity:DNA restriction and modification
lac	10	lactose utilization
lon	11	mutation leads to filament and slime formation, and to radiation-sensitivity
lys A	55	lysine synthesis
nuc R ^C	19	regulatory gene, mutation leads to constitutive synthesis of enzymes involved in catabolism of nucleosides
pro _b B	9	proline synthesis
pup _b	89	deoxyribonucleoside catabolisms, purine nucleoside phosphorylase
pur E	14	purine synthesis
pyr A	0	pyrimidine synthesis
pyr F	25	pyrimidine synthesis
ser B	89	serine synthesis
str A	64	streptomycin sensitivity/resistance/dependence
thr	0	threonine synthesis
thy A	54	thymidylate synthetase

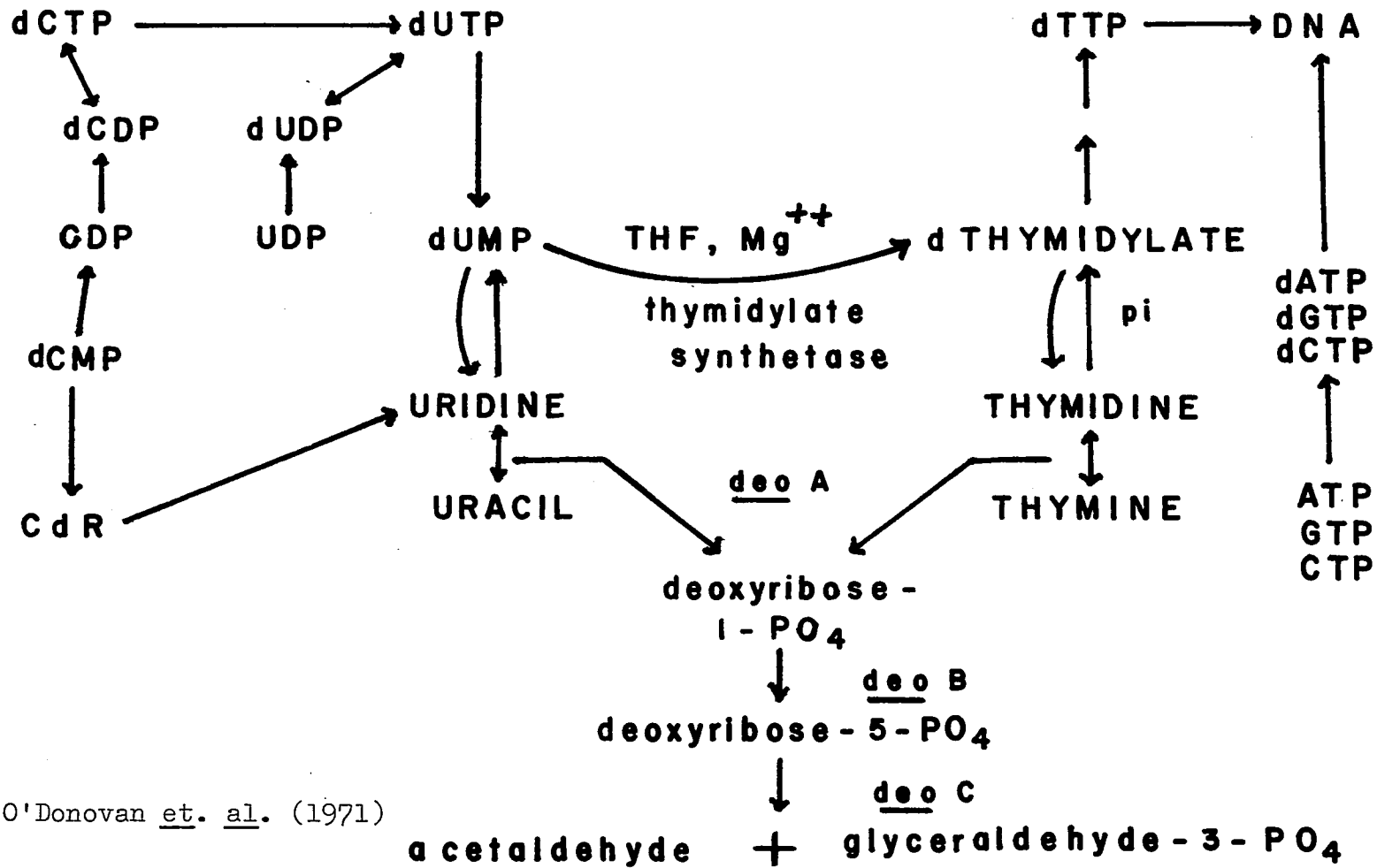
Symbol	Map Position (min.)	Enzyme or Function
ton _b	24	phage T1 resistance/sensitivity
tpp _b	89	deoxyribonucleoside catabolism, thymidine phosphorylase
trp	25	tryptophan synthesis
trp R	89	tryptophan synthesis, regulatory gene
tsx	12	phage T6 resistance/sensitivity

- a. Taylor (1970)
- b. map order dra-tpp-drm-pup proposed by Ahmad and Pritchard (1969)
- c. Ahmad and Pritchard (1971)

Appendix 2. Pathway for the Metabolism of Thymidine.

ATP	adenosine triphosphate
CDP	cytidine diphosphate
CdR	deoxyribose cytidine
CTP	cytidine triphosphate
dATP	deoxyribose adenosine triphosphate
dCDP	deoxyribose cytidine diphosphate
dCMP	deoxyribose cytidylate
dCTP	deoxyribose cytidine triphosphate
dGTP	deoxyribose guanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxyribose thymidine triphosphate
dUDP	deoxyuridine diphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
GTP	guanosine triphosphate
pi	inorganic phosphate
THF	N^5, N^{10} -methylenetetrahydrofolate
UDP	uridine diphosphate
deo A	thymidine phosphorylase
deo B	deoxyribomutase
deo C	deoxyriboaldolase

METABOLISM of THYMIDINE



O'Donovan *et. al.* (1971)