PLEIOTROPIC EFFECT OF DnaA GENE ON INITIATION OF DNA REPLICATION AND CELL DIVISION IN *ESCHERICHIA COLI*

by

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ABSTRACT

Cell duplication in Escherichia coli involves complex events, coordinated with chromosome replication. Because of the importance of chromosomes in perpetuating the normal cell cycle the initiation of their replication must be coordinated with cellular division. Following initiation, the cell must replicate and segregate its chromosomes, create a site necessary for septation and divide. These events could be coordinated by either; (1) biochemical reactions involving diffusible enzymes, or (2) multienzyme complexes which are localized at the site of DNA replication and cell division. In the latter case, the cyclic events of replication, segregation and cell division may be coordinated by physical-chemical or biochemical means. In any case, physical association implies pleiotropic effects.

To test this hypothesis, cell division of the initiator mutant of E. coli, isolated by Kohiyama (1968) was studied. The temperature-sensitive initiator mutant E. coli CR 34T83 (ts DnaA) grew normally at 30 C, and at the restrictive temperature (42 C). The DNA replication as measured by radioactive precursor uptake, stopped after approximately 40 minutes and was equivalent to completion of rounds of replication started. Measurement of ribo- and deoxyribonucleotide triphosphate pools by thin-layer chromatography at 30 C and 42 C indicated residual DNA synthesis was not due to a limitation in the DNA precursors.

Using a combination of density and differential radioactive labelling for the starts and ends of chromosomes, a preferred place for re-initiation of new replication cycles was shown. It was shown that DNA replication at 42 C terminated at a fixed region of the chromosome, and was identical to the 150 µg/ml chloramphenicol sensitive step involved in the process of initiation of chromosome replication in E. coli.

A cessation of cellular division was noted by measurement of cell growth by Coulter Counter, at a shift from 30 C to 42 C, resulting in filamentous growth. Upon a return to 30 C, the cells resume division after approximately 15 - 20 min. The pleiotropic behaviour, that is, the cessation of cell division and initiation of DNA replication was a result of a point mutation in the gene DnaA, coding for a membrane bound protein involved in initiation. This mutation was mapped by transduction and was located at the isoleucine-valine region of the E. coli map. When this gene was transduced to different strains of E. coli K_{12} the same pleiotropy was observed. This pleiotropy could be uncoupled, however, at 30 C by inhibitors of DNA synthesis or initiation.

During recovery at 30 C from growth under 42 C, expression of cell division was proportional to cell equivalents generated at the restrictive temperature. RNA and protein synthesis, for 10 minutes during the recovery period, was obligatory for initiation of new rounds of replication, but not for the expression of cell division. A cell

division "potential" protein was present under the restrictive growth condition. This "potential" was made at a derepressed rate and underwent a rapid degradation if kept at 42 C. At any given time, when returning from 42 C to 30 C, this "potential" allowed expression cell division based on DNA/mass or normal cell equivalents generated at 42 C. The half-life for decay of the division "potential" was estimated to be 1.4 minutes.

The results were interpreted, in terms of an enzyme complex, which is common to the initiation of DNA replication and cellular division.

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ABBREVIATIONS

(IUPAC-IUB-CBN 1970 Recommendations)

T	Thymine
dThd	Thymidine
BrdUrd	Bromodeoxyuridine
BrUra	Bromouracil
dTTP	Deoxythymidine triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
ATP	Adenosine triphosphate
CTP	Cytidine triphosphate
GTP	Guanosine triphosphate
UTP	Uridine triphosphate
TCA	Trichloroacetic acid

LITERATURE REVIEW

The cell cycle of bacteria is a complex, but well-coordinated process. This process, which allows the cell to generate its progeny, is an accumulative one. Thus, sequentially, starting with chromosome replication and ending with physical separation of the daughter cells, we see a number of events which, under normal conditions aim at one ultimate goal - the "duplication" of the cell.

The duplication of E. coli is a sum of two well controlled events: (1) the DNA replication; and (2) the cell division cycle. The separate aspects of the DNA replication cycle have the following expressions: (i) attachment; (ii) initiation (an event); (iii) the origin (a site); (iv) polymerization (chain elongation); and (v) segregation. The separate aspects of cell division are: (i) formation of division proteins; (ii) physiological division and physical separation of the two cells.

Much of the earlier work on the regulation of bacterial growth and duplication has been reviewed in the extensive treatise by Maaløe and Kjeldgard (1966).

The state of current knowledge concerning all aspects of replication of DNA in microorganisms has been amply discussed in the Cold Spring Harbour Symposia of Quantitative Biology of 1968. Since that time, the subject of DNA replication has been reviewed by Bonhoeffer and

Messer (1969) and Lark (1969b), and the cell division cycle by Helmstetter (1969a, b), Donachie and Masters (1969) and a Symposia of the Society for General Microbiology (1969). Since the appearance of these reviews, some observations have been made which should be added to the present reviews. The following literature survey is an attempt to make the information current.

I. The DNA replication cycle in *E. coli*

The model accepted for replication supposes the Watson-Crick structure of hydrogen-bonded assembly of four nucleotides to make up the *E. coli* chromosome and replication by a semiconservative mechanism.

A. The membrane attachment of DNA in *E. coli*

Jacob, Brenner and Cuzin (1963) suggested a chromosome attachment site on the cell membrane. This unit systematically could hold the replicase unit, through which the chromosome circulated, and could act, as well, as a vehicle for moving daughter chromosomes apart. Several groups have provided additional evidence and extensions of this hypothesis. These studies are either directly electron microscopic, or fractionational separation and biochemical studies. The fractionational separation studies are variations of the M-band method (Tremblay *et al.* 1969). Essentially, the method consists of lysing the cells with sarkosyl and banding the lysate in a sucrose gradient containing Mg^{++} ions. The magnesium ions induce the crystalization of sarkosyl

in situ. Ten to thirty percent of the cell membrane, 75% of RNA in the cell and 90% of the DNA are found associated with the crystals. Because nucleic acids alone have no affinity for sarkosyl, the simplest inference is that the complexed DNA and RNA is attached to fragments of the membrane containing the chromosome attachment site and the RNA in turn, is presumably attached to the DNA from which it was being transcribed.

Recently, Shachtele *et al*. (1970) and Daniels (1971) using the M-band method have shown specific attachment of *E. coli* DNA. Fielding and Fox (1970) provided evidence for a stable attachment at the replicating origin in *E. coli*. The consensus from these studies is that the DNA at the attachment region is protected from sonic iscillation and represents approximately 0.6 percent of the total DNA of the cell which would correspond to a length of 6 μ.

Fuchs and Hanawalt (1970), using a 5 - 20% linear sucrose gradient layered over a 60% sucrose "shelf", have succeeded in isolating a "growing point" complex from *E. coli*. The latter contained 0.5 - 1% of the entire *E. coli* genome; that is, at least 5 μ of DNA.

Direct electron microscopic studies involving thin sectioning of *E. coli* strains and subsequent staining, has indicated association of the nuclear regions to the membranes (Altenburg *et al*. 1970; Pontefract and Thatcher, 1970). In general, these studies do not permit an adequate demonstration of the attachment organelle. Furthermore, in one case, the strain 0111-a of *E. coli* was used which is known to form

"extra membranes" (Weigand et al. 1970), and could thus invalidate the observations of Altenburg and co-workers.

B. The initiation of normal chromosome replication in E. coli.

The replicon theory (Jacob, Brenner and Cuzin, 1963) implicated that the replication of the bacterial chromosome was controlled by a gene whose product was "the initiator". The initiation was defined as the interaction of the initiator with a particular site of the replicon, the replicator. Under normal growth conditions, the "initiator" synthesis is continuous and the material includes protein. However, it involves more than one step (Lark and Ranger, 1969; Ward and Glaser, 1969). The steps are dissociable by different levels of chloramphenicol. One step occuring at the time of, or slightly before, initiation, is sensitive to high (150 µg/ml) and resistant to low (25 µg/ml), concentrations of chloramphenicol, and is the same as the amino acid sensitive step of initiation. A different step, occurring much earlier is sensitive to low levels of CAM. The low levels of CAM are 2 µg per ml and 25 µg per ml for E. coli B/r and TAU$^-$ respectively. Cooper and Weusthoff (1971) however, suggest that there are either more than two steps involved in CAM sensitivity of initiation, each defined by its sensitivity to a different (1 to 20 µg per ml) concentration of CAM, or as suggested by Lark (1969b), there are only two steps, with a spread of "probability" that the step will be inhibited by CAM in a particular cell. There no longer can be much doubt that amino acid starvation can bring the DNA replication to a

fixed end point, and, on restoration of amino acids, synthesis will take up again from this, the natural starting point (Bird and Lark, 1968; Kohiyama, 1968).

The starting point for the chromosome replication, the origin, is not a point but a region. The origin of replication has been defined on the genetic map of E. coli K12 and B/r/1, and is located between arg G and xylose loci (7 and 8 o'clock). This conclusion is backed by three different experimental approaches: (1)transduction (Abe and Tomizawa, 1967; Caro and Berg, 1968; Masters, 1970); (2) enzyme induction (Donachie and Masters, 1969; Wolf et al. 1968; Helmstetter, 1968); and (3) synchronized mutagenesis (Cerda-Olmeda et al. 1968; Wolf et al. 1968). Essentially, all experiments are based on gene-dosage effects assuming doubling of a gene, or its product, upon replication of that gene.

Original studies by Nagata (1963) and recently those of Vielmetter et al. (1968), disagree with the idea of fixed chromosome replication origin. This contradiction arose from studies of Hfr strains, where it was shown that the site of integration of the F factor served as the origin and the polarity of the insertion determined the direction of replication (Nagata, 1963). Vielmetter et al. (1968) also obtained results favouring this conclusion. In view of these findings it remains confusing as to the site of the origin for replication in E. coli. The idea that once initiated, the circular molecule of DNA replicates sequentially along its length

has been re examined by Nagata and Meselsohn (1968). In their experiments, a pulse of ^{3}H-TdR was given to exponentially growing cells. Subsequently, if cells were pulsed with 5-bromouracil, it was found that the tritium label was associated with the 5-bromouracil only when the interval between addition of the radioactive and density label pulse equalled a multiple of the generation time. Thus, the original observations of Cairns (1963) and Lark et al. (1963) were confirmed on the sequential replication.

C. The replication of the E. coli chromosome.

Two important questions are to be asked here: (1) how does the replication start; and (2) how does the chain elongate in E. coli? Several models for the latter have been proposed since 1968, by Gilbert and Dressler (1968) and by Okazaki et al. (1968); by Haskel and Davern (1969); by Richardson (1969); by Morgan (1970); and by Werner, (1971). Many of the current models are variations on the simple model proposed by Watson and Crick (1953) suggesting a separation for the strands at the growing point, addition of nucleotides by Watson-Crick base pairing, and joining by enzymatic means (Guild, 1968; Hurwitz et al. 1968; Okazaki et al. 1968; Kornberg, 1969; Richardson, 1969). Gilbert and Dressler have proposed a general model, the "rolling circle" to account for the feature of both E. coli and other bacteria or bacteriophage replication. Briefly, the model proposes that synthesis begins by opening one strand of the original circle at a specific point by introducing a single-strand break

displaying a 3'-hydroxyl and a 5'-phosphoryl end-group. In order to prevent ligase from repairing this break, the 5'-end is transferred to some site, perhaps on a membrane. DNA polymerase then initiates synthesis by elongation of the 3'-end and uses the intact circular strand as a template. The old positive strand is peeled off the circular template for DNA polymerase to synthesize the new negative strand. As the growing point continues around the circle, a daughter molecule is peeled off endlessly.

Kubitschek and Henderson (1966) and Morgan (1970) have proposed mechanisms in which nucleotide precursors are paired in Watson-Crick base pairs before being incorporated into the daughter strands. Although the two models differ in many respects regarding the reaction mechanism, they both have the important fundamental property that only one parental DNA strand is copied in the event of a mismatch. To resolve some of the paradoxes created by all of the above models, Werner (1971) has suggested a new mechanism for DNA replication in E. coli, and has introduced a reasonable doubt about what has been accepted as plausible mechanisms for DNA replication. In brief, using short pulses of tritiated thymidine, he found that high molecular weight DNA is formed prior to the appearance of low molecular weight DNA, suggesting that large DNA is the precursor of DNA fragments, and implying that Okazaki pieces are not the result of discontinuous synthesis. During short pulses, the relative amount of label found in Okazaki pieces varied with the nature of the precursor used.

Twenty percent of the incorporated ^{3}H-thymine was found in the pieces, and, in the presence of unlabelled thymidine, the incorporation of ^{3}H-thymine into Okazaki pieces was entirely suppressed. He proposed that the pieces arose from single strand nicks in both parental and newly synthesized DNA to act as swivel points for the rotation of the DNA helics during replication and transcription. He also suggests that the labeling of the pieces during short pulses of ^{3}H-thymidine represents repair synthesis, while thymine is used for DNA replication.

Haskel and Davern (1969) have presented the "pre-fork synthesis model for DNA replication". In summary, DNA synthesis is continuously initiated from parental strand nicks and occurs ahead of the fork. The nicks thus act as initiation sites for chain synthesis and small chains, like Okazaki fragments, are synthesized via a polymerase. The fork serves as a locus for unwinding and separating the already replicated strands of the two double helices.

The predictions of the models proposed above have been tested in E. coli, and, in every case, certain drawbacks are present. Lark (1969a) and Caro (1970) have looked at the question of symmetry with respect to the Gilbert and Dressler model (1968). It was concluded that in E. coli, the DNA is replicated by a symmetrical process and initiation takes place on both daughter chromosomes at once (Caro, 1970; Lark, 1969a). Lark, in a very sophisticated way, excluded both the age and the polarity of the template as a source for selective asymmetry and thus absence of inherent asymmetry in the DNA molecule,

which could restrict the selection of template for replication. Finally the "rolling circle" model was tested in a nutritional shift-up of synchronous B/r/1 cells. Results did not fit with the asymmetrical model predicted (C. Bagwell, unpublished results). Thus, the "rolling circle" question remains open for examination.

The Haskell and Davern (1969) model has not been tested experimentally yet and probably should be classified under other schemes that have been compiled in the Journal of Theoretical Biology by Erhan (1969) and Phillips (1969).

The Werner (1971) model has already been challenged (Lark, personal commun.), particularly when his proposed recognition of thymine for replication and thymidine for repair could not be substantiated by the known sequence of the pyrimidine biosynthetic pathway (O'Donovan and Neuhard, 1970; O'Donovan, personal commun.). Finally, the strain used in this study (15 TAMT, Cairns and Denhardt, 1968) was grown at 14 C to establish a generation time long enough (400 minutes) to allow for short pulse labelling. It has been well documented by Ingraham's group that macromolecular synthesis and growth of E. coli at low temperatures is severely affected (Ng, Marr and Ingraham, 1962; Shaw and Ingraham, 1967; Shaw, 1968; Ng, 1969). Thus, Werner's observations could well be an artifact of growth at 14 C.

The remaining models which fit into the original suggestions of Watson and Crick (1953) are viable and differ only in the mechanistic sense (Okazaki et al. 1968; Richardson, 1969; Kornberg, 1969).

The first question, "how does the replication start"? still remains as a grey, rather than the black box of mystery for molecular biologists. Although the origin and terminus of a circular chromosome structure are the ends of a linear double strand, untwisting the molecule, when it is in helical form, would be greatly enhanced by occasional breaks in the chains (Wang and Davison, 1968). However, the mechanism for the creation of a swivel, which would be the growing point or the fork, is still obscure.

In order for the replication to start, special enzyme(s) or replicases are needed. These have been polymerase I (Kornberg polymerase) II, (Cairns polymerase) and III (Gefter polymerase) (de Lucia and Cairns, 1969; T. Kornberg and Gefter, 1970), but the Kornberg polymerase no longer is qualified in replication and seems to be a repair replicase (Kelly et al. 1969). The chromosomal replicases could, upon the formation of the fork, synthesize the new chains. Since all DNA polymerases have been shown to have an absolute requirement for adding nucleotides to a free 3'-OH group of preexisting primer (Richardson, 1969), the newly formed chains at the fork must extend themselves in an antiparallel fashion by synthesizing short segments of DNA at a given time. The synthesis would be restricted to the fork region, and the segments formed would subsequently be joined together. Recent evidence supports this system and will be given here.

In vivo studies by Yudelevich *et al.* (1968), with *E. coli* CR34, and Iyer and Lark (1970), with *E. coli* 555-7 ($TAMT^-$) and *E. coli* Pol A^- have indicated a preferential location of newly synthesized DNA at the 3'-OH end of a large deoxynucleotide strand. Thus, regardless of the polymerase system used, the asymmetry in starting the replication by the replicase mimics that of the *in vitro* synthesis of DNA by the Kornberg polymerase.

Studies initiated by several workers on *E. coli* (Okazaki *et al.* 1968; Yudelevich *et al.* 1968; Sadowski *et al.* 1968; Bird and Lark, 1969; Iyer and Lark, 1970), has supported the discontinuous mode of replication. Collectively, the following conclusions have been reached: (1) with pulses of thymidine (or thymine, Lark, personal commun.), incorporated label is mainly found as small (1 micron) and large (3 to 300 microns) single strand pieces extractable by alkali; (2) at any instant, there are about 5 short pieces in cultures with chromosome replication time of 40 minutes and 3 with replication time of 80 minutes; (3) the time required for the synthesis of a 1 μ piece at 37 C is 2 seconds, and this is the same for fast and slow growth rates; (4) studies with exonuclease I, which specifically hydrolyses single stranded DNA from the 3'-end, confirms the extension of the chain from the 3'-end.

As pointed out by Iyer and Lark (1970), two explanations could validate the observed occurence of the short and long pieces; (1) synthesis occurs by the continuous extension from the 3'-end and, for

short pieces, in the 5' and 3' direction; (2) synthesis occurs symmetrically from 3' to 5' on both strands but a symmetrical fragmentation occurs only on the 5'-strand, such that it contains the single strand breaks. These points are not clear in the E. coli system. In B. subtilis, pieces are complementary to only one strand of parental DNA (Kainuma and Okazaki, 1970) while in T4 and Lambda phages, the short pieces anneal equally to both strands (Ginsberg and Hurwitz, 1970; Okazaki et al. 1970).

Recent studies on toluenized E. coli cells (Moses and Richarson, 1970; Mordoh et al. 1970; Kohiyama and Kolber, 1970) have shown replicative synthesis of DNA under in vivo conditions, the presence of all four deoxyribonucleotide triphosphates, and have indicated a stimulation in synthesis by ATP alone. Furthermore, the replicative synthesis could be abolished in DNA temperature-sensitive mutants. Moses and Richardson (1970) have characterized further the newly-made DNA by sedimentation and pycnographic analysis, and there is little doubt that this incorporation does correspond to chromosomal DNA. This system has not been explored enough to allow analysis of the steps involved in the replication nor of the question of discontinuous replication.

The model of discontinuous synthesis implicates the need for the DNA ligase to covalently join the fragments. In vitro (Modrich and Lehman, 1971; Sadowski et al. 1968) or in vivo (Pauling and Hamm, 1969) studies on DNA ligase clearly document the presence of this enzyme and purported functions.

Preliminary approaches to the dissection of the active replicating machinery, which would clarify whether or not the polymerase-ligase-nuclease complex exists in E. coli, are promising (Smith et al. 1970; Knippers et al. 1970; Kohiyama and Kober, 1970). Hopefully, these systems should demonstrate exactly how the initiation of DNA replication in E. coli is started.

D. Separation of daughter chromosomes: Segregation.

Replicated DNA and chromosomes eventually become evenly partitioned between daughter cells. Morphological studies coupled with autoradiography have shown that the E. coli chromosome is indeed associated with the cell membrane (Ryter, 1968; Rubenstein et al. 1970). Studies of Ryter, Hirota, and Jacob (1968) suggested that segregation is random, that is, at each cell division, each of the old strands has the chance of distribution into either progeny. Lark (1966) had obtained different results which suggested that a definite pattern of segregation existed. More recently however, Chai and Lark (1970), upon reexamination of the previous model, support Ryter's random segregation model.

II. Cell division cycle of E. coli

A. RNA and protein synthesis in the normal division cycle of E. coli

During its duplication cycle, RNA and proteins are synthesized simultaneously with replication of the chromosome and thus the cell

increases in size, mass and cellular constituents. In the case of general RNA synthesis, the rate is apparently proportional to the amount of template DNA available (Cultler and Evans, 1967; Helmstetter, 1969b). The RNA precursor pools, on the other hand, indicate fluctuating patterns during the cell cycle (Mychalowska, 1970).

Although a requirement for protein synthesis has been demonstrated (Mathison, 1968) in cell division, no specific proteins have been identified unique to cell division. However, changes in the "division proteins" have been implicated by several groups (Inouye and Guthrie, 1969; Green et al. 1969; Inouye and Pardee, 1970; Reeve et al. 1970; Smith and Pardee, 1970; Inouye, 1971) but the work suffers two criticisms: (1) if these proteins are enzymes, their substrates are not known; and (2) in no case have those proteins been shown to be the gene product of the genetic lesion. A role in septation has been postulated for the "division proteins" in the above listed works in general.

In the absence of protein synthesis, limited division has been observed (Pierucci and Helmstetter, 1969). When protein synthesis was entirely blocked during DNA replication, in spite of the completion of rounds, no division was observed. However, if protein synthesis was inhibited subsequent to the completion of a round, division was observed. These results were consistent with the "trigger" for division idea previously suggested by Clark (1968) and Helmstetter and Pierucci (1968). This concept is probably correct,

but it does not entail the additional requirements needed for division (Donachie et al. 1968), that is, the critical mass-to-DNA ratio. Changes in the patterns of these division proteins during the normal cycle remains to be studied.

B. Physiological division and septation.

The importance of separating the duplicated cell, by compartmentalization, into two physiologically separate entities has been emphasized by Clark (1968). After the end of a round of replication, two cells are physiologically separated by the formation of a weak septum and then by a strong cross-wall (Clark, 1968). Evidence has implicated a burst in the synthesis of membranes in E. coli at the point of the physiological division (Daniels, 1969).

It is possible that septation enzymes and building blocks need membrane-proteins complexes for their attachment and priming before they can erect the septum. In such a case, physiological division would allow formation of a light barrier which would act as an anchorage site for deposition of binding proteins for septum enzymes. Recently, a membrane protein has been isolated in S. faecalis whose function is organizational rather than catalytic in that it serves as a binding site for ATPase (Baron and Abrams, 1971).

III. Regulation of the cell duplication cycle in E. coli.

A. Control of the DNA replication in E. coli.

Hirota et al. (1968) suggested two modes of regulation for

the initiation of the chromosome replication in E. coli. The first is a negative system, that is, the presence of a repressor which would block the replicator (Jacob, Brenner, and Cuzin, 1963) from being accessible to replicating enzymes. The derepression could occur by sudden critical variations in the concentration of some molecule, the regulation of which was through DNA replication or cellular growth. The second is a positive regulation which implicates that a molecule is produced in each DNA cycle which could directly initiate new rounds. No firm experimental data in support of either hypothesis is available.

Studies of replication under conditions in which gross protein synthesis is inhibited (Lark and Ranger, 1969), or in which synthesis of specific initiator proteins (Levine and Sinsheimer, 1968) is inhibited have indicated that such initiator proteins are present in stoichiometric amounts and that additional protein synthesis is required in order to initiate new rounds of replication. Rosenberg et al. (1969) by starving E. coli 55-7 (a 15 T^-) for T, obtained accumulation of initiation potential. If amino acids were withdrawn, following the T starvation, net protein synthesis was halted. Under amino acid starvation, however, if T was added back, DNA replication continued for a round and then stopped. When CAM (300 µg per ml) was added to the latter culture, "uncontrolled" DNA replication was observed over several hours. It was concluded that once a new cycle had been initiated, still other unregulated initiations could occur and, to prevent this, small amounts of protein was needed. Similar conclusions have been reached

by Kogoma and Lark (1970) where, following a period of inhibition of replication with NAL, temperature sensitive mutations, or T starvation, DNA synthesis can proceed for many hours in spite of the inhibition of protein synthesis.

Pritchard, Barth and Collins (1969) have proposed an alternative model for the control of DNA replication. Their model suggests that an initiator protein is made in the cell constituitively which constitutes a constant fraction of total protein of the cell at all growth rates. An inhibitor protein is coded by a gene located adjacent to the chromosome origin, or part of the origin itself, and is transcribed only during the replication of that gene. Each inhibitor gene, and consequently each chromosome origin, is responsible for the synthesis of a fixed number of the inhibitor proteins at all growth rates. There is a cooperative interaction between the inhibitor protein and either the initiator or chromosome origin. This interaction is such that a two-fold change in its concentration effects an inhibition of initiation over a range from complete to zero. Thus, when the cytoplasmic volume increases by growth of the cell, the concentration of the inhibitor will be diluted progressively. When the level of inhibitor falls below the critical level, reinitiation takes place, which will be associated with the production of a new pulse of inhibitor. The frequency of the initiation will be determined by the dilution rate of the inhibitor, that is, the reciprocal of the growth rate. An important feature of this model is that (1) it

is self-regulatory and (2) assumes a negative control of replication.

Simon (1968) proposed a semiquantitative model for the cell cycle in E. coli, where it was assumed that the DNA replication starts when a threshold dTTP concentration was reached. This model was not tested by the author. The dTTP levels during the cell cycle (Mychajlowska, 1970) however, could substantiate this model.

The two models of regulation of DNA replication are not without precedent. The positive model could exist with a negative control operating on the synthesis of the initiator protein (Hirota, et al. 1968; 1970). The mutant, CR34T46, is assumed to have an alteration in the protein, which in some way, acts on the chromosome to allow replication to start. Furthermore, this component would act only when required for initiating a new cycle.

Hirota et al. (1970) have also considered the regulation of the initiator mutant CR34T46 by the negative control, the synthesis of a repressor during growth (Pritchard et al. 1969), or of the antirepressor (Rosenberg et al. 1969). In this case, it is argued that the T46 mutation is not an alteration in the regulatory mechanism, since only 10 minutes at 30 C is enough for reinitiation to occur, but would not be enough time for the dilution of the repressor to occur. If the antirepressor is the element affected, it would be compatible with a model where the antirepressor is made immediately after DNA replication has stopped.

It should be possible to resolve these questions by study of

several "initiator" mutants. In the past, several such mutants have been isolated in E. coli (Kohiyama et al. 1966; Fangman and Novick, 1968; Kuempel, 1969; Carl, 1970). Phenotypically, these mutants are characterized by their capacity for residual DNA synthesis. Genotypically, they do not fall into the DnaA class (Fangman and Novick, 1968; Carl, 1970). It is also possible that the conditional lethal mutants isolated, which affect initiation, deal specifically with different stages of initiation and the regulation of initiation. To date this is not clear.

B. Regulation of cell division in E. coli

The only fact known for certain about normal E. coli cell division is that it is coupled to DNA replication (Clark, 1968b; Helmstetter and Pierucci, 1968). Inhibition of DNA replication normally allows for 25 percent residual division (Clark, 1968b; Helmstetter and Pierucci, 1968), or causes an immediate cessation of cell division (Donachie, 1969; Inouye, 1969), and DNA-less cells are not produced. Even production of DNA-less minicells by a mutant of E. coli was blocked upon stopping of DNA replication (Clark, 1968b). However, it is possible to get temperature sensitive mutants that could produce DNA-less cells at 40 C when they have lost their ability to synthesize DNA (Hirota et al. 1968).

Inouye (1971) has shown that cell division can be uncoupled from DNA replication by introducing a defective recA gene, which is involved in recombination, into E. coli when DNA replication was blocked.

Hence DNA-less cells result. The product of the recA gene has been shown to reduce the amounts of nucleases produced by the recB and recC genes (Willets and Clark, 1969; Barbour *et al.* 1970), but the correlation between the recA gene product and the septum is not yet understood. Interestingly enough, whenever the DNA replication and cell division are uncoupled by mutations so that DNA-less bacteria are produced, these mutants behave as if rounds of replication are completed at regular intervals.

A rigorous analysis of the regulation of *E. coli* cell division was reported by Donachie and Begg, (1970), in which the coordination between DNA segregation and cell division were explained in terms of a unit cell concept. However, the mechanism for cell division in *E. coli*, and the steps involved in the regulation of expression of division, remain speculative (Pardee, 1968; Previc, 1970).

MATERIALS AND METHODS

I. Bacterial and Phage Strains

A. Bacterial strains

Bacterial strains of Escherichia coli used in this study are given in Table I.

B. Bacteriophage strains

For the transduction experiments, the generalized transducing bacteriophage P1kc-L4 (Caro and Berg. 1971) was used.

II. Media and chemicals

A. Media

E. coli CR34T83 was grown in a v/v mixture of tryptone broth medium A of Kaiser (1955) and the minimal salts medium E of Vogel and Bonner (1956). The following supplements were added: glucose, 0.5 µg per ml; thymidine, 20 µg per ml; deoxyadenosine, 50 µg per ml; vitamin B_1, 7.5 µg per ml. These media will be referred to as A and E, respectively. When grown under minimal conditions, the required amino acids were added at 20 µg per ml to minimal salts medium E of Vogel and Bonner (1956).

E. coli B/r/1 was grown in 007 minimal salts medium with 0.5% glucose added (Clark and Maaløe, 1967).

Table I. Bacterial strains.

Strain Number	Mating Type	Relevant Genetype	Derivation and source
KG 55	F^-	thr^-, leu^-, pro^-, his^-, thi^-, arg^-, lac^-, gal^-, ara^-, xyl^-, man^-, T_6^r, str^r	AB 1157 from E.A. Adelberg
KG 71	F^-	thr^-, leu^-, thi^-, ilv^-, $thyA^-$, $DnaA^-$	CR34T83 from M. Kohiyama
KG 71-2	F^-	Same as KG 71 but low thymine requirer	This work
KG 74	F^-	thr^-, leu^-, thi^-, ilv^-, $thyA^-$, $DnaA^+$	This work
KG 77	F^-	thr^-, leu^-, thi^-, ilv^+, $thyA^-$, $DnaA^-$	This work
KG 78 (1-40)	F^-	KG 146, ilv^+, $DnaA^-$	This work
(141-216)		KG 163, ilv^+, $DnaA^-$	
KG 99	F^-	Auxotroph	B/r/1 from D.J. Clark
KG 110	F^-	$thyA^-$, arg^-, ura^-	15 TAU^- from P. Hanawalt
KG 142	F^-	Prototroph. T_3^r, $^-$, str^s, T_6^s, P1kc indicator	X289 from R. Curtiss III
KG 146	F^-	thi^-, arg^-, ilv_{300}^-, $metB^-$	JC-533 from John Clark
KG 162	F^-	trp^-, lac_2^-, tna^-, str^r	#BE235 from N. Otsuji
KG 163	F^-	trp^-, lac_2^-, tna^-, ilv^-, str^r	#BE269 from N. Otsuji
KG 173		arg^-	*E. coli* C-122 from L. Katz

For the transduction experiments, the media used were as specified by Caro and Berg (1971).

B. Chemicals.

Thymidine 2-^{14}C, Schwarz Bioresearch Inc., Orangeburg, New York; thymidine methyl-^{3}H, ^{14}C-5-bromo-2-deoxyuridine, Amersham/Searle Co., Des Plaines, Illinois; $^{32}P_i$ was obtained from Tracer Laboratories.

Chloramphenicol, Sigma Chemical Corporation, St. Louis, Missouri; Nalidixic acid, Sterling Winthrop Research Foundation, Rensselaer, New York; Daunomycin-HCl, Calbiochem, Los Anageles, California; Rifampin, Calbiochem, Los Angeles, California.

III. Culture Methods

A. Growth conditions for liquid cultures.

Stock cultures of the strains used were maintained on agar slants. Starter cultures were prepared by inoculation into A and E medium to a density of 10^8 cells per ml. These cultures were kept at 4 C until needed.

Batch cultures were incubated by shaking at the desired temperature.

B. Plating methods.

Viability assays as well as transduction assays were

carried out on 1.5% agar plates which were incubated at 30 C to dry the plates and check for contamination. The plates were stored at 4 C until needed.

C. Temperature shift conditions.

All temperature shifts were accomplished by transferring cultures into flasks prewarmed at the new temperature. If the prewarmed flask had a volume of ten times that of the volume of medium being transferred, temperature equilibration was reached in less than two minutes.

IV. Measurement of macromolecular synthesis and cell growth.

A. Measurement of cell numbers.

The cell numbers were determined by a Coulter Counter (Nuclear Data, Palatine, Illinois) and their size distribution was shown by an attached Nuclear Data 512 channel pulse height analyser (model 2200). Cells passed through a 30 μ orifice for a time interval of ten seconds. This system allows monitoring of the quality of the growth by measuring the cell numbers and the distribution of the cell volumes within the population of cells (Clark and Maaløe, 1967; Painter and Marr, 1968).

B. Measurement of cell mass.

Spectrophotometric measurement of growth was performed using either a Klett-Summerson photoelectric colorimeter with a No. 65 filter, or a Perkin-Hittachi spectrophotometer at 500 nm using a cuvette with a 1 cm light path.

C. Measurement of tryptophanase activity.

Tryptophanase assay was performed for testing transductants with the unselected tna marker. ilv^+ transductants were purified on a selective medium and then inoculated into 3 mls of liquid tryptone broth. Tryptophanase activity was assayed by formation of indole in an overnight culture of a strain grown in tryptone broth, by adding 2 ml of Ehrlich solution and overlaying the mixture with 1 ml of xylol (Difco Manual, 9th ed., p. 53).

D. Measurement of DNA synthesis.

1. Total DNA synthesis.

DNA synthesis was measured by the incorporation of radioactive thymidine into the TCA insoluble fraction of the cells. A typical uptake experiment involved growing the cells in radioactively labelled medium containing the label at the desired specific activity with unlabelled carrier at a final concentration of 20 µg per ml. Deoxyadenosine was present in all cases at a concentration of 50 µg per ml. At regular intervals, one ml samples were removed and

added to 3 ml of 7.5% TCA with 200 µg per ml unlabelled thymidine. Each sample was filtered through a 0.45 µ Millipore filter and washed four times with 5% TCA containing 200 µg per ml thymidine. The filters were washed two times with hot water, following the TCA washes (Lark, Repko, and Hoffman, 1963). This method gave lower backgrounds than the use of TCA alone. After heat drying, the filters were placed into polyethylene spectravials (Amersham/Searle Corp.) containing 10 ml of liquid scintillation counting solution (Liquifluor:toluene; 40 ml:litre, Amersham/Searle Corp.). Samples were counted for ten minutes in a Unilux Scintillation Counter (Nuclear Chicago Corp.) and counts were corrected for background.

In some experiments, for the total incorporation of the radiolabel chemicals, the samples were processed by the batch method described by Byfield and Scherbaum (1966).

2. Measurement of the rate of DNA synthesis.

The rate of synthesis of DNA was estimated by pulse-labelling with ^{14}C- or ^{3}H-thymidine, again in the presence of 50 µg per ml deoxyadenosine. A typical uptake experiment involved transferring one ml samples of cells to 13 x 100 mm tubes containing 100 µl of the labelled thymidine. After three minutes the incorporation was stopped with three ml of 7.5% TCA containing unlabelled thymidine. Each sample was washed and processed as above.

E. Measurement of acid soluble nucleoside triphosphate pools.

The chromatographic analysis of the triphosphates was based on the chromatographic separation of the nucleoside triphosphates by Randerath and Randerath (1967) as modified by L. Mychajlowska (1970 M.Sc. thesis, UBC).

1. Preparation of the samples.

Cells were grown in low phosphate buffer (10^{-6} M phosphate) and were allowed to equilibrate for the pools at least one generation (40 minutes at 30 C) after addition of the $^{32}P_i$. To measure the nucleoside triphosphate pools, 250 µl cell samples were mixed with 100 µl of 2N formic acid. The contents were then mixed and allowed to stand in an ice-bath for ten to fifteen minutes for the lysis and release of the pool material. These samples were centrifuged in a Beckman Microfuge and the supernatants were saved for spotting on thin layer chromatographic plates.

2. Chromatography.

Polyethyleneimine-impregnated cellulose plates were made according to Randerath and Randerath (1967) by Miss Mychajlowska. These plates were used within a week after preparation. The sample, supernatant of the acid hydrolysis, was added in 5 x 20 µl quantities to the plates in a slow manner to avoid flooding the surface

of the plates. After the spotting was completed, the plates were dried off and then washed with methanol (anhydrous) for 20 to 30 minutes to remove unnecessary salts from the medium and contaminating label.

Two dimensional chromatography was performed according to the method of Irr and Gallant (1969). The first dimension was 1N Acetate:1M LiCl (1:1, v/v), until about one and one-half inches from the top. A second run was performed immediately after this in a 1N Acetate: 1.5M LiCl (1:1, v/v) solvent and allowed to run in the same direction for five and one-half hours. To allow for a better separation of the deoxyribonucleoside triphosphates from the ribonucleoside triphosphates, wicks were made out of Whatman No. 3 filter paper which allowed the solvent to "run over" the plate. At the end of the first run, the plates were taken out and dried and washed with methanol to remove the LiCl of the first run. The second solvent system was made of 3M NH_4Acetate and 4.3% Borate, pH 7.0, and the plates were allowed to run for four and one-half hours in this system. At the end of this run, the plates were dried and prepared for autoradiography.

3. Autoradiography.

The dried plates containing the $^{32}P_i$-labelled triphosphates were placed over Kodak Royal Blue medical X-Ray films, placed in a light-proof box, and allowed to process for three days.

These films were developed for 3.5 minutes in Kodak X-Ray developer, rinsed in water, and fixed ten minutes in Kodak X-Ray fixer. The films were then rinsed again with water and dried.

The areas which showed radioactivity were clearly seen as dark (exposed) spots on the film. These films were then placed over their corresponding plates and with a pin the exposed spots were punched out on the plate. The radioactive area was then scraped off the plate, with the aid of a razor blade, and the scraped anion-exchange resin with the absorbed nucleotides were then collected from the plates and put in scintillation vials. These vials were properly marked as to the nature of the spot (ATP, etc) and ten millilitres of scintillation fluid was added. The radioactivity was counted in a Unilux Scintillation Counter for ten minutes. Counts per minute were corrected for backgrounds.

V. Density Gradient Sedimentation Analysis.

A. Measurement of BrUra incorporation in the DNA.

Strains 15-TAU and KG-71-2 were grown in the presence of thymidine and deoxyadenosine until a density of 5×10^7 cells per ml was obtained. The cells were then filtered and washed free of thymine and resuspended in ^{14}C-BrUra (Schwartz-Bioresearch Co.) with a final specific activity of 0.033 µc per ml (6 µg per ml final concentration). These cells were incubated at 30 C and, at timed intervals, one ml samples were taken and processed as described in Section V.A. For the gradient analysis, 10 mls of cells were rprocessed

as described below in Section VI.D.

B. Density labelling.

Procedures for the labelling of the starts and ends of the chromosomes with radioactive thymidine and subsequent BU density pick-up of the desired section of the chromosome was done according to Lark, Repko and Hoffman (1963). Overnight grown cells of KG-71-2 were grown in the presence of ^{3}H-thymidine, specific activity of 20.6 Ci per mM, final concentration of 1 mCi per ml, at 30 C in a 50 ml flask. The cells were shifted to 42 C for an hour in the ^{3}H-thymidine medium. Then, these cells were filtered free of ^{3}H-thymidine, washed twice with prewarmed A and E medium, and resuspended in fresh medium with supplements and ^{14}C-thymidine, specific activity 52.8 mCi per mM, final concentration 0.5 µCi per ml (Schwartz Bioresearch Co.), in 20 ml medium for 25 minutes. The cells were filtered and allowed to randomize for five generations. After an hour at 42 C, the cells were filtered and put into 50 mls of medium containing 10 µg per ml BrUra and allowed to grow for fifty minutes. At the end of this time the cells were collected for lysis.

C. Preparation and analysis of DNA samples by density gradient centrifugation.

1. Extraction of DNA

Cells collected for the density gradient technique were

put directly onto frozen A and E medium and saved until needed. Frozen cells were thawed at room temperature and then spun down at 8000 rpm for five minutes. This pellet was resuspended in one ml of Tris-EDTA buffer (0.01 M each, at pH 8.2). Lysozyme (Calbiochem) was added at a final concentration of 200 µg per ml and the mixture was incubated at 37 C for 20 minutes. After 20 minutes, 1 ml of a 0.1% solution of sodium lauryl sulfate was added to the cell suspension. Immediately, the lysates became viscious. The lysates were brought to a total of 3.5 ml with distilled water and were treated with 50 µl of pronase (Worthington), at final concentration of 200 µg per ml, to digest the bacterial proteins.

2. Centrifugation and Fractionation.

To 3.26 ml of the lysate, 4.36 gm of CsCl (Harshaw, optical grade) was added. The mixture was incubated at 37 C to dissolve the CsCl. This combination gave a final density of 1.76 gm per cm^3 (ρ = 1.405). The mixture was poured into cellulose nitrate centrifuge tubes and overlaid with mineral oil. Centrifugation was performed in a Beckman L2-65B ultracentrifuge at 37,000 rpm for 40 hours at 20 C.

Following centrifugation, the base of the cellulose nitrate tube was punctured in a Beckman fraction collector. Ten drop samples were collected per fraction into 13 x 100 mm tubes. From every fifth fraction a drop was removed for densitometry analysis

by an Abbe refractometer at 23 to 25 C (room temperature).

3. Measurement of radioactivity.

Samples were diluted with 5% TCA, ice cold, and filtered through 0.45 μ HA Millipore or Reeve Angel Fiber Glass filters. They were dried and counted as described previously. Samples were corrected for background and adjusted for the double label counts.

VI. Genetic analysis of the mutants.

A. Isolation of the temperature resistant revertants.

The standard technique for isolating the temperature resistant revertants consisted of inoculating 0.1 ml of an overnight culture diluted 1:100 to contain 1×10^7 cells per ml, directly into A and E broth or onto supplemented agar plates prewarmed to 42 C. After incubation overnight at 42 C, colonies were picked up and examined for their genetic background and for the DnaA mutation phenotype. Growth in the tubes was checked microscopically for snake formation.

B. Transduction experiments.

1. Bacteriophage donor lysate preparation.

Desired bacterial donor strains were grown from an overnight culture in 9 mls of Luria broth with supplements and 2.5×10^{-3}M CaCl with aeration, and at 30 and 37 C, depending on the strain.

Two to four hours incubation was necessary to give a density of about 2 x 10^8 cells per ml. Phage P1Kc was diluted to about 6 - 8 x 10^7 pfu (plaque-forming units) per ml, and 0.1 ml of this was added to 1.9 ml of bacteria in a sterile Wasserman tube at 37 C. After twenty minutes of preadsorption, 0.2 ml of this adsorption mixture was added to 2.5 ml of Luria soft agar and poured onto Luria agar plates. After five minutes required for solidification of the overlay, the plates were incubated at 37 C for six hours. To harvest the donor phage, 5 mls of Luria broth (without CaCl) was added per plate and the plates were allowed to sit overnight in the refrigerator. Next morning, the soft agar layer was broken up with a glass spreader and was scraped into a centrifuge tube.

About 0.1 ml of chloroform was added for every 5 mls of lysate and the mixture was vortexed for 1 minute to burst any unlysed cells. This mixture was centrifuged at 5 - 6000 x g for 15 minutes to pellet the cell debris and the supernatant was saved for phage titration.

2. Transduction experiments.

Desired recipients were grown as described for the indicator strains above. Donor P1kc was diluted to give an MOI (multiplicity of infection) of 1 - 3 phages per bacterium. After 20 minutes of preadsorption, the contents of the tube were spun down and the cells were resuspended in 10 mls of phage 0.1 or 0.05 mls of

the suspension were plated on selective plates. The supernatant was assayed for unadsorbed phage. The phage suspension was always checked for contaminating bacteria, and the recipient strains were checked for reversion as controls.

RESULTS AND GENERAL DISCUSSION

I. Properties of CR34T83

A. Analysis of macromolecular synthesis and cell division.

1. Temperature shift conditions.

Whenever one is working with temperature sensitive mutants, the definition of the sensitive temperature, as well as the physiological temperature range at which the behaviour of the mutant could be examined, becomes one of utmost importance. The response of the T83 cells to a temperature transition from 30 C to a range of temperature from 27 to 45 C was tested in a temperature gradient block.

In a typical temperature shift experiment, T83 cells grown for several generations at 30 C in Klett tubes were transfered to the temperature block. At timed intervals, cell numbers were determined using the Coulter Counter. Figure 1 represents the results of such an experiment. The control cells grown at 30 C, in this system, go through two doublings during the 85 minute incubation period. At temperatures above 42 C, there was no cellular division and, hence no net increase in the cell numbers for shifted culture. At temperatures between 30 and 33.5 C, when compared to the cells growing at 30 C, a significant increase in the final cell numbers was observed. The latter changes were accounted for, by the fact that between the 30 and 33.5 C, a faster growth rate was achieved, without affecting the

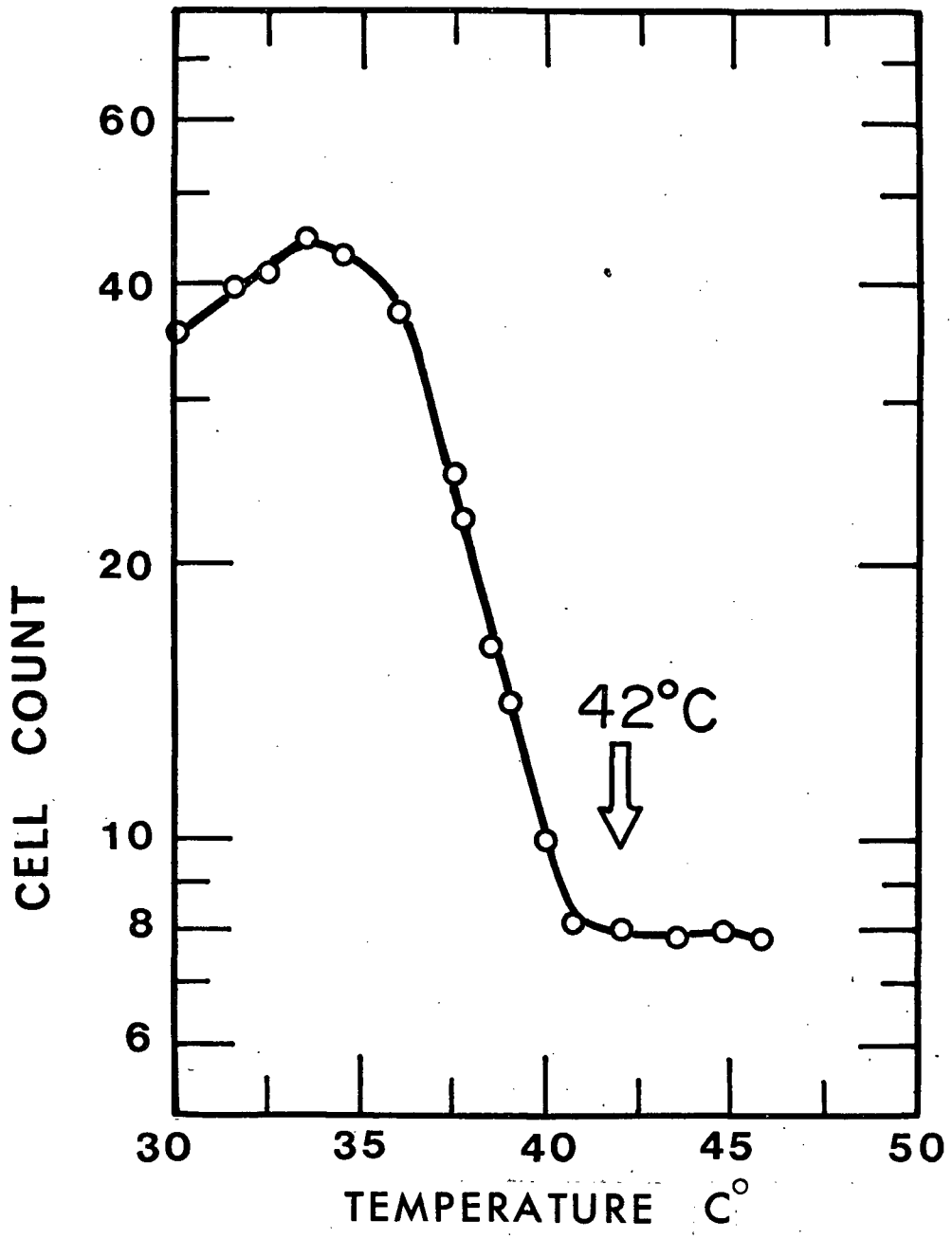


Figure 1. Response of T83 grown at 30 C to a change in temperature.

T83, grown for several generations at 30 C, was distributed into 16 tubes (18 x 150 mm) at a cell density of 5 x 10^7 cells per ml, and was placed in a temperature gradient block. Cell counts were monitored by a Coulter Counter for the next 85 minutes (two generations). Final cell numbers (relative cell counts) are plotted against the temperature of incubation.

temperature sensitive condition. However, at temperatures above 33.5 C, there was a general retardation of growth and a complete cessation of cell division between 41 and 45 C. Forty-two degrees was thus chosen as the ultimate non-permissive temperature.

When T83, at different cell densities between 1.5 and 10 x 10^7 cells per ml, were shifted from 30 to 42 C, the response of the cells was the same, that is, there was no significant residual division at 42 C. Thus, regardless of the cell density, the response was the same.

2. Cell division and DNA replication in a shift to non-permissive temperature.

a. Cell division. The possibility of measuring bacterial growth electronically with a Coulter counter and the accuracy of this method for monitoring the quality of the bacterial growth has been demonstrated by several laboratories over the past decade (Kubitschek, 1969a).

In principle, the cell volume distributions for cultures of E. coli, and bacteria in general, growing under steady state conditions permit the determination of the cell volume change during the cell cycle, (Harvey and Marr, 1966). Harvey et al. (1967), and Kubitschek (1968a), gave a more rigorous derivation of the cell volume to cell growth relationship by the electronic measurement of average cell growth ratios. The instrumental resolution of the Coulter counter is such that one could detect the smallest changes in the quality and quantity of bacterial growth possible.

In steady state, exponential growth conditions, in A and E medium at 30 C, CR34T83 divides with a generation time of 42 minutes which corresponds to a specific growth rate of 1.4 $Hour^{-1}$. Under such conditions, when cell numbers increased in an exponential manner, the cell size, as indicated by the peak channel position, for 30 C grown cells, remain constant at channel 75 (Figure 2b). Upon a shift to 42 C, there is a cessation in cell division (Figure 2a).

Results obtained by Clark (1968a) and Helmstetter et al. (1968) indicated that cell division depends upon the completion of a round of DNA replication. In an exponential population, broth grown cultures of E. coli, about 28% of the cells which have completed their DNA replication cycle but have not separated yet (Kubitschek, 1969b), are in doublet forms. These cells have been shown to undergo division when their DNA replication has been blocked (Clark, 1968b; Helmstetter, 1968). It is clear that this event does not take place in CR34T83.

Coincident with the cessation of the cell division there is an increase in the cell mass which corresponds to mass increase expected of an exponential culture at 42 C (Figure 2b). The cells increase in size, as viewed microscopically, and filamentous forms result.

Viability counts for T83 cells at 42 C indicate no loss in viability for the first fifty minutes. Upon longer incubation, however, there is a gradual decline in the percent of survivors to fifty percent by 90 minutes, and to 100 percent by 180 minutes of incubation at 42 C.

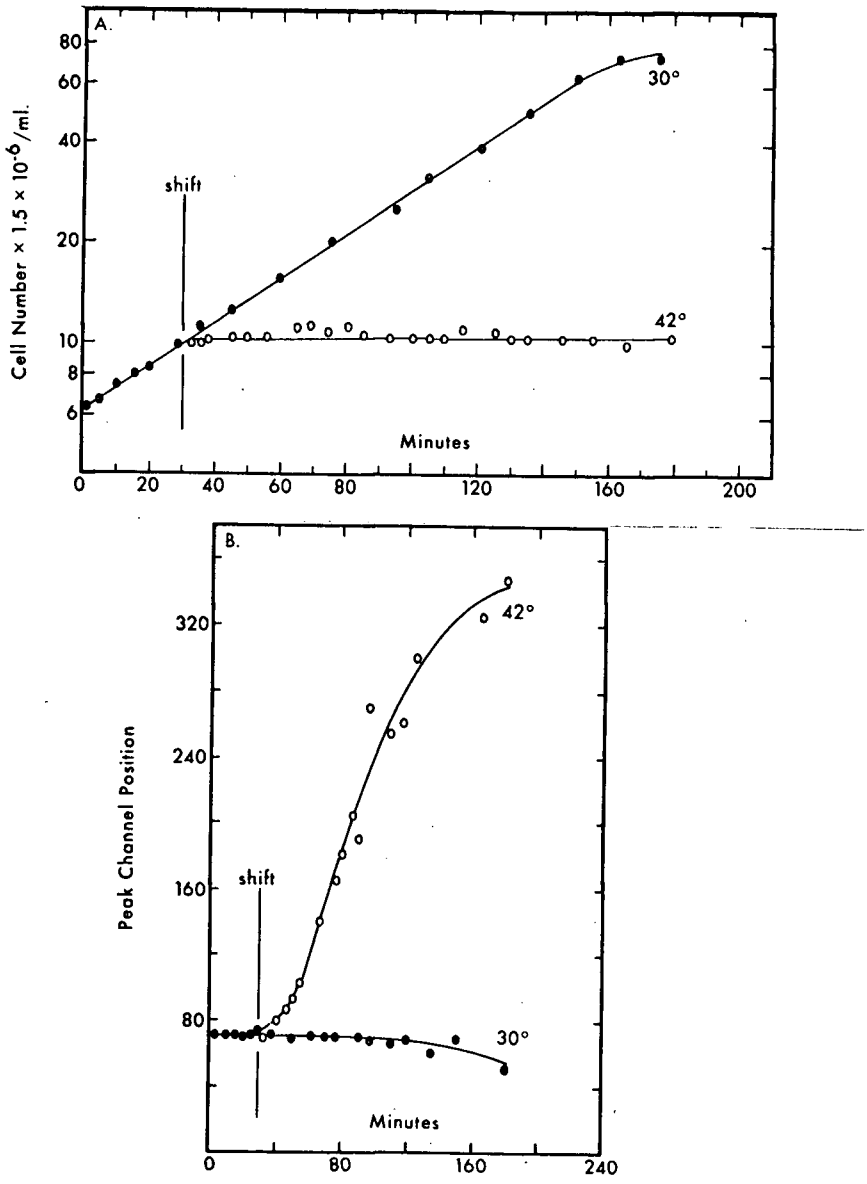


Figure 2. Growth of T83 under permissive and non-permissive conditions.

T83 grown in A and E medium at 30 C (●) was shifted to 42 C (0) as indicated by the vertical arrow. Cell numbers (panel A) and cell size (panel B) were monitored as a function of time during growth at the permissive (30 C) and non-permissive (42 C) temperatures.

b. Studies on DNA replication in CR34T83.

i. Total uptake of TdR.

Shifting a culture of CR34T83 from 30 to 42 C results in residual amounts of DNA being synthesized under non-permissive conditions (Figure 3). This residual DNA synthesis proceeds at a gradually decreasing rate for approximately forty to sixty minutes. No further detectable synthesis occurs upon longer incubation at 42 C. The percent increase in the amount of residual DNA synthesized at 42 C in rich medium is 40 to 60%. In minimal medium, under identical conditions, the residual synthesis is 20 to 30%. The same observations have been made for the other initiator mutant CR34T46 (Hirota et al. 1970). These results fit nicely with the Helmstetter-Cooper model of DNA replication under different growth conditions (Helmstetter and Cooper, 1968). The model indicates that cells grown in rich medium contain multiple forks whereas, in minimal medium, cells have single forks. Accordingly, CR34T83 will show a higher percentage of residual DNA synthesis when grown in broth as compared to the minimal conditions.

ii. Rate of DNA replication.

At a given instant of time, the rate of incorporation of ^{14}C-thymidine into DNA by a growing culture is a function of the number of replicating forks in the cells and the replication velocity. Under steady state conditions in an exponential population, the ratio of rate of DNA replication/cell numbers should remain constant (Maaløe and Kjedgard, 1966). If any disturbance of anomaly from this rule was

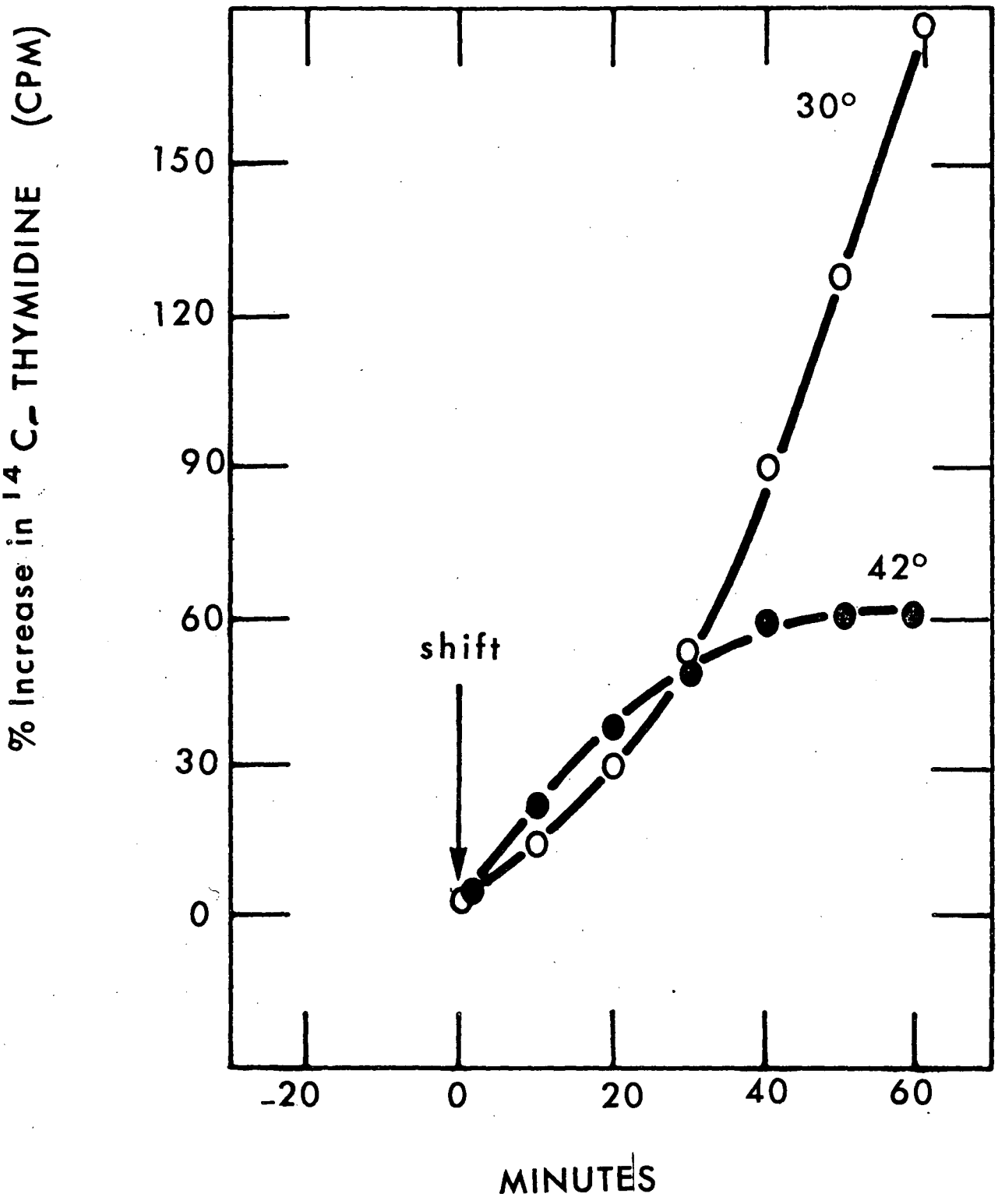


Figure 3. Uptake of C-14 thymidine into CRT-83 growing at 30 C and 42 C.

T83 growing in the presence of C-14 thymidine at a final concentration of 20 µg per ml and a specific activity of .3 µc/µM was shifted from 30 C to 42 C. The cell density at the time of shift was 1.5×10^8/ml. The incorporation of C-14 thymidine into the cold TCA insoluble fraction of the 30 C culture (O) and 42 C culture (●) is given as a function of time.

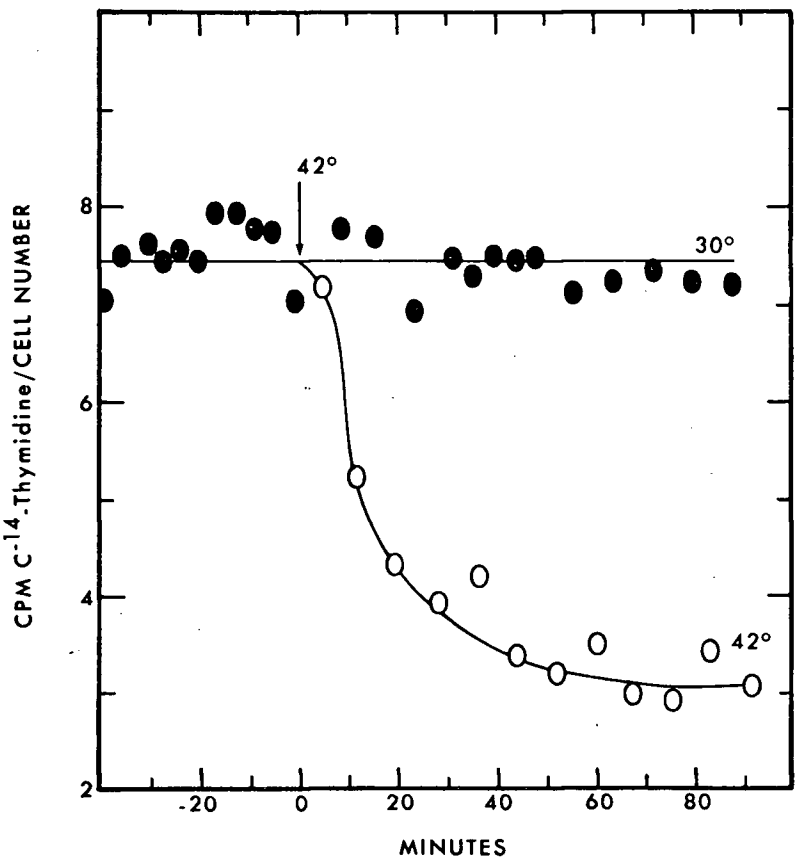


Figure 4. Rate of DNA synthesis in CR34T83 after a shift to 42 C.

T83, growing at 30 C at a cell density of 2.2×10^7 cells per ml, was shifted to 42 C at zero time. The rates of incorporation of ^{14}C-TdR into cold TCA insoluble fraction was determined in a series of 3 minute pulses, using 1 ml cell samples incubated with ^{14}C-TdR at a final concentration of 20 µg per ml, and with 50 µg per ml AdR. Counts per minute of ^{14}C-label per cell is plotted against time for the culture shifted to 42 C (O) and control at 30 C (●).

to take place, one should see for example, an increase in the DNA per cell ratio, that is, a multinucleate snake, or a decrease, as in a temperature sensitive DNA replication mutant.

At 30 C and at 42 C, the rate of DNA replication in CR34T83 was measured by the incorporation of ^{14}C-thymidine into cold TCA insoluble material of the cell. The results are shown in Figure 4.

The DNA per cell ratio calculated from the counts per minute of ^{14}C-TdR incorporated per ml over the cell number per ml. For 42 C grown cells, the ratio of rate per cell decreased until a plateau value was reached, due to a continual but limited DNA synthesis in the absence of cell division at 42 C. The 30 C cells maintain a constant DNA per cell ratio. The conclusion that DNA synthesis comes to a halt after sixty minutes at 42 C could be reached from the pulse experiments.

II. Recovery of CR34T83 at 30 C after growth at 42 C.

A. Single shift experiments.

The design of the experiments: Exponentially grown cells at 30 C were transferred to 42 C, as described in section III. C. A portion of the original culture (1/5 the starting volume) was maintained at 30 C throughout the rest of the experiment as the control reference culture. After appropriate incubation time at 42 C, about 3/5 of this was shifted down to 30 C and the remaining 1/5 was kept at 42 C throughout the rest of the experiment. The terminology used

in describing these results are as follows: shift up, a shift from 30 C to 42 C; shift down, the reverse case; pulse, incubation of a culture at 42 C for a short time as given by the length of the pulse; and recovery, the resumption of cell growth at 30 C after a pulse at 42 C.

During recovery from a pulse at 42 C, the cell division pattern shows three distinct phases. These are: (phase i), a lag in cell division; (ii) an accelerated division for a short time; and (iii) continued division, corresponding to that of the normal 30 C culture. In analysing such results, the lag refers to the length of time needed for the culture to start dividing, and the time needed for a 50% increase in the cell numbers from the base line, or the lag is referred to as the half-step time.

1. Cell division during recovery from a 42 C pulse.

Figure 5 describes the response of exponential cells of CR34T83 to varying pulses of growth at 42 C. In every case, the cells stopped dividing after a shift up, and recovery in the shift down contained (1) a lag (2) accelerated division, and (3) normal division phases. A comparison was made between the recovering cultures pulsed for different periods at 42 C and the control cultures. The time during recovery for 42 C grown cells, when the accelerated division curve changed its rate to normal rate, was used as the reference. At this time, the value for the number of cells for the control culture was assumed to be 100% division, and the residual division at the inflection point was calculated accordingly. As shown in Figure 5, for a short

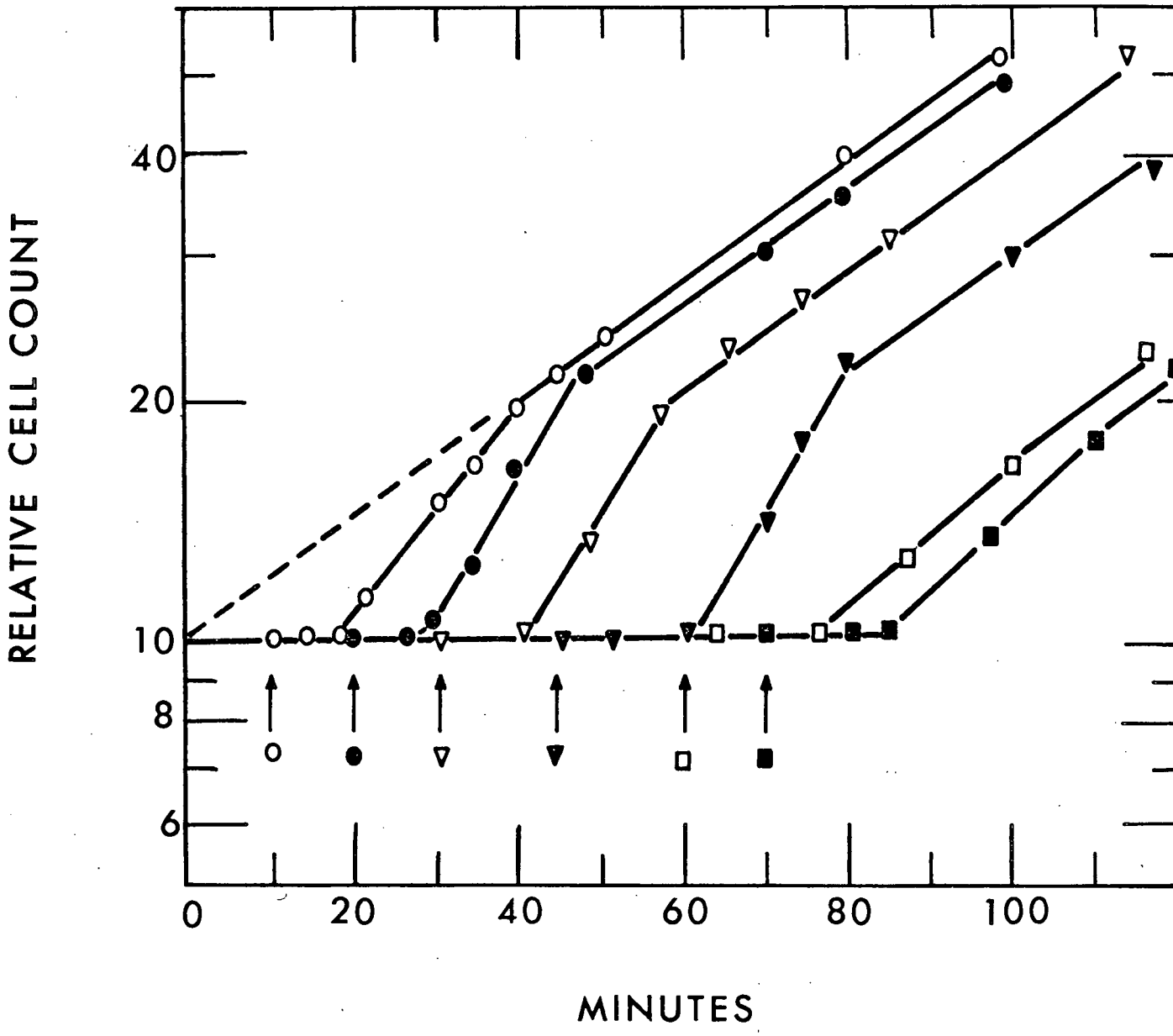


Figure 5. Recovery of CR34T83 at 30 C following growth at 42 C.

A culture of T83 growing exponentially at 30 C, was shifted to 42 C at a density of 1.5×10^7 cells per ml. At 10 (○), 20 (●), 30 (▽), 45 (▼), 60 (□), and 70 (■) minutes as indicated by vertical arrows, subcultures from 42 C were transfered to 30 C and allowed to recover. Cell counts are plotted as a function of time. The control culture, maintained at 30 C, is represented by the dashed line.

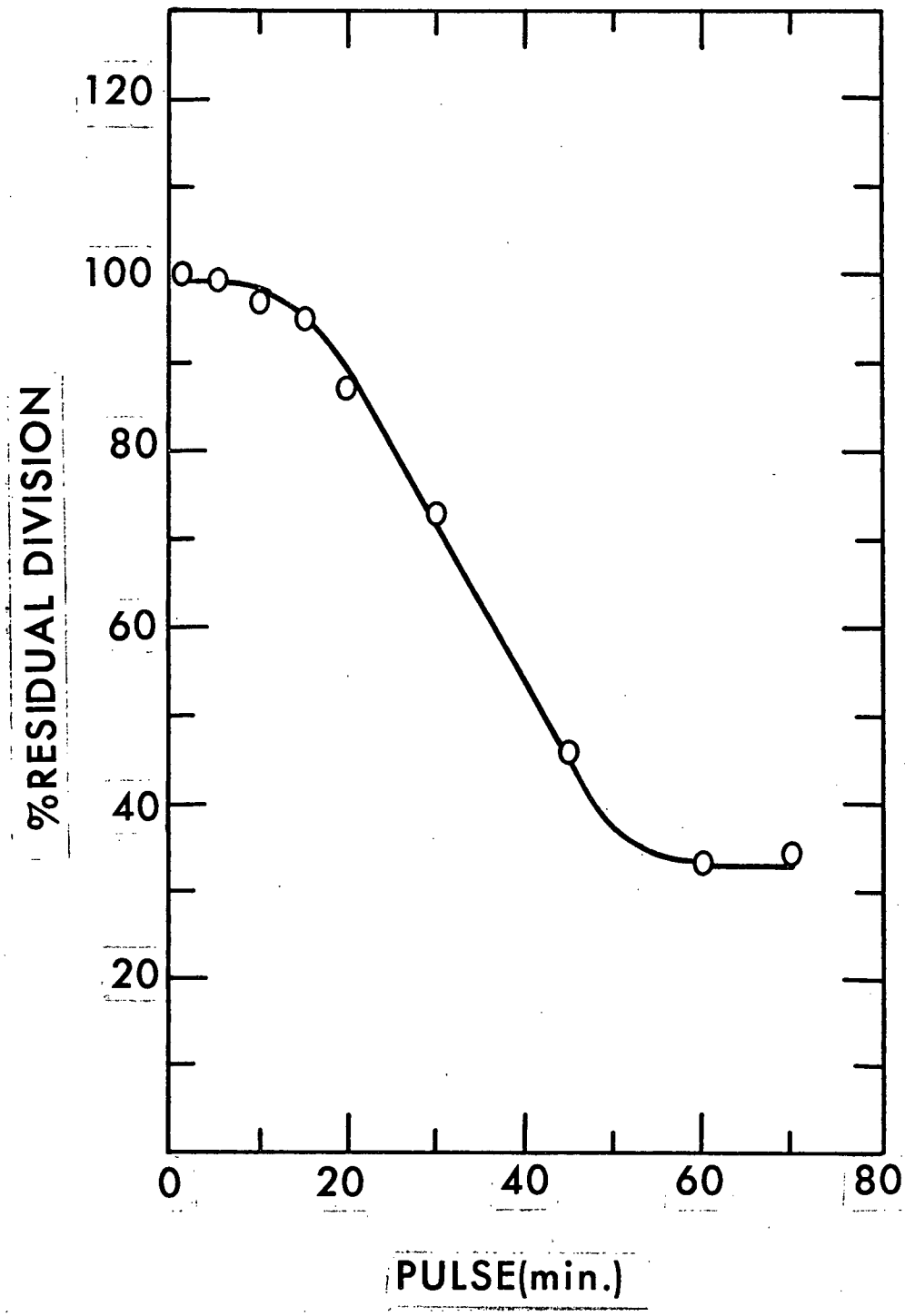


Figure 6. Relationship between cells recovering from 42 C to their control cultures kept at 30 C.

Data from Figure 5 and numerous other similar experiments were used to construct this graph. The ordinate represents the ratio of cell counts for cells recovering from a temperature block at 42 C, to the control cells left at 30 C, as expressed in percent values. The ratios used were taken at the time when the recovering cells entered their normal division period, that is, the inflection points of the recovery curves. The abscissa represents the duration of the temperature block at 42 C.

pulse (up to 10 minutes), the cells showed a 100% recovery in numbers to that of the control 30 C grown cells. However, this figure declines rapidly, for pulses up to 50 minutes, to the 35% level. For pulses longer than 50 minutes, the same 35% level was maintained.

The results from viability data clearly ruled out the possibility of a loss in viable cells during the first 50 minutes. Thus, the cells at 30 C, which had continued the regular initiation cycles for their DNA replication, gained a priority over those at 42 C. In other words, possible cells at 42 C, by staying at the non-permissive condition, lost the nuclear equivalents directly proportional to the length of the block, whereas the cells at 30 C initiated regularly and gained nuclear equivalents. In the final analysis, it appeared that there had been a loss in residual cell division, or, expressed in terms of DNA cycles, a loss in initiation. Furthermore, the DNA synthesis between 40 and 60 minutes maintained the number of divisions obtained during the recovery to a constant, that is, cells which had a complete nuclear equivalence.

Analysis of the relationship between the pulse in 42 C and the length of the lag and time for 50% increase in the cell numbers after recovery are given in Figure 7. The distribution of lag for pulse length between 5 and 30 minutes was maintained at a relatively constant length, averaging 14 ± 3 minutes. The corresponding half-step was 19 ± 4 minutes. With longer than 30 minute pulses, this relationship became protracted to larger values. It appeared, thus, that the length

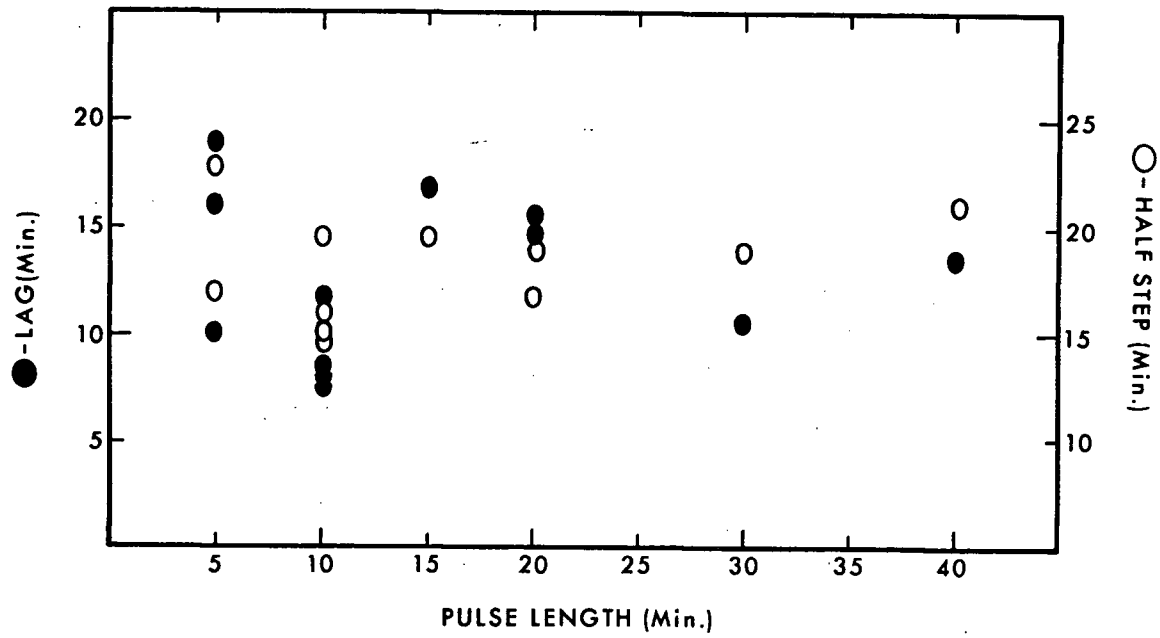


Figure 7. The relationship between the lag in recovery to the period of growth at 42 C.

Results from Figure 5 and similar experiments were used to construct this graph. The lag (●), and the half step (○), that is the time required for a 50 percent increase in cell numbers after the lag, are plotted as a function of the pulse of non-permissive growth.

of shorter temperature pulse was unimportant in deciding the behaviour of the cells in recovery.

2. Cell volume distributions for T83 at recovery.

The change in size distribution of T83 during a shift up and recovery is illustrated in Figure 8. Under balanced growth conditions, at 30 C T83 has a size distribution typical to exponential cultures of E. coli (Painter and Marr, 1966). This distribution moved toward higher channel numbers or larger cell volume upon shift to 42 C. The general shape of the size distribution curve changed to a flatter curve and there was a considerable shift in the mean cell volume.

The filaments resulted in phase I which, upon a shift back to the permissive condition, fragmented to a population of heterogeneous sizes during phase II, the accelerated division to give three peaks at 105 minutes. Eventually, the smaller normal size gained dominance as the cells were in their normal growth phase III, from 145 to 160 minutes.

3. Rate of DNA synthesis during recovery from a 42 C pulse.

The rate of thymidine incorporation into cells of T83 recovering from pulses of different length at 42 C was determined by pulse labelling the cells with ^{14}C-thymidine and counting the radioactivity incorporated into the cold TCA insoluble material. Figure 9 shows the result of such experiments. Upon shifting T83 from 30 to 42 C, the rate of thymidine incorporation into DNA decreased gradually and stopped. During the course of this drop, the cells went through their

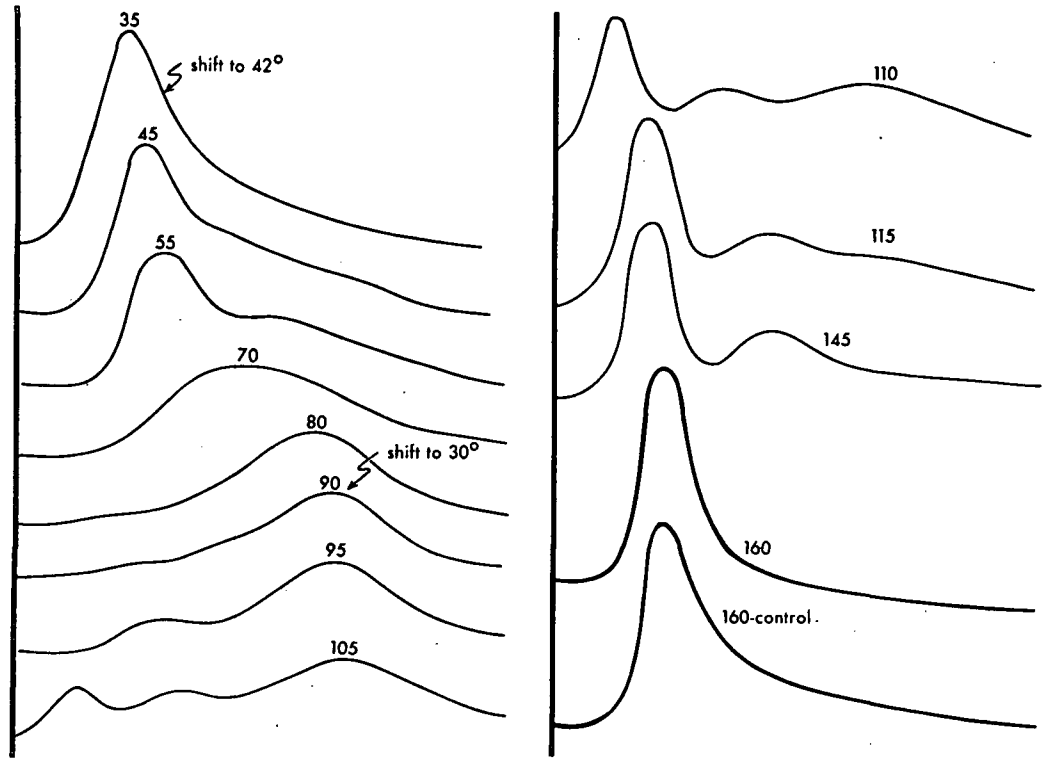


Figure 8. Analysis of the cell size during recovery from growth at 42 C.

T83, growing at 30 C was shifted to 42 C at 35 minutes and returned to 30 C at 90 minutes. Cell volume distributions were obtained from plots of the pulse height analysis of the cells. The ordinate represents the cell number, and the abscissa, the pulse height analyser channel number (0-511). Each curve is numbered as to the sampling time.

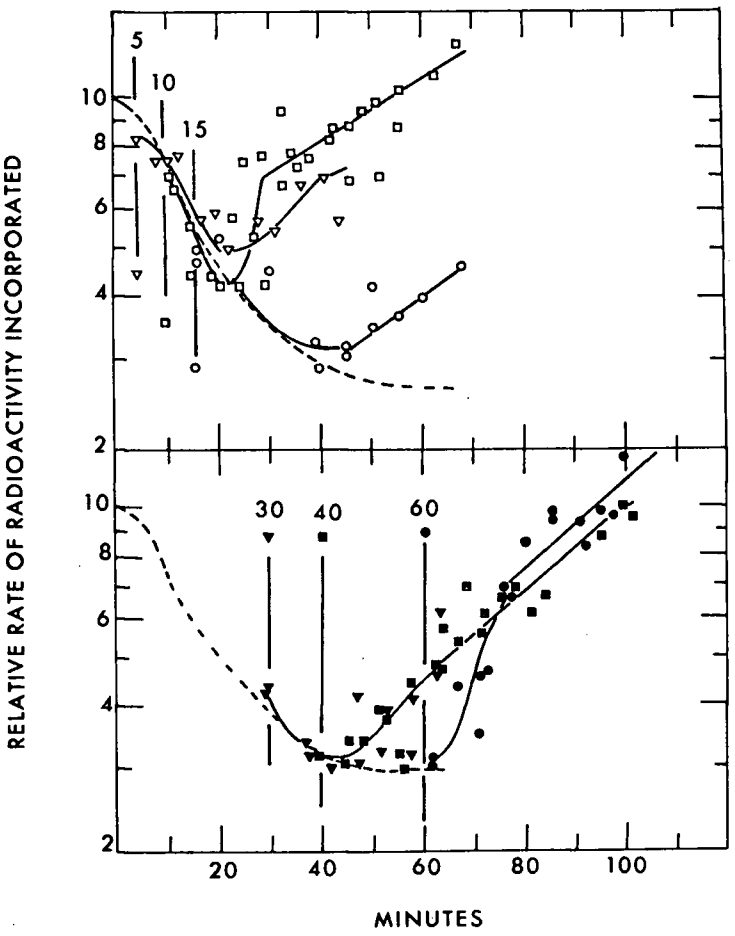


Figure 9. Rate of labelled thymidine incorporation into T83 during recovery.

Cells grown at 30 C in A and E medium at a density of 5 - 8 x 10^7 cells per ml (zero time). After 5 (▽), 10 (□) and 15 (○) minutes (upper panel) or 30 (▼), 40 (■), and 60 (●) minutes (lower panel), cells were shifted back to 30 C as indicated by vertical lines. Cells were pulsed with 0.025 µc per ml ^{14}C-TdR or 2.5 µc per ml ^{3}H-TdR, at a final concentration of 20 µg per ml TdR, and with 50 µg per ml AdR, for 3 minutes. Relative rate of radioactive thymidine incorporated (CPM) is plotted against time. The curves represent the best smooth lines fitted through the experimental values of two separate experiments. The broken line represents the uptake for cells grown and maintained at 42 C since zero time.

residual DNA replication between 45 and 60 minutes. When the cells were shifted to 30 C, the permissive temperature, after 60 minutes of incubation at 42 C, the rate of incorporation of labelled thymidine into DNA rose rapidly for 20 minutes and then continued at a rate compatible with that of the 30 C control culture. The cells returning from a 40 minute nncubation at 42 C, on the other hand, displayed a lag of approximately seven minutes before resuming the uptake at an increasing rate. The 15 and 30 minute pulsed cells, when returned to 30 C, went through a drop in the rate of incorporation of labelled thymidine into DNA until 40 to 45 minutes. After this point, the rate of uptake increased. For cells incubated for five and ten minutes, similar continued drops in the rate were observed which, after a short while, picked up to the normal rate of the 30 C cells.

Several points were noteworthy. Firstly, for short temperature pulses of five and ten minutes, the rate of incorporation at 30 C following a shift to the 42 C temperature, did not get to normal for approximately seven to twelve minutes after the shift down, and in fact, kept dropping like that of the 42 C controls. Similarly, following 15, 30, and 40 minute incubations at 42 C, the rate of uptake dropped despite the cells being incubated at 30 C. These cells started a new rate of uptake at respectively, 30, 15 and 5 minutes after shift down. Interestingly enough, once shifted to permissive temperature, the cells had two places for start of replication from the zero time origin at 42 C. The first one occurred

at 22.5 minutes and the second at 45 minutes. The relationship of pulse and the start point seemed to be 22.5 minutes start point for pulses shorter than ten minutes, and 45 minutes for pulses 15 to 40 minutes. The cells with 60 minutes of incubation at 42 C, when returned to 30 C, however, initiated their replication within 3 minutes.

4. Measurement of ^{32}-P-labelled nucleoside triphosphates in CR34T83.

In a mutant, where there is a limited ability for synthesis of DNA, it is possible for the limitation to reside in the availability or conversion of the ribonucleotides to the deoxyribonucleotide triphosphates.

The DNA-RNA precursor pools of T83 were measured according to the presence of ^{32}P-labelled P_i in the ribo- and deoxyribonucleoside triphosphates. Figure 10 summarizes the results of distribution of four ^{32}P-labelled ribo- and deoxyribonucleoside triphosphates under permissive (30 C) conditions, at a shift up (42 C), and during the recovery stage.

Analysis of the deoxyribonucleoside triphosphate pools in T83, under the permissive growth conditions, indicated in decreasing levels, the following order: dATP; dCTP; dGTP; and dTTP. There was no detectable increase in the dTTP levels. Similar order is established for exponential cultures of E. coli B/r/1 when grown in glucose minimal medium at 37 C, that is in decreasing levels of dATP, dCTP, dGTP and dTTP.

During the 60 minutes of incubation under a shift up (42 C)

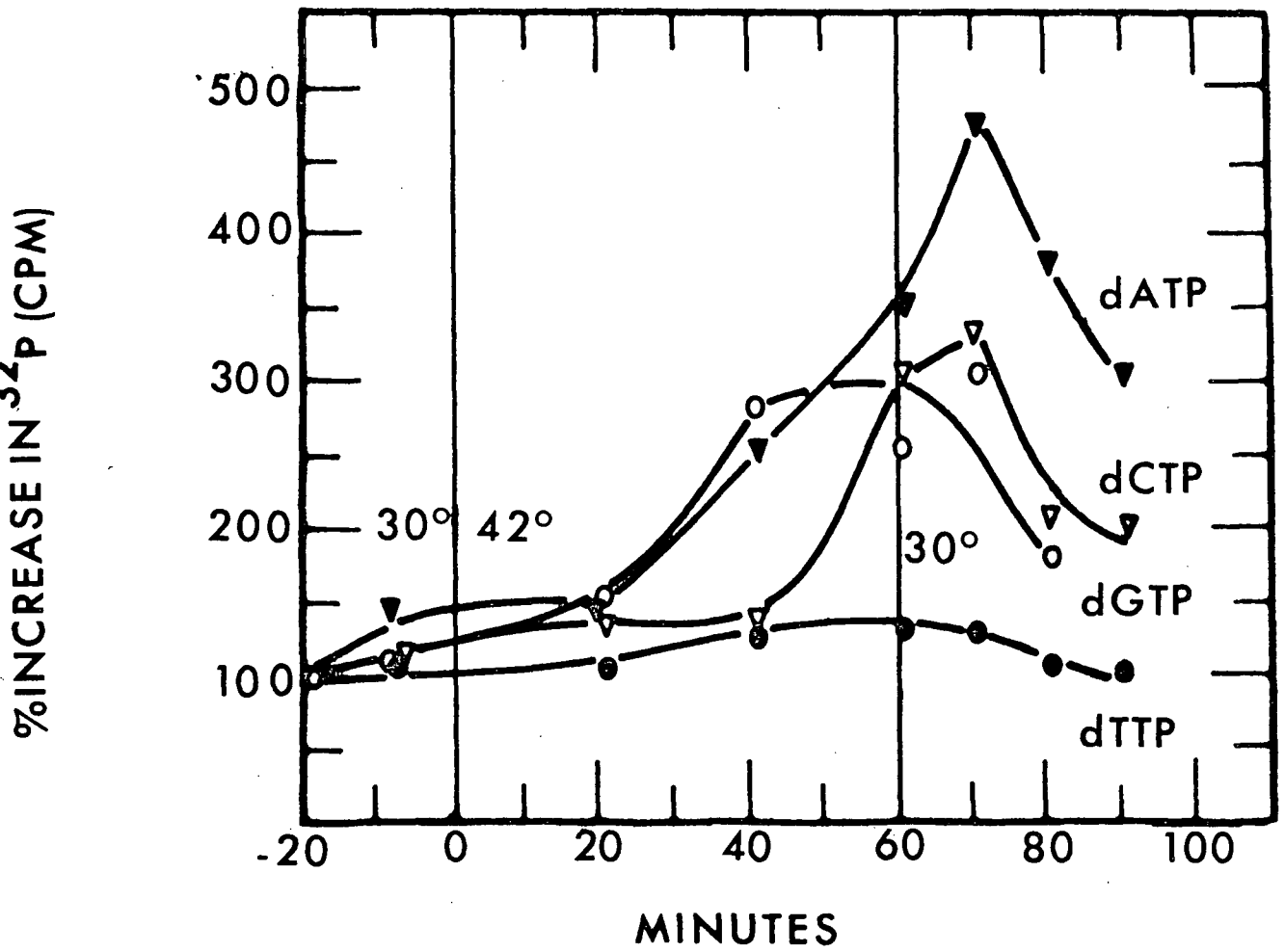


Figure 10. Distribution of ^{32}P-labelled deoxyribonucleoside triphosphates in CR34T83.

T83 was grown in A and E medium with a final phosphate concentration of 1.75×10^{-6} M. $^{32}P_i$ was added at 100 µc per µmole of PO_4 and 60 minutes were allowed for complete equilibration of the label into the pools prior to sampling. Distribution of ^{32}P-labelled deoxynucleoside triphosphates during recovery from growth at 42 C was measured as described in Materials and Methods. The ordinate represents the percent increase in the CPM over the initial CPM observed, and the abscissa represents time.

condition in T83, the pool levels showed a gradual increase. The order of the pools matched with that of B/r/1 grown in the presence of 150 µg per ml chloramphenicol (Mychajlowska, 1970). The distribution of ^{32}P-labelled ribonucleoside triphosphates in T83 is shown in Figure 11.

B. Multiple shift experiments.

The observation that T83 stopped dividing when shifted from 30 to 42 C indicated that a division substance was temperature sensitive and was required for the expression of cell division until the moment before cell separation. Since this effect was an immediate one, one might expect that cell division could be blocked immediately during the recovery period (at 30 C) when such recovering cells were returned to 42 C. However, it could be equally possible for recovering cells to be altered so that they may be irreversibly committed to cell division, that is, during the rapid division period, in which case they would be immune to the non-permissive temperature effect. Experiments were designed to test these possibilities. Results of such an experiment are shown in Figure 12. When recovering cells were returned to non-permissive conditions at 7.5 minutes after the shift down, there was approximately 5% residual division. For cells returned to 42 C, 15 and 22.5 minutes after shift down, that is, cells at their rapid division phase, 22 and 35 percent residual division was observed respectively. Finally, upon shifting the recovering cells just prior to their return to normal division phase to 42 C, approximately 18 percent residual division was observed.

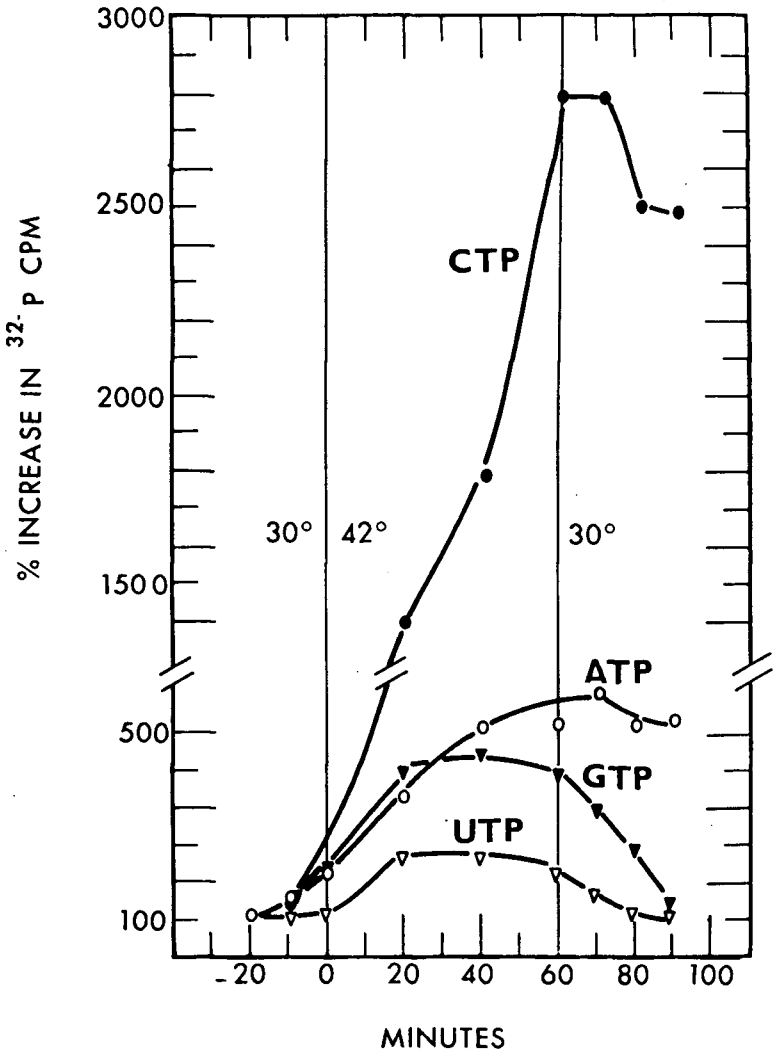


Figure 11. Distribution of ^{32}P-labelled ribonucleoside triphosphates in CR34T83.

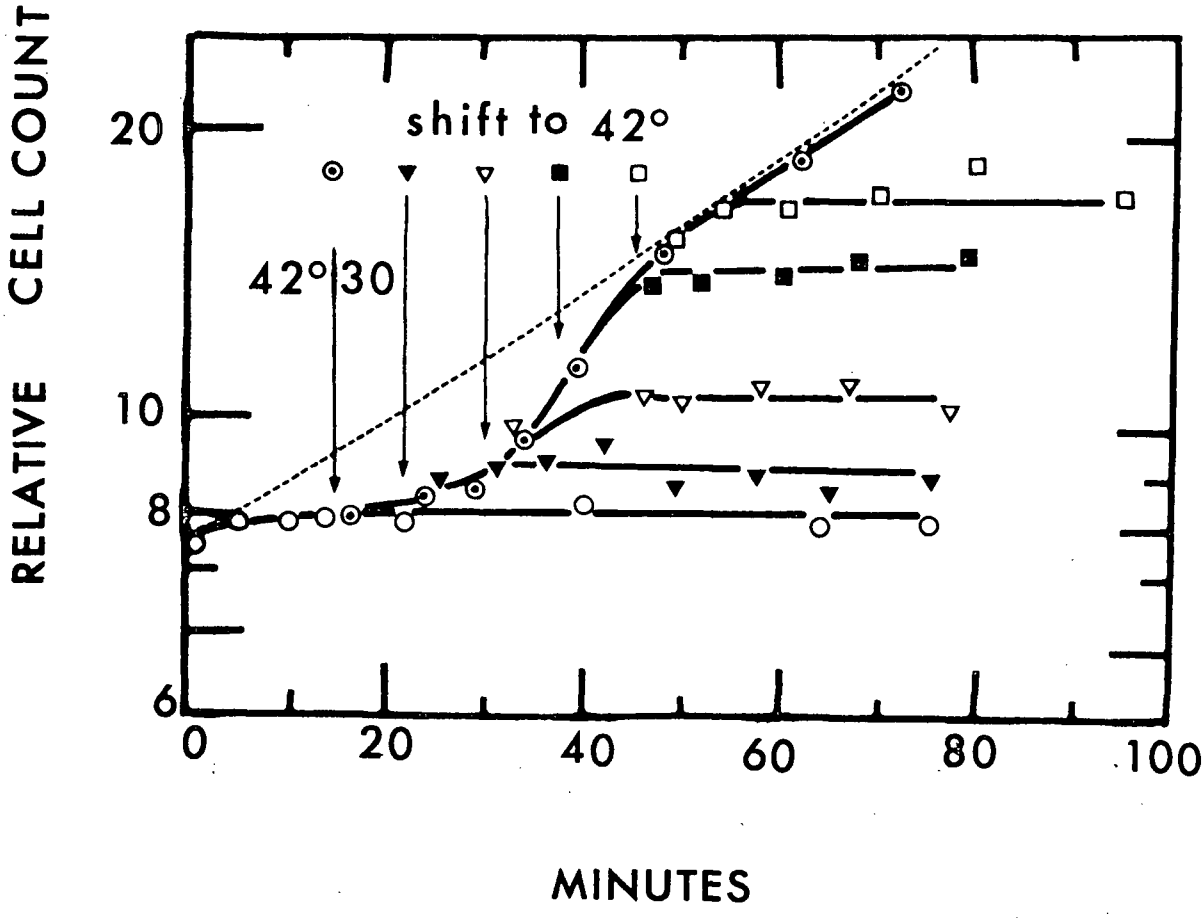


Figure 12. Inhibition of cell division during the recovery period by shifting to the non-permissive temperature.

A culture of T83 growing exponentially at 30 C was shifted to 42 C at zero time. After 15 minutes, the culture was returned to 30 C (⊙). At 7.5 (▼), 15 (▽), 22.5 (■), and 30 (□) minutes, as shown by vertical arrows, samples were removed from the 30 C flask (--), and shifted to 42 C. Cell counts were followed by Coulter Counter (ordinate) for the next 100 minutes. Controls are 30 C grown cells (○), and 42 C grown cells (○). Initial cell density was approximately 2×10^7 cells per ml.

It was concluded that during the recovery period at 30 C the cells were most labile to the shift up when at their lag phase. On the contrary, cells at their accelerated division phase were more immune to the non-permissive temperature effect and showed a greater escape than when they resumed the normal division phase. Subsequent to the expression of the slight escape from the temperature block at 42 C, all classes of cells clearly stopped further division. Apparently, the minimum lag period at 30 C was essential for the escape from the temperature block and the expression of division at 42 C. A rigorous prediction that all systems were as expected led to an experiment in which the response of T83 to multiple temperature escillation was examined (Figure 13). The results indicated that cell division was inhibited when the cells were passed through oscillations between 30 and 42 C. As long as the shift to the non-permissive temperature occurred earlier than the lag needed for the expression of division (12 minutes) during recovery, the cell division was inhibited. There was a slight amount of increase in cell numbers during the course of these osciallations. The cells, at the last shift down, however, showed a longer than normal lag and had lost the rapid division phase of their recovery. Instead, they resumed division at the rate compatible to that of the 30 C grown cells.

C. Physiological requirements of the recovery from a single shift.

1. Role of DNA synthesis in recovery..

The coordinated relationship between DNA replication and cell division has been well established (Lark, 1969b; Helmstetter, 1969b).

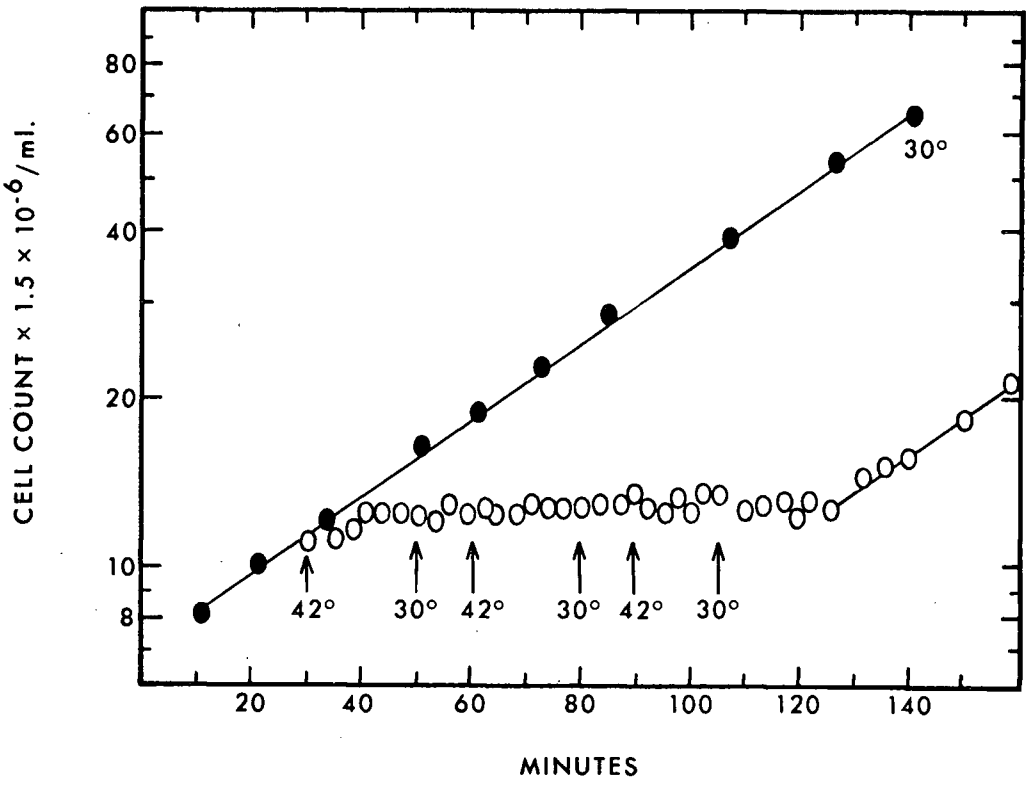


Figure 13. Inhibition of cell division by periodic exposure to the non-permissive temperature.

A culture of T83 growing exponentially at 30 C was shifted to 42 C. At times indicated by vertical arrows, this culture was oscillated between 30 C and 42 C cycles. After the last shift to 30 C, at 105 minutes, cells were kept at 30 C to allow expression of cell division. Growth of the control (●) and experimental (○) cultures is plotted as a function of time.

Termination of a round of replication is a necessary condition for cell division (Clark, 1968b; Pierruci and Helmstetter, 1968). It is known that any arrest in chromosome replication resulted in formation of filaments (Bilen, 1969; Donachie, 1969; Boyle et al. 1967). This coordinate regulation between DNA replication and the various processes involved in cellular division has been examined by Inouye and Pardee (1970) and results indicated that blocking of DNA replication by chemical agents or by mutations caused a change in membrane proteins that were involved in cell division.

Nalidixic acid has been shown to stop semiconservative DNA replication immediately (Dietz et al. 1966; Goss et al. 1965)as well as repair replication (Eberle and Masker, 1971) in E. coli. Using Nalidixic acid, Clark (1968 a,b) and Helmstetter and Pierruci (1968) showed that it did not affect the cellular division of those cells which had completed their DNA replication.

The various aspects of the coordination between DNA replication and cell division was examined in T83. Nalidixic acid, at a final concentration of 10 µg per ml, was added to T83 during incubation at 42 C. Since during the incubation at 42 C, cells continue to make a residual amount of DNA, the blocking of DNA replication by Nalidixic acid should have allowed for cellular division proportional to the amount of DNA replication that had already occurred. Figure 14A represents the results of an experiment where Nalidixic acid was added at the time of shift up and at fifteen minute intervals during

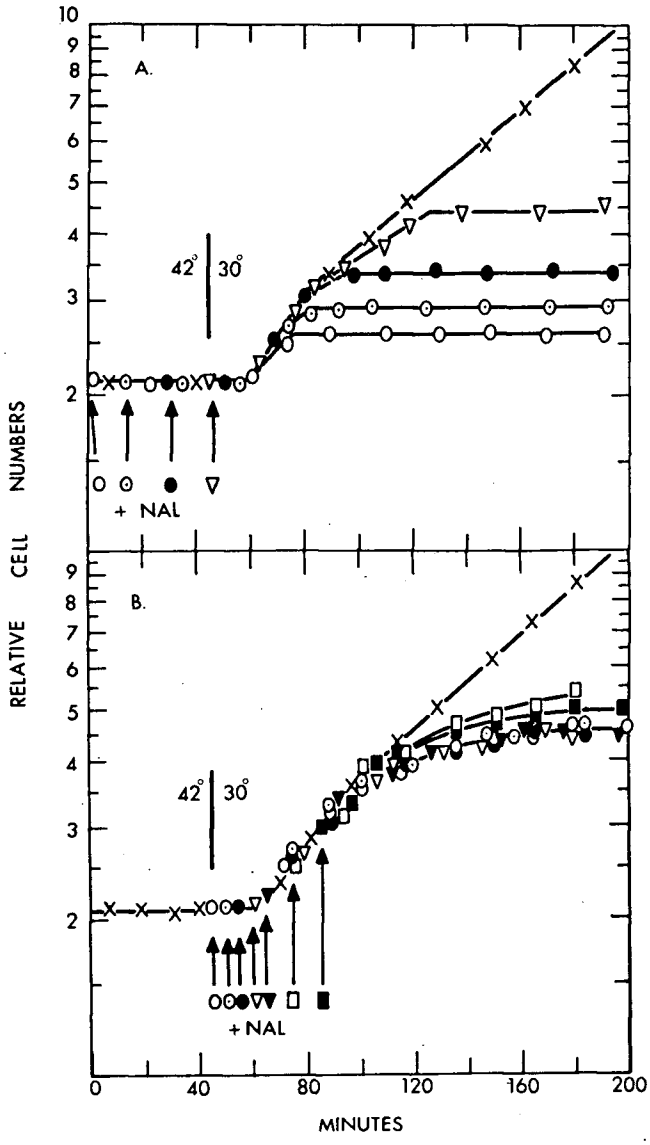


Figure 14. Effect of inhibition of DNA synthesis on cell division at recovery at 30 C.

T83, grown at 30 C, at a cell density of 3×10^7/ml, was shifted to 42 C at zero time. Nalidixic acid was added to subsamples during the 45 minute incubation at non-permissive temperature at the time of shift up (0), or 15 (0), 30 (●), and 45 (▽) minutes thereafter (panel A), or after return to the permissve temperature for recovery at zero (0), 5 (0), 10 (●), 15 (▽), 20 (▼), 30 (□), and 40 (■) minutes after shift donw. Vertical arrows indicate the times of addition of Nalidixic acid to a final concentration of 10 µg per millilitre.

the non-permissive treatment. When the cells were returned to 30 C, cellular division resumed at the same time as the control cells. There was a direct correlation between the amount of DNA synthesized at 42 C and the division observed at 30 C. Cells that were treated right at the shift up time (zero minutes) resulted in 27% residual division. If fifteen minutes of DNA replication was allowed at 42 C, the residual division at 30 C went up to 42% and reached 59% for thirty minutes of residual DNA synthesis. When DNA synthesis was allowed for 45 minutes, there was nearly a one hundred percent increase in cell number during recovery. Control cells at 30 C, when given the same amount of Nalidixic acid, resulted in 30% residual division. When Nalidixic acid was added during the recovery period, no increase in cell numbers was observed for additions between 0 and 20 minutes.shift down. For addition at 30 and 40 minutes post-shift down, however, residual division levels rose to 140 and 160 percent respectively.

2. Role of RNA synthesis in recovery.

By binding to RNA polymerase, Rifampicin specifically blocks initiation of transcription but not the completion of those in progress (Mizuno et al. 1968; Burgess et al. 1969). Addition of Rifampin, just prior to the initiation of new cycles of DNA replication, does not interfere with completion of rounds started but prevents the initiation of new ones (Silverstein and Billen, 1970).

When the response of exponentially growing cells of E. coli B/r/1 and T83 were tested for different concentration of Rifampicin, it was

found that inhibition of ^{3}H-uracil uptake into the cells occurred relatively slowly. Up to five minutes was required for 50 percent inhibition at concentrations of 1 to 25 µg per ml of Rifampicin, but when high concentrations were used (50 to 500 µg per ml), effective inhibition was observed. However, when the percent residual division was compared with the concentration of Rifampicin, it was noticed that B/r/1, in glucose minimal medium, reproducibly gave 30, 13, 6.5, and 1.2 percent residual division respectively, for concentrations of 10, 25, 50 and 100 µg per ml. At still higher concentrations, less residual division was obtained. Thus, 10 µg per ml was selected as a suitable concentration which effectively inhibited RNA synthesis without having any ill effects on the cells.

CR34T83, when grown in medium A and E at 30 C with 10 µg per ml Rifampicin, gave 50 percent residual division which was consistent with the B/r/1 data. When T83 was shifted to 42 C for varying lengths of time, from 10 to 60 minutes, and Rifampicin was added to the culture at the recovery time, all the cells recovered and underwent residual division (Figure 15). It was concluded that RNA synthesis subsequent to the shift down was of little consequence to cell division at recovery. Presumably, mRNA in general, and division messengers at the shift down time, were undergraded and available for translation. Once such translations were completed, residual division, based on the amount of DNA and cell equivalents, resulted. After a 40 minute incubation at 42 C, cells that were shifted down to 30 C in the presence

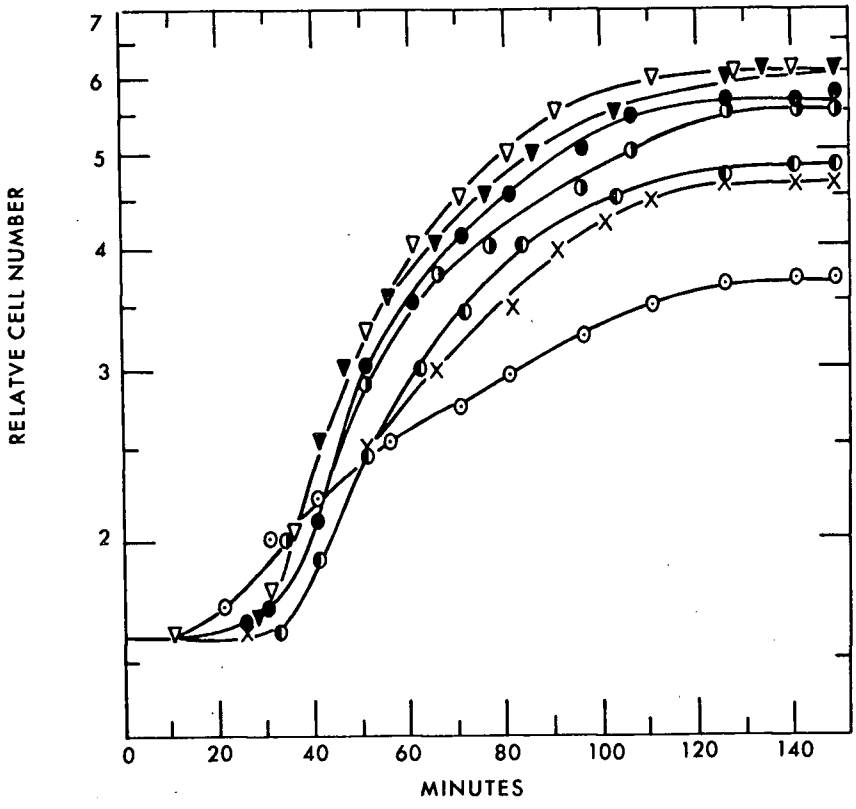


Figure 15. The effect of inhibition of RNA synthesis at recovery on the expression of division.

Exponentially grown cells of T83, at a density of 2.5×10^7 cells per ml, were shifted from 30 to 42 C. After incubation at 42 C for 10 (0), 18 (0), 25 (0), 32 (●), 40 (▽), 45 (▼), and 60 (X) minutes, they were returned to 30 C. Return to 30 C was taken as the zero time for recovery. Rifampicin (10 µg per ml) was added to recovering cells at zero time. Cell numbers were followed as a function of time.

of Rifampicin resulted in residual division that produced an increase of approximately 20 percent in cell numbers. Longer incubations than 40 minutes resulted in reduction in residual division.

Similar experiments were conducted in which Rifampicin was added at different time intervals during a 45 minute pulse at 42 C or during recovery at 30 C (Figure 16). A 45 minute incubation in the presence of Rifampicin at 42 C virtually destroyed the capacity of the cells to recover at 30 C. Shorter blocks of total RNA synthesis gave increasing amounts of residual division. Thus, in order for the cells to express the division of their cell equivalents at recovery, RNA synthesis at 42 C was needed. However, once shifted to permissive temperature from a 45 minute growth at 42 C, no great change in residual division was observed if Rifampicin was added at 5, 10 or 15 minutes after the shift down to 30 C.

If the accumulation type of behaviour was proportional to the duration of growth at 42 C, presumably, with longer incubation, more potential should be accumulated. Figure 17 shows that this is not the case. Between 15 and 45 minutes incubation at 42 C, significant changes in residual division were observed. However, the change between 45 and 60 minutes was negligible. It was also noted that Rifampicin treatment at 42 C, for as short as 10 minutes prior to recovery, was sufficient to reduce the maximum division potential seen at the time of shift down by 50 percent.

3. Role of protein synthesis on cell division and DNA replication during the recovery period.

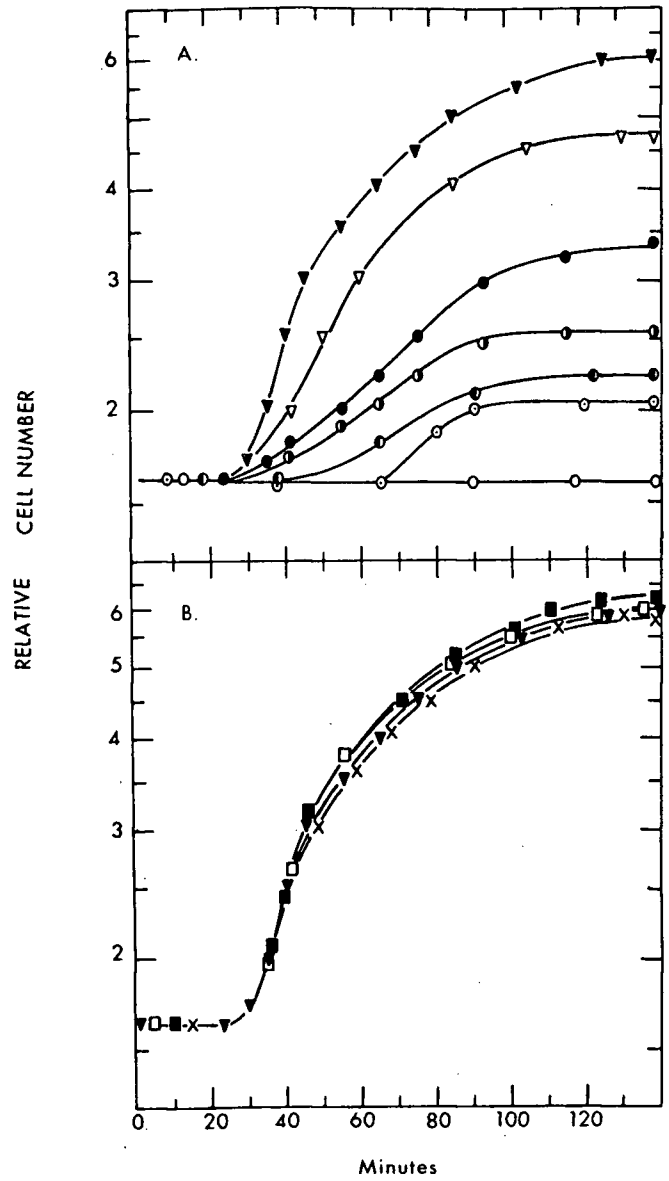


Figure 16. Effect of inhibition of total RNA synthesis on recovery.

Subcultures of T83, at a density of 2.3×10^7 cells per ml, were shifted to 42 C for 45 minutes and Rifampicin was added at various times during the pulse. Recovery from the treatment was examined at 30 C. Rifampicin was added (10 µg per ml) at 0 (0), 10 (0), 18 (0), 25 (0), 32 (0), 40 (∇), and 45 (▼) minutes. All of the subcultures were kept at 42 C until 45 minutes, at which time they were returned to 30 C (zero time). This is shown in panel A. Panel B is the same experiment except that Rifampicin was added at 0 (▼), 5 (□), 10 (■), and 15 (X) minutes after a 45 minute pulse at 42 C.

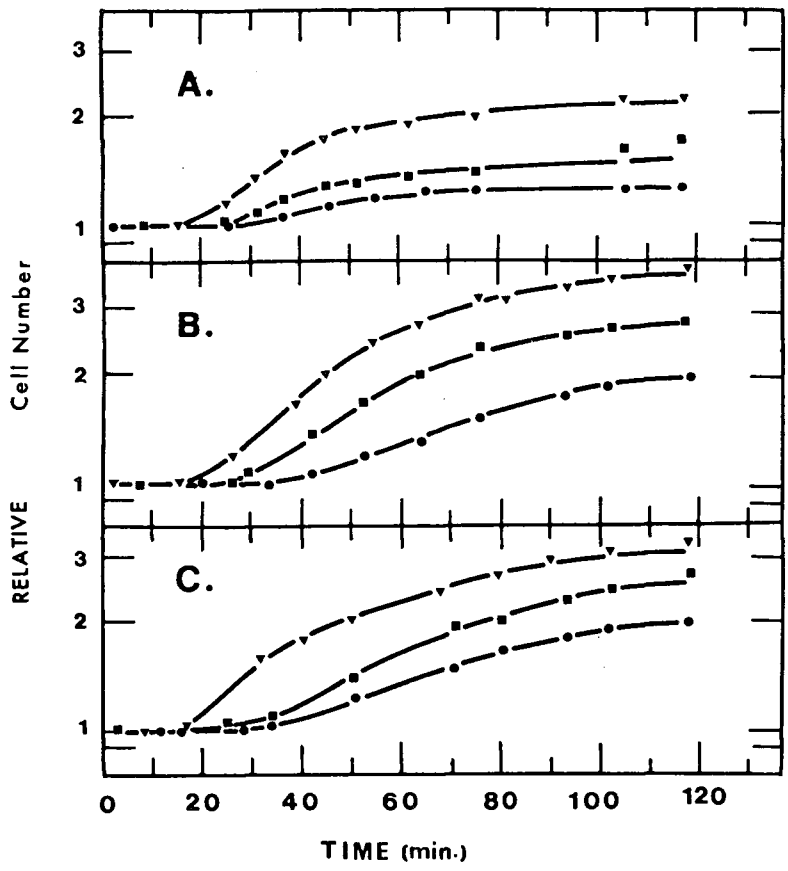


Figure 17. Measurement of the accumulation of division potential in T83.

CR34T83, at a density of 2.3 x 10^7 cells per ml, was shifted from 30 C to 42 C and kept there for 15 (A), 45 (B) and 60 (C) minutes. In each case, at 10 (●), 5 (■) or 0 (▼) minutes before shift down (zero time), Rifampicin was added to all the cells. Cell counts were followed during recovery time.

a. Cell division.

The data presented so far indicates that in T83, the temperature sensitive block affected a step required in cellular division and chromosomal replication. It seems that the structure or substance(s) needed in the vital functions of the cell's cycle is involved in this condition. Accordingly, the temperature effect could involve a complete and irreversible denaturation of the temperature labile components and the lag thus represents time needed for the resynthesis of the new parts, the reorientation of the affected structure, or perhaps for the renaturation of the protein in question. The following transitions could be entertained for such temperature sensitive condition: (a) the temperature sensitive product or structure is native and active at the permissive temperature, but is rendered denatured and inactive at the non-permissive temperature; or (b) it is rendered inactive, though remains in the native form at the non-permissive temperature. The following experiments were undertaken to explore the above discussed possibilities as likely candidates for the temperature sensitive condition.

In the first series of experiments, T83 was subjected to a 42 C temperature pulse for ten minutes. To one half of the 42 C cells, chloramphenicol, at final concentration of 150 µg per ml, was given five minutes after shift to 42 C. The rest of the culture was not treated. At the end of 10 minutes at 42 C, both cultures were returned to 30 C. As shown in Figure 18, the cells that received

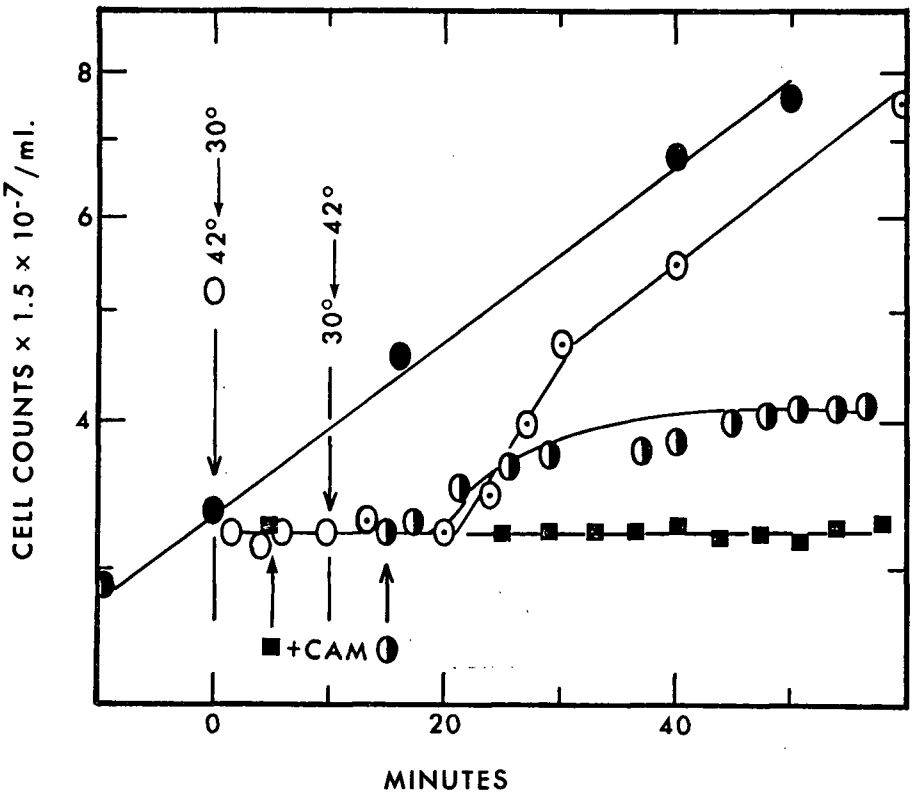


Figure 18. Effect of inhibition of protein synthesis on recovery of T83.

Part of an exponential culture of CR34T83 growing at 30 C (●) was shifted to 42 C (○). At 5 minutes prior (■) and post (◑) shift down, CAM (150 µg per ml) was added to subcultures (○). Cell counts are plotted as a function of time.

chloramphenicol did not recover.

To test for the necessity of new protein synthesis for the expression of subsequent cell division during the recovery period at 30 C, chloramphenicol was added five minutes after the shift down. The results are seen in Figure 18. After a short pulse of 10 minutes at 42 C, when cells were returned and allowed to undergo protein synthesis, full division was expressed after the 10 minute lag period. However, if protein synthesis was blocked, such that there was only a limited amount of protein synthesis at 30 C, there was a decreased amount of division in recovery. Apparently, a short period of protein synthesis was essential for the expression of the division after a 10 minute growth at 42 C.

Similar tests were performed, except with an extended pulse of 45 minutes at 42 C (Figure 19). T83 was grown for several generations at 30 C and was distributed into eight identical flasks and shifted to 42 C for 45 minutes after which chloramphenicol was added to the flasks at 10, 18, 25, 32, 40, and 45 minutes at 42 C, and at 5 or 10 minutes after shift down. It was concluded that, if protein synthesis was inhibited for 5 to 35 minutes, in T83 grown at 42 C for 45 minutes, no cell division was observed during recovery period. However, if chloramphenicol was added at the time of shift down (45 minutes), or after the shift back to 30 C, exactly one doubling was observed.

It was of interest to find out the mode of acquisation of the division potential and see if it was an accumulative one. If so,

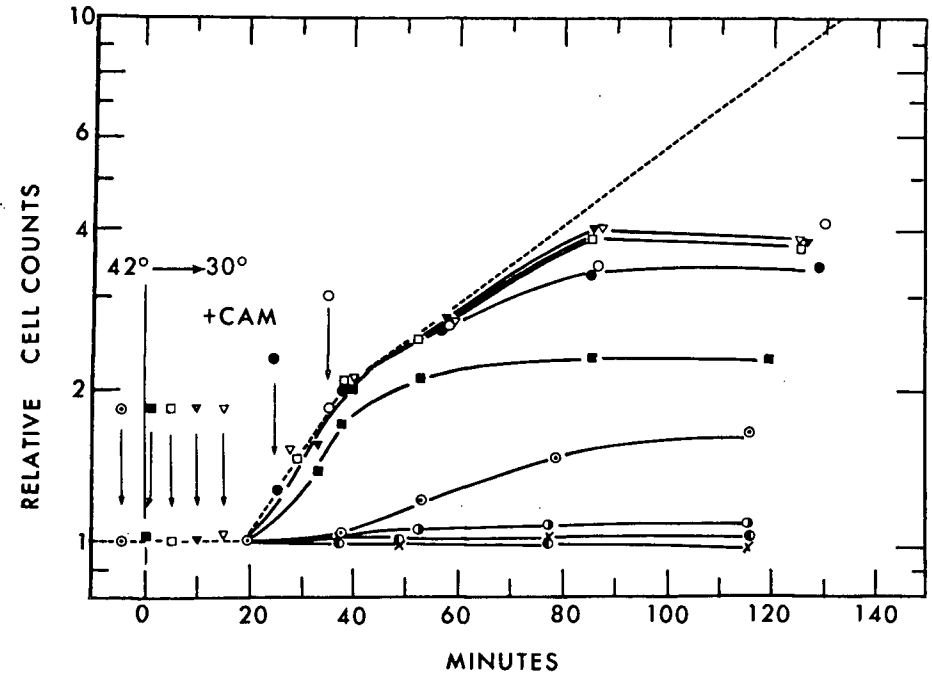


Figure 19. Effect of inhibition of protein synthesis during and after a pulse at 42 C, on recovery

T83 grown at 30 C in A and E medium to a density of 1.5×10^7 cells per ml, was shifted to 42 C for 45 minutes. CAM (150 µg per ml) was added to subcultures, as indicated by vertical arrows, after 18 (X), 25 (◐), 32 (◑), or 40 (⊙) minutes incubation at 42 C, or at 0 (■),5(□), 10 (▼), 15 (▽), 25 (●), and 35 (○) minutes after return to 30 C. The return to 30 C after a 45 minute pulse at 42 C was taken as zero time for recovery. Relative cell counts are plotted against time. The dashed line represents the untreated control.

gradually increasing levels of residual division was produced upon longer incubation at 42 C. The accumulation hypothesis led to an experiment where 150 µg per ml chloramphenicol was added at the time of shift down to the T83 cells grown at 42 C for varying lengths of time. Results of such experiments are presented in Figure 20. For a 10 minute shift to 42 C, and return to 30 C in the absence of any further protein synthesis, there was a 33 percent increase in cell counts. With longer incubation at 42 C, the amount of division observed at recovery under chloramphenicol inhibition rose. Thus, with 18, 25, 32 and 40 minutes of incubation at 42 C, respectively, 40, 67, 87 and 100 percent increase in cell number was observed. Incubations of 45 to 60 minutes yielded approximately a 110 percent increase in the cell counts. The percent increase in the cell numbers was found to be linearly related to the pulse at 42 C, which plateaued off at 40 minutes. When compared with the amounts of residual DNA made at 42 C, the plots of percent residual DNA synthesis and division data revealed a parallel relationship between the amount of DNA made at 42 C to that of cell division. Thus, the general implication of these data was that (a) the transition for cell division potential was that of a native and active protein at 30 C going to native but inactive at 42 C, and its reactivation upon return to 30 C; and (b) at the time of shift down from any length temperature block, there existed enough potential in the cell to express division or cellular recovery based on the amount of DNA per cell equivalents.

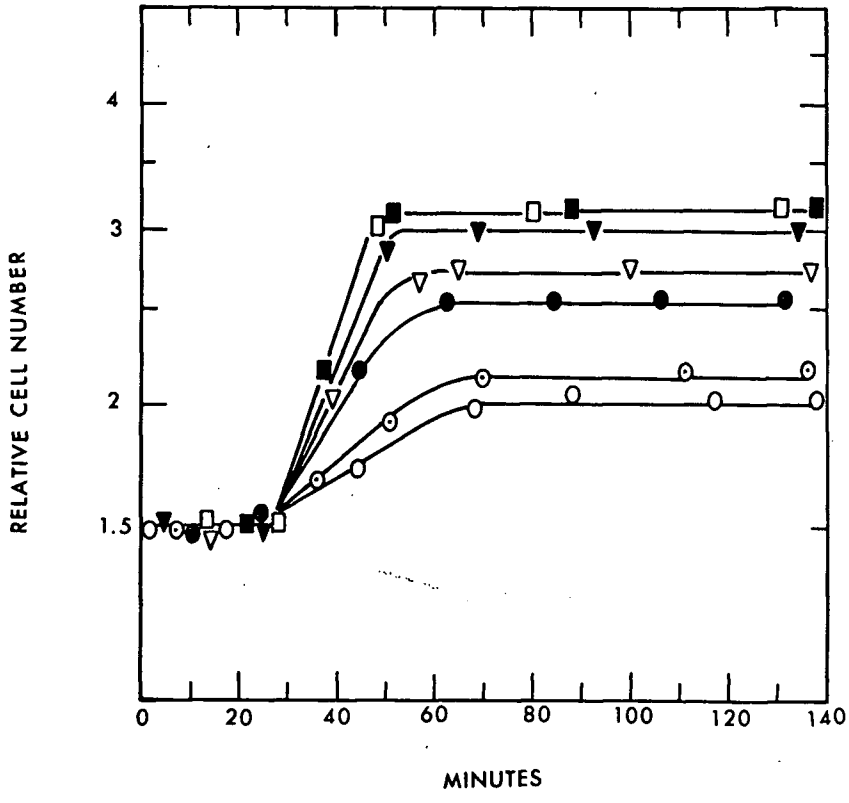


Figure 20. Effect of inhibition of protein synthesis on the recovery of cells at 30 C.

CR34T83 cells were pulsed at 42 C for variable lengths of time and, at the time of shift down to 30 C (zero time), 150 µg per ml chloramphenicol was added to the culture. Cell counts are plotted against time since the return to 30 C. Symbols representing pulses at 42 C are: (○), 10 minutes; (⊙), 18 minutes; (●), 25 minutes; (▽), 32 minutes (▼), 40 minutes; (□), 45 minutes; and (■) 60 minutes.

The division rates at which the recovery cells from a 42 C block underwent, were compared with control cells and with those given chloramphenicol at shift down (Figure 21). There was a change in the rates of cell division during the recovery phase II (rapid division) and a small increase in the rates between 20 and 45 minutes which declined to a slower rate upon longer incubation. On the contrary, the cells which had lost their capacity for synthesis of proteins at recovery, showed a gradually increasing rate of division upon longer incubation at 42 C. There was a four-fold change in the rate of division between cells incubated at 42 C for 45 to 60 minutes when recovering at 30 C in the presence of chloramphenicol (Figure 21).

It was obvious that, aside from the formation of DNA equivalents at 42 C, with the longer incubations, the stage was readied for rapid division, presumably by allowing accumulation of other division proteins which had enough of a potential to allow expression of division even in the absence of any protein synthesis.

b. DNA synthesis.

From the cell division data during recovery, it was concluded that protein synthesis at 42 C was not needed for division based on cycles of DNA replication completed at the time of shift from 42 to 30 C. It was important to correlate the effects of inhibition of the protein synthesis needed for new rounds of DNA replication to that of recovery from the growth under non-permissive conditions.

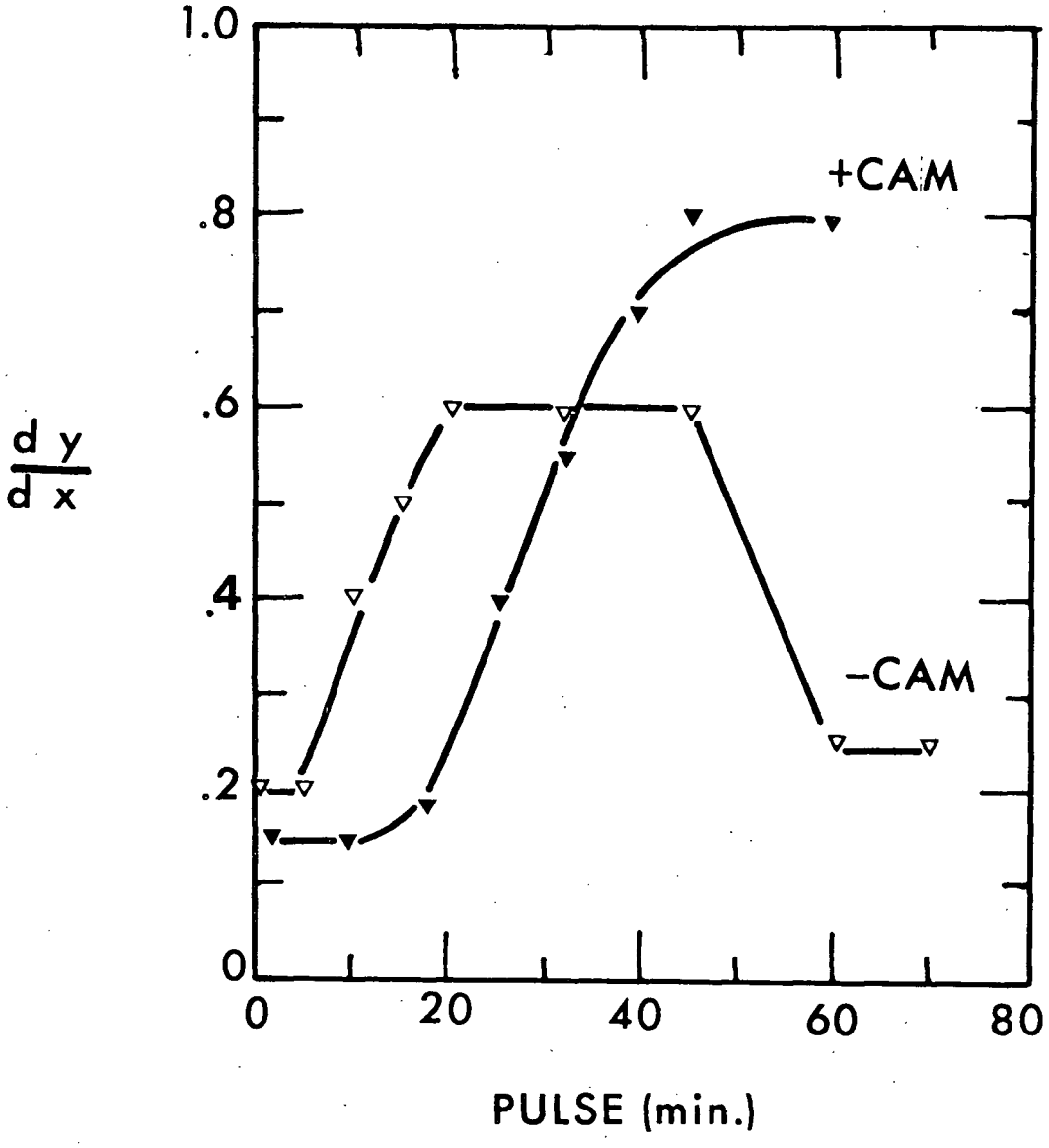


Figure 21. Changes in the rate of cell division during recovery from a 42 C block in the presence of chloramphenicol.

The rate of cell division (dy/dx) was calculated during the rapid division phase of recovery in the presence (∇) and absence (▼) of protein synthesis. T83 cells, grown at 42 C, were shifted to 30 C for recovery. To one half of the culture 150 µg per ml CAM was added at the shift down time (▼). The abscissa represents the length of time at 42 C.

The fact that the mutant was defective in further initiation of DNA cycles at 42 C implied that blocking protein synthesis at 42 C should result in no net DNA synthesis at recovery. However, net synthesis subsequent to addition of CAM would have implied a recycling of the old initiated cycle. The results of such experiments are shown in Figure 22. No new DNA synthesis was observed for 60 minutes after shift down to 30 C. Meanwhile, the control culture showed resumption of new synthesis in approximately 8 minutes after shift down. There was no relationship between the duration of continued protein synthesis at 42 C and the amount of residual DNA synthesized. Thus, unlike the division at recovery, the initiation of new rounds of replication needed new protein synthesis at the recovery time as indicated by the control cells.

The relationship between the role of protein synthesis at the 30 C recovery period and of the initiation of new rounds was examined for pulses of 10 and 30 minutes at 42 C. Results are shown in Figure 23. Cells recovering from 10 minutes of incubation at 42 C required more than 5 minutes of protein synthesis for resumption of DNA synthesis, since addition of chloramphenicol at 0 to 5 minutes subsequent to the shift down resulted in a 0 to 5 increase in residual DNA synthesis over 90 minutes at 30 C. During this time, the control cells increased their levels of DNA synthesis to over 100%. Similarly, if the cells were returned to 30 C from a 30 minute block at 42 C, the same rule held true. Noticeable levels of new replication took place only when

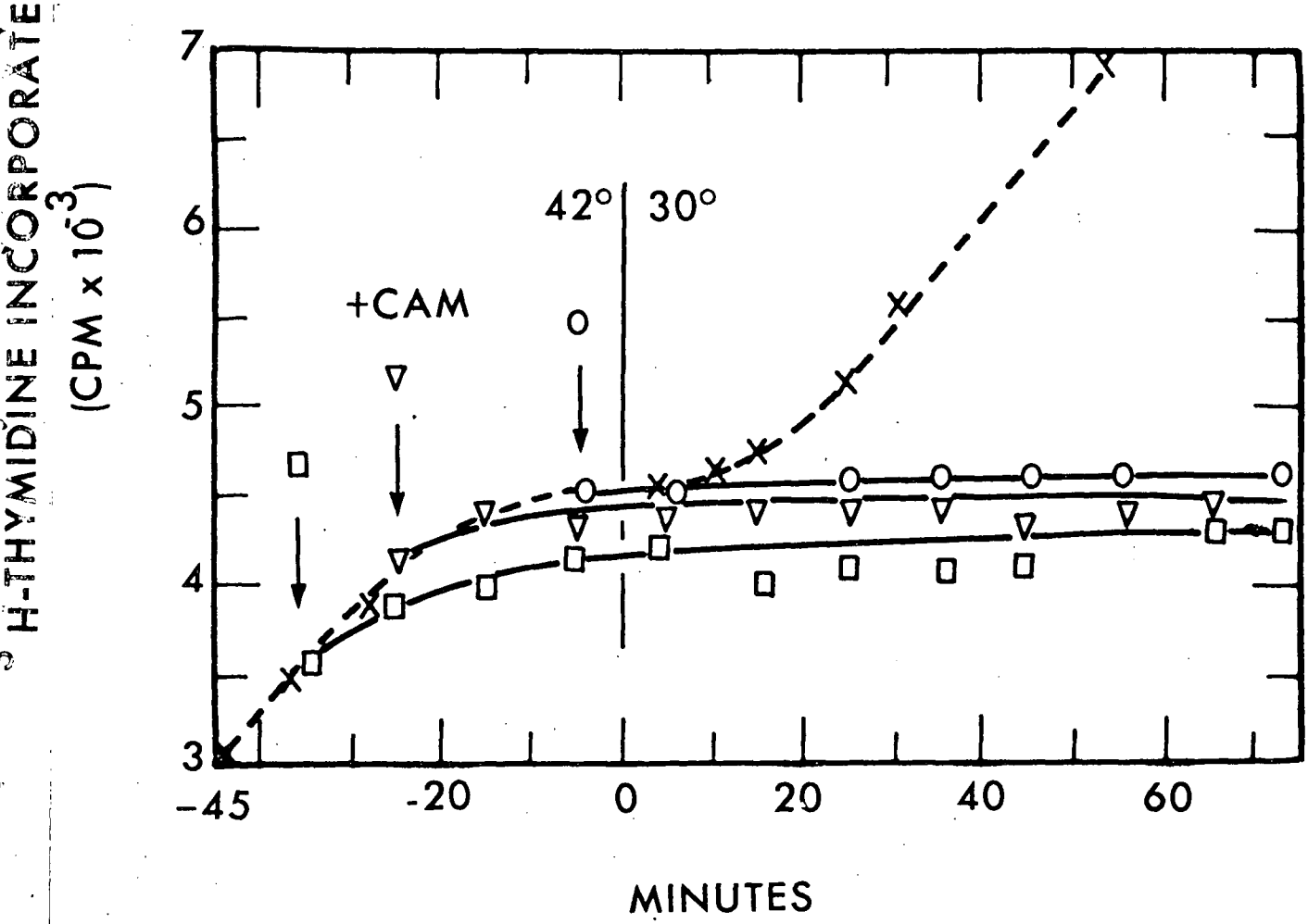


Figure 22. The effect of inhibition of protein synthesis at 42 C on the DNA replication at recovery.

T83 grown in the presence of 0.5 µc per ml ^{3}H-TdR, 20 µg per ml cold TdR, and 50 µg per ml AdR, was shifted to 42 C for 45 minutes. To subcultures at 42 C, CAM (150 µg per ml) was added at the times indicated by the vertical arrows. After 45 minutes, the cells were returned to 30 C, and allowed to recover. The CPM of ^{3}H-TdR incorporated for the control (X) and the experimental cultures at 5 (O), 25 (∇), and 35 (□) minutes with CAM at 42 C are plotted as a function of time.

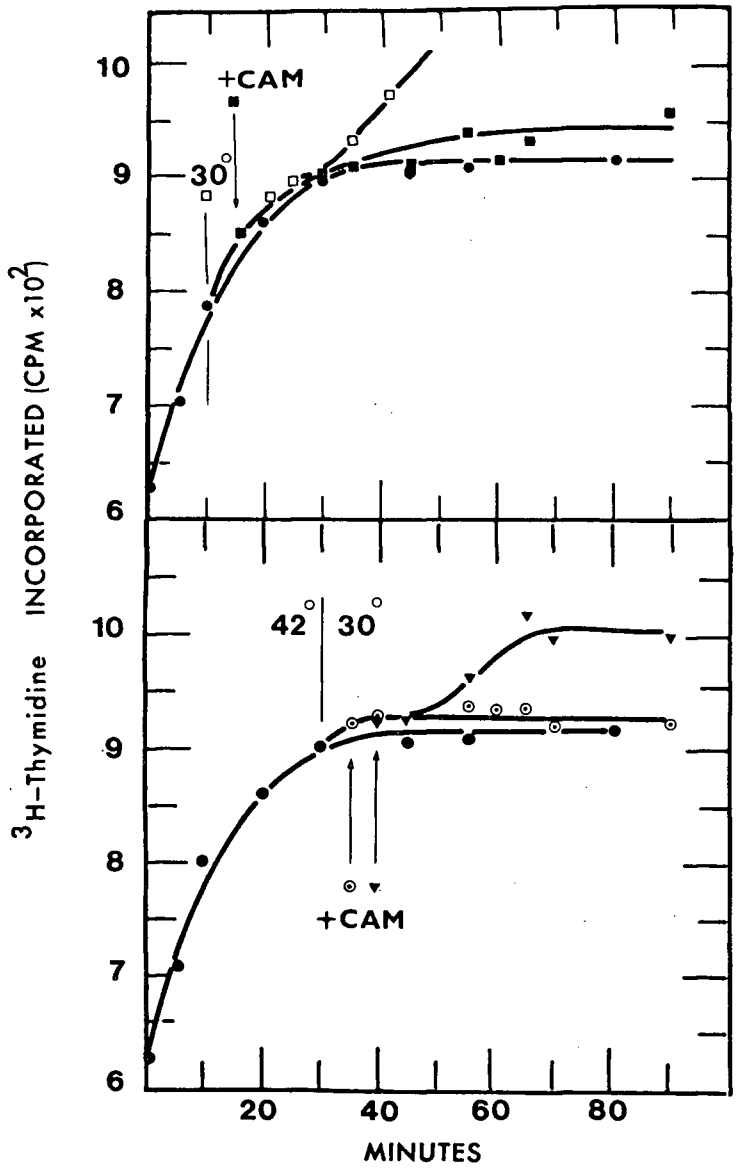


Figure 23. Role of inhibition of protein synthesis on initiation of new rounds during recovery.

T83 was grown in the presence of ^{3}H-thymidine (0.5 µc per ml) 20 µg per ml cold thymidine, and 50 µg per ml deoxyadenosine, at 30 C. The cells were shifted to 42 C and were kept there (upper panel). Ten minutes later, a portion of these cells was transferred back to 30 C (□), and, 5 minutes after shift down, CAM (150 µg per ml) was added to half of the 30 C cells (■).

After 30 minutes, a part was returned to 30 C (lower panel). Five (θ) and 10 (▼) minutes after shift down, CAM (150 µg per ml) was added to subcultures, and the incorporation of thymidine (^{3}H-methyl-thymidine, 0.5 µc per ml and 20 µg per ml cold TdR) into the TCA insoluble fraction was followed. CPM of radioactivity is plotted against time.

approximately 10 minutes of protein synthesis was allowed.

The nature of DNA made subsequent ot 5 minutes of protein synthesis, was determined by looking at the rate of replication. If, during the 5 minutes, new initiator molecules were inaugurated, the rate of DNA replication should have shown an increase. When the rate of the DNA replication was determined in a series of 3 minute pulses using ^{14}C-thymidine, a burst in the rate of replication was noticed approximately 18 minutes after shift down. Presumably, this burst had resulted from the protein synthesis that had taken place at recovery since blocking protein synthesis at 42 C immediately prior to a shift down did not produce a burst in DNA synthesis. It was concluded that the protein synthesized at recovery was necessary for the initiation of new rounds of DNA replication (Figure 24).

D. Attempts to uncouple DNA replication and cell division at 30 C.

To test the possibility of uncoupling the DNA replication from cell division, three experiments were carried out.

1. Inhibition of initiation of new rounds by phenethyl alcohol.

Phenethyl alcohol (PEA), at a 0.25% level, has been shown to allow residual DNA synthesis in E. coli (Berrah and Konetzka, 1962) by interferring with the initiation of new cycles of replication (Treich and Konetzka, 1964). Results from several laboratories indicated that PEA had multiple immediate effects on the cell such as on

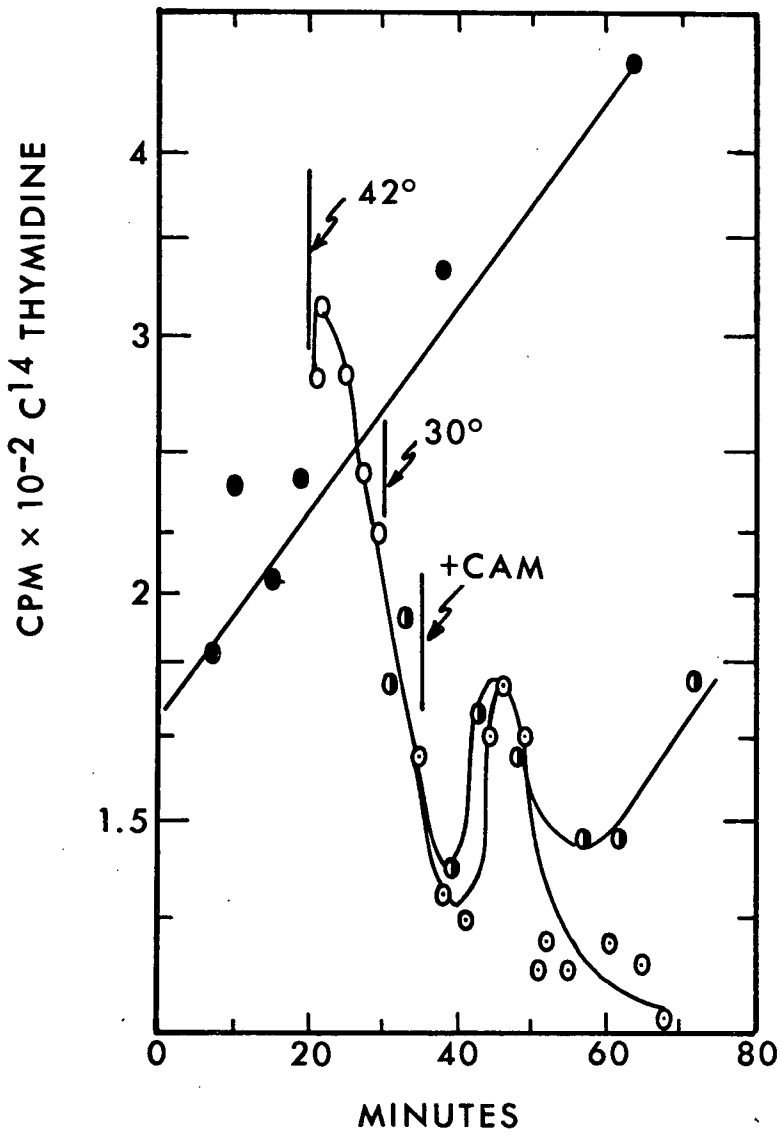


Figure 24. The effect of chloramphenicol on the rate of DNA synthesis during the recovery period.

An exponential culture of T83 growing at 30 C (●) was shifted to 42 C (○). A part of the culture was returned to 30 C (◐) and, after 5 minutes at 30 C, CAM (150 µg per ml) was added to a portion of the recovering cells (◑). To determine the rate of DNA synthesis, cells were pulsed for 3 minutes with ^{14}C-TdR at 20 µg per ml in the presence of 50 µg per ml AdR. CPM of ^{14}C-TdR incorporated are plotted as a function of time.

the activity of cell membrane (Silver and Wendt, 1967), on transport reactions and nucleotide pools (Plagemann, 1970), and on respiration and oxidative phosphorylation (Cosgrove and Treich, 1970). Studies by Lark and Lark (1966) confirmed the results of Treich and Konetzka (1964) and their results ascertained that, in E. coli, PEA blocked the DNA replication at the same position on the chromosome as amino acid starvation.

When 0.25% PEA was added to exponentially growing cultures of T83 at 30 C in A and E medium, approximately 55% residual DNA synthesis occurred (Figure 25A). This figure matches nicely with the residual DNA synthesized by 42 C grown cells. Furthermore, the time needed for the completion of the residual DNA synthesis was 40 to 50 minutes, after which time no further increase was observed. Based on this evidence, the temperature effect and PEA had singular action in allowing completion of started rounds.

Examination of cell division data subsequent to addition of 0.25% PEA revealed residual division. As shown in Figure 25 B, during 25 minutes after addition of PEA, cellular division continued at the normal rate. The amount of residual division calculated was approximately 50%. Thus, it was possible to uncouple the two processes, cell division and DNA replication, under permissive temperature conditions by blocking one process, since in the absence of any new initiation, the cells could express only residual division. The amount of residual division observed was increased by an additional 10% if NaCl (0.65%

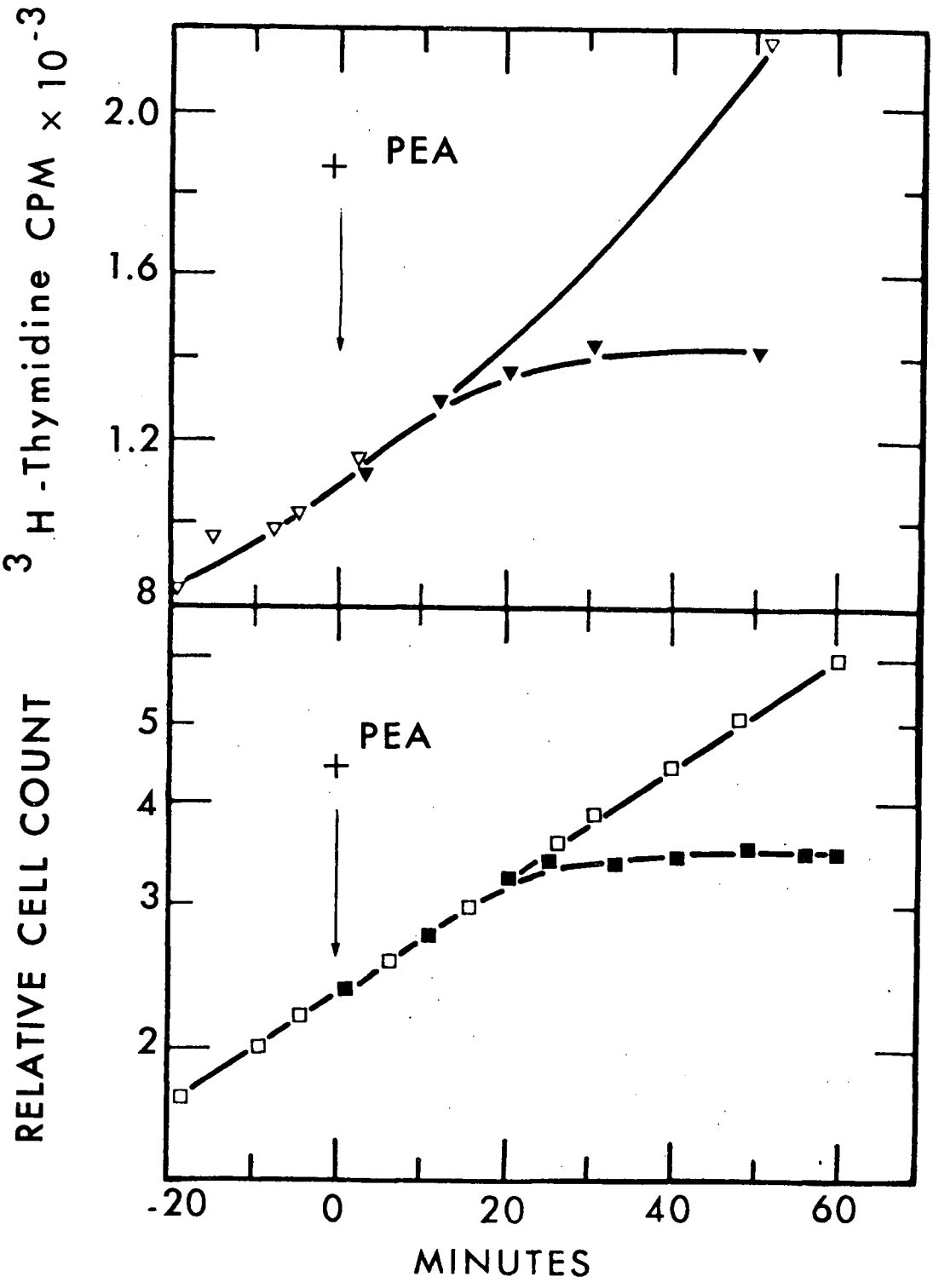


Figure 25. Uncoupling of cell division from DNA replication by phenethyl alcohol.

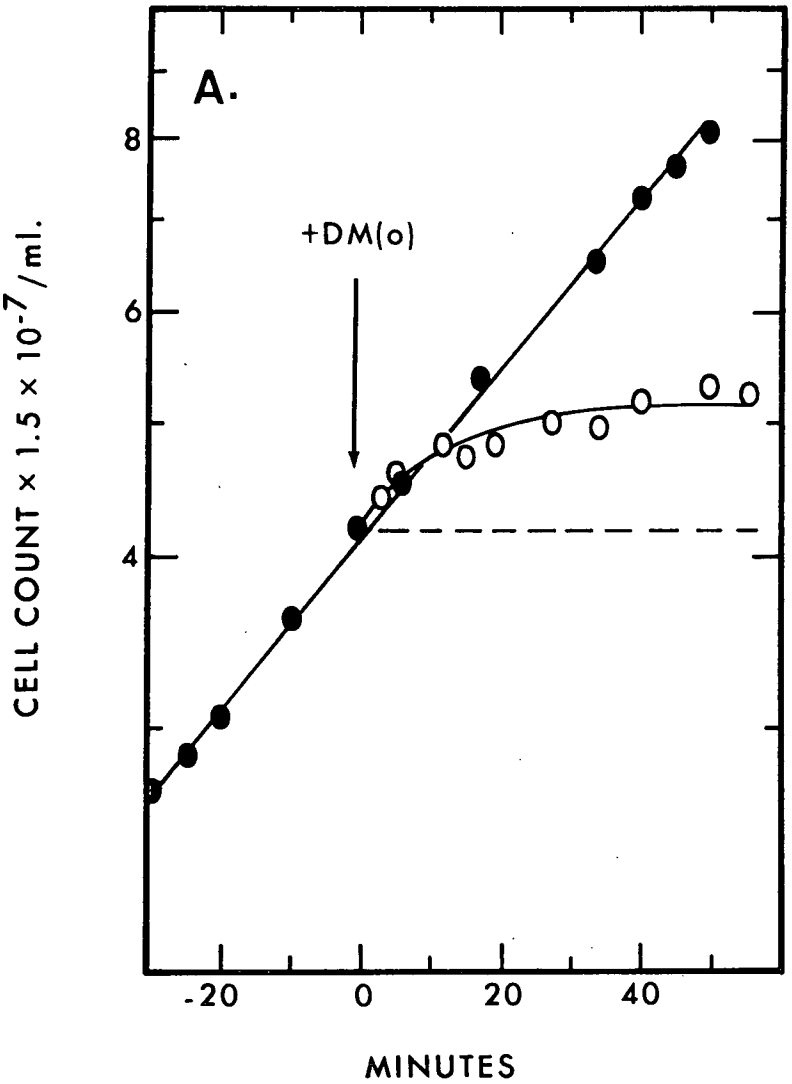
T83, growing at 30 C for several generations in the presence of 20 µg per ml of cold and 0.5 µc per ml ^{3}H-thymidine and 50 µg per ml deoxyadenosine, was treated with phenethyl alcohol at 0.25% final concentration, as shown by the vertical arrow. Uptake of radioactive TdR (triangles) and cell counts (squares) were followed for control (empty symbols) and PEA treated cells (full symbols). The cell density at the time of PEA treatment was 4×10^7 cells per ml.

final concentration) was added simultaneously with PEA. However, addition of salt had no stimulatory effect on the residual DNA replication.

2. Uncoupling cell division from DNA replication by Daunomycin.

Daunomycin (DM), an antibiotic in the anthracycline group isolated from a culture of Streptomyces peucetius, was shown to inhibit both DNA-dependent RNA-polymerase and DNA polymerase in E. coli in vitro (Hartman et al. 1964). The in vivo enzymes associated with DNA synthesis (thymidine kinase, deoxycytidine monophosphate deaminase, and DNA polymerase) in HeLa cells were not affected by DM (Kim et al. 1968). The inhibitory action of this antibiotic on DNA replication lies in its ability to bind to the DNA template (Calendi et al. 1965; Kerstein et al. 1966) and to interfere with the availability of the template to the replicating enzymes. The possibility of using DM for the uncoupling of cell division and DNA replication in T83 was explored.

DM, at 20 µg per ml, was found inhibitory to DNA synthesis in E. coli B/r/1. RNA synthesis continued, however for 30 minutes at the normal rate. When DM was added to an exponentially growing culture of T83 at 30 C, cell division stopped after 40 minutes. The amount of residual division obtained after addition was 25 percent as shown in Figure 26B. There was a gradual decline in the rate of DNA replication at 30 C in the presence of DM. In the first 10 minutes of incubation, there was a 50 percent reduction in the rate of ^{14}C-TdR incorporation into the DNA. The rate of DNA synthesis was promptly reduced to 17


A.
+DM(o)
CELL COUNT x 1.5 x 10⁻⁷/ml.
8
6
4
-20
0
20
40
MINUTES

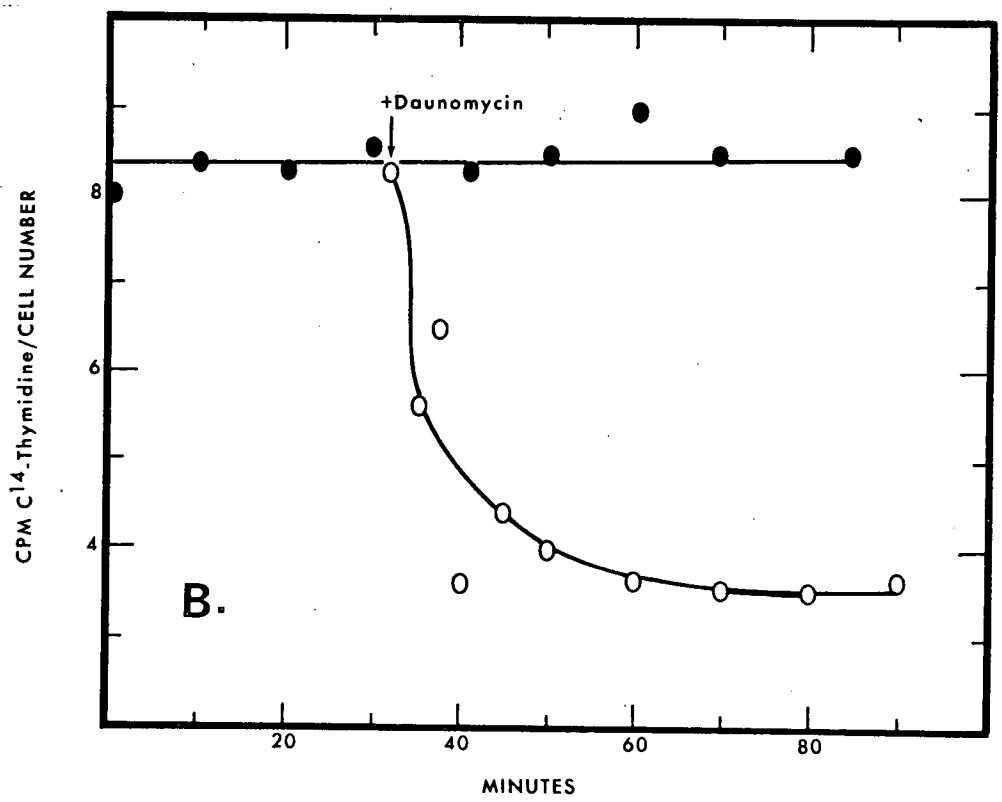

+Daunomycin
CPM C¹⁴-Thymidine/CELL NUMBER
8
6
4
B.
20
40
60
80
MINUTES

Figure 26. Uncoupling of cell division and DNA synthesis by Daunomycin.

Daunomycin (DM) was added to part of a culture of CR34T83 growing at 30 C, at a final concentration of 20 µg per ml. Growth of the culture, with (O) and without (●) DM is plotted as a function of time in panel B. The rate of DNA synthesis was measured by the incorporation of ^{14}C-thymidine at a final concentration of 20 µg per ml and a specific activity of 0.61 µc per µM into TCA insoluble material. The rate of DNA synthesis per cell for the experimental culture with (O) and without (●) DM is plotted as a function of time in panel A.

percent of the control within an hour after drug treatment. The results of this experiment are shown in Figure 26A. Interestingly enough, the kinetics of DM inhibition resembled that of the 42 C effect in T83.

This study showed that, by inhibition of polymerization of DNA (and RNA) in T83, one could observe residual division at 30 C. For the duration of DM treatment, cells remained viable, and recovered immediately when the drug was removed.

3. Uncoupling of cell division from DNA replication by Nalidixic acid.

The coupling between DNA replication and cell division was examined in another experiment, during which coordination of the two events was tested in relation to a temperature block imposed between the Nalidixic acid effect. T83 cells grown at 30 C were treated with Nalidixic acid for 20, 15 and 10 minutes before being put at 42 C. Ten minutes after incubation at 42 C, the cells were returned to 30 C and allowed to recover from the temperature block. If Nalidixic acid interfered, in conjunction with temperature, with the cell division by any means, then the cells should not have divided to their committed 30% level. The results show that indeed, this was not the case as is shown in Figure 27. Culture A, that had been treated with Nalidixic acid for 20 minutes prior to shift up, by expressing its 30 percent residual division, showed no net increase in the cell number after shift down. Culture B could express only 27 percent before stopping division

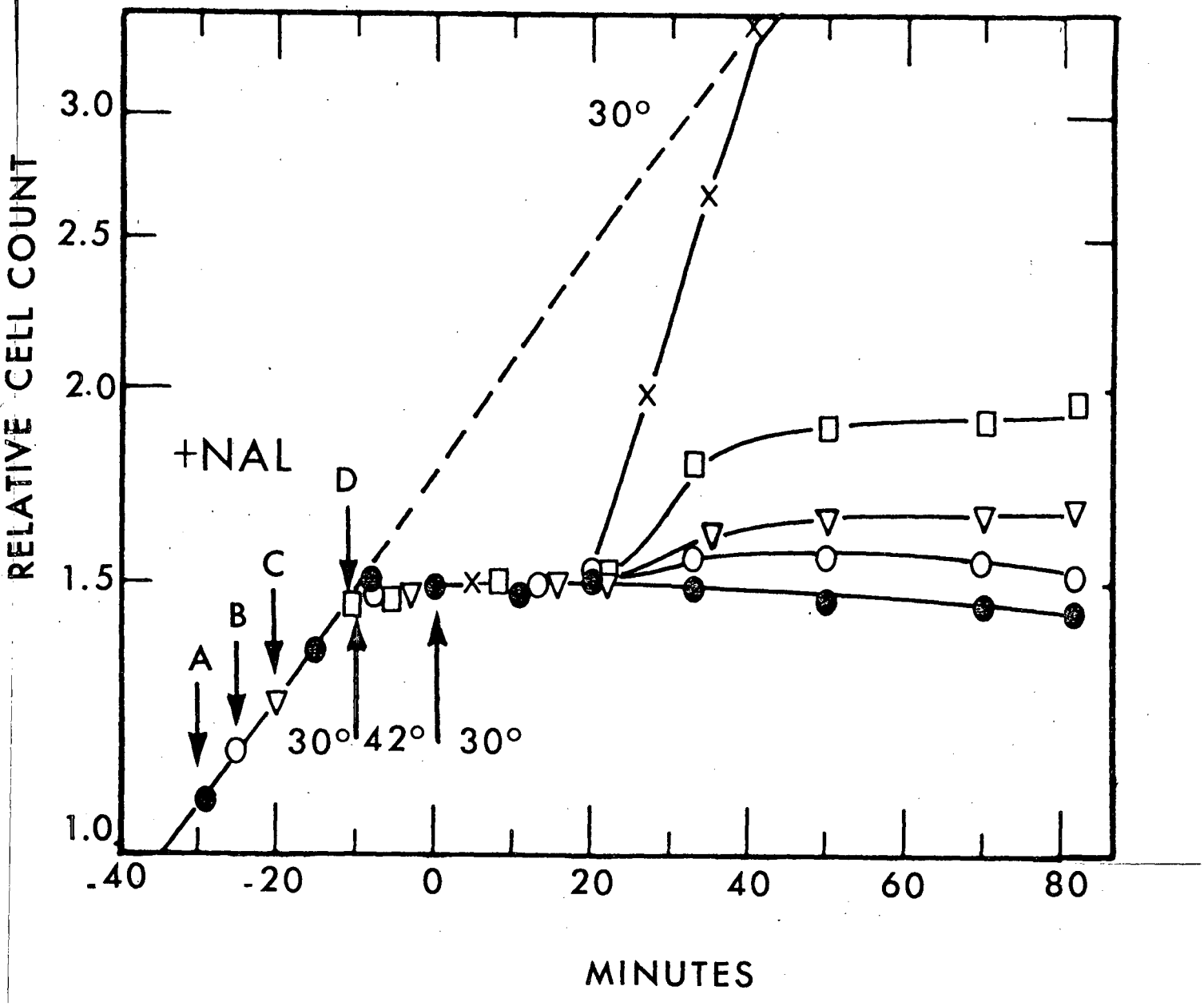


Figure 27. Relationship of inhibition of DNA synthesis and cell division during temperature shifts in T83.

To subsamples of T83 grown at 30 C, Nalidixic acid was added at 20 (A), 15 (B), and 10 (C) minutes prior to and at the time of a shift to 42 C. Cells were returned to 30 C and cell division was assayed. Recovery of the control without addition of Nalidixic acid is represented by X.

due to the 42 C condition. Culture C expressed 20 percent of 30 C before shift and another 12 percent at recovery, and finally, culture D expressed all of its division after being returned to 30 C.

The obvious interpretation of these results was that the temperature sensitive condition that affected the cellular division at 42 C did not obliterate the potential of those cells that could divide. Furthermore, those cells that had the necessary condition for cellular division would have divided successfully to the level expected, and their recall of the division owed to be expressed at recovery was not reduced. This division, however, needed protein synthesis since, if chloramphenicol was added at the same time with Nalidixic acid, there was no division.

III. Control of DNA synthesis in T83.

A. Identification of the place of resumption of DNA synthesis at recovery.

In E. coli the fact that rounds of chromosome replication start at a fixed point on the genome called the "replication origin" is now well established (Lark et al. 1963; Abe and Timizawa, 1967; Caro and Berg, 1968; Wolf et al. 1968; Helmstetter, 1968a), and is located between the arg G and xylose loci. Replication starts from here and continues sequentially in a clockwise direction.

Experiments were designed to determine the place of the resumption of rounds of replication in T83 at recovery. (a) If T83 was tempera-

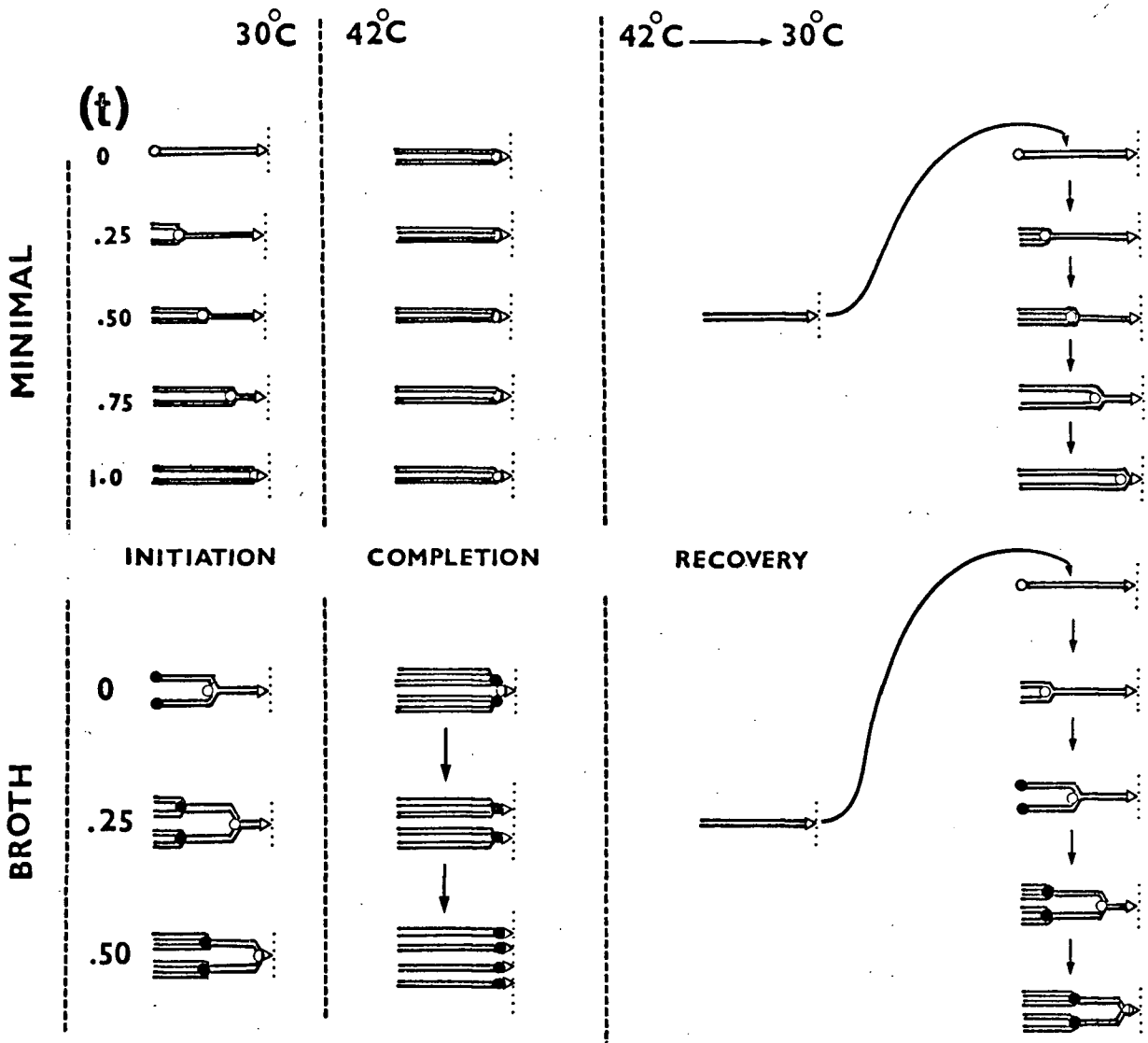

30°C
42°C
42°C ⟶ 30°C
(t)
0
.25
.50
.75
1.0
MINIMAL
INITIATION
COMPLETION
RECOVERY
BROTH
0
.25
.50

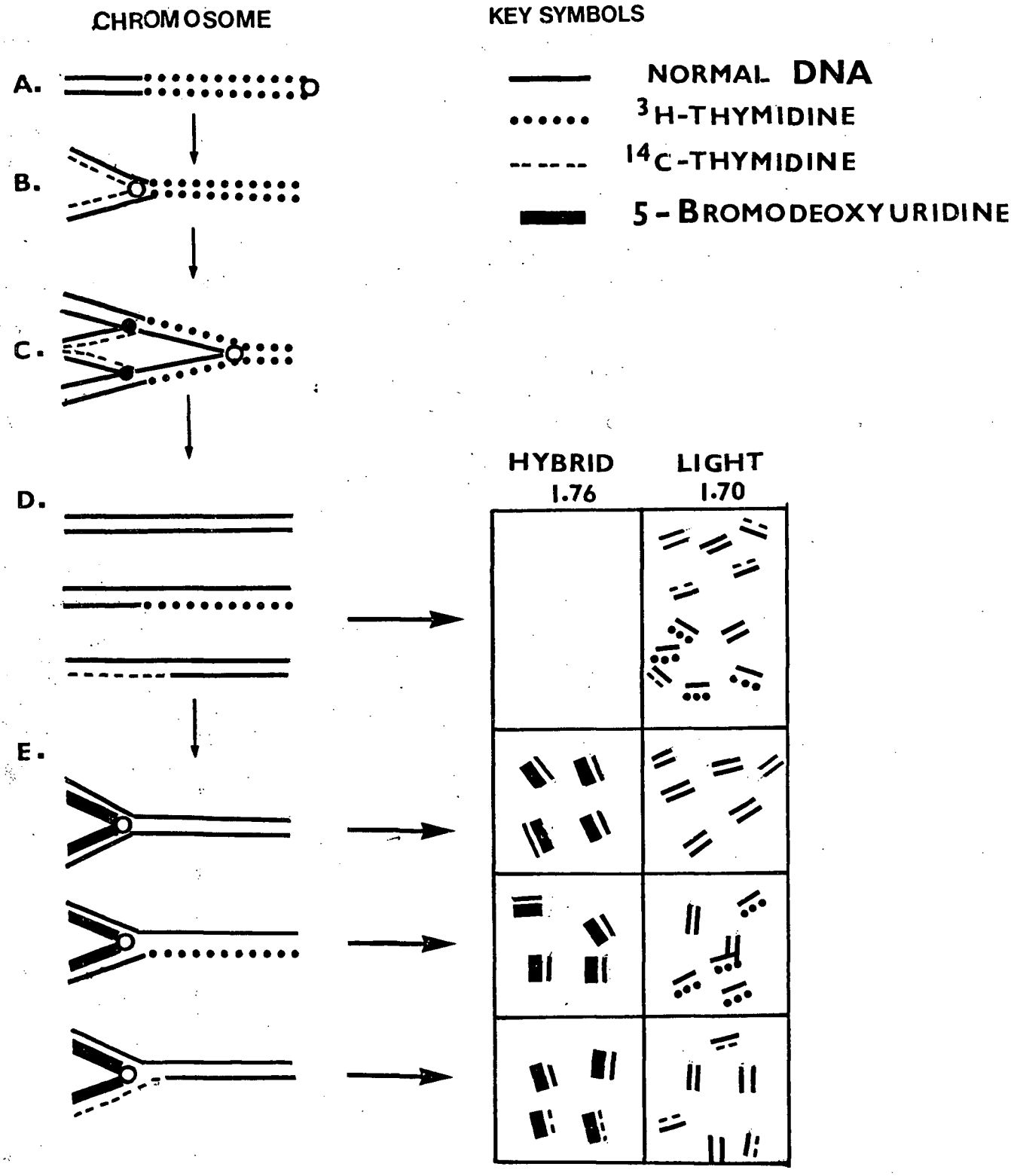
Figure 28. Construction of the model for DNA replication in CR34T83.

The construction is based on the Helmstetter and Cooper Model for DNA replication for cells grown under minimal and broth conditions. Time (t) needed for a growing point to traverse the genome is shown on the left and is expressed as a function of chromosome length. Open circles represent first forks and filled circles represent most recently established forks for broth medium. Triangles represent attachment point for DNA to the membrane (dots).

The sequence begins with random population of chromosomes at various stages of replication at 30 C. Upon a shift to 42 C, they will complete the initiated rounds. Three possible segregation patterns predicted for broth grown cells are shown. Finally, during the recovery at a 42 to 30 C shift down, new initiations are commenced in a synchronous fashion.

ture sensitive for the initiation of DNA replication, at recovery, the place of reinitiation would be at a specific site on the chromosome and (b) if the temperature sensitive step was blocking the initiation at the same place as the 150 µg per ml CAM step, then recovery from a 42 C pulse followed by a CAM block should identify the origin.

The prediction of a shift up on an exponential population from 30 to 42 C is shown in Figure 28. Chromosomes after 45 minutes, based on residual DNA synthesis data, align themselves and, at recovery, new rounds are initiated. If, at the time of a shift from 30 to 42 C, ^{3}H-TdR was added, the completion of rounds of replication would be labelled (Figure 29A). During the recovery, one could add a different label, for example ^{14}C-TdR, for a short time, withdraw the label, and chase with cold TdR (Figure 29B). Thus, the starts of rounds would be labelled. If cells were grown exponentially in unlabelled TdR for several generations and randomized, there would be a fixed number of cells whose chromosomes were labelled at starts and ends (Figure 29D). In order to identify the unique origin of recovery in DNA cycles, the cells could be realigned by a new temperature block (42 C) and, at recovery, be given a density label, for example 5-BrdUrd for a short time (Figure 29E). The results of density centrifugation of such DNA would yield a light (1.70 gm per cc) DNA and hybrid BrUra-T (1.76 gm per cc) DNA. If the place of the reinitiation after the second 42 C pulse was at the same region of the chromosome as the first one,


CHROMOSOME
KEY SYMBOLS
NORMAL DNA
^{3}H-THYMIDINE
^{14}C-THYMIDINE
5-BROMODEOXYURIDINE
A.
B.
C.
D.
E.
HYBRID
1.76
LIGHT
1.70

then the only label associated with the hybrid density material would be the label used at the origin (^{14}C-TdR). Similarly, if the cells were exposed to 150 µg per ml CAM at 30 C after the randomization from the first 42 C pulse, again they should align their chromosomes. If CAM was removed and cells were resuspended in fresh medium with 5-BrdUrd to allow new cycles to reinitiate, the density label should be associated with the ^{14}C-TdR label.

The rate of DNA synthesis decreases when BrUra is substituted for T. Approximately 120 minutes is required for a round of replication in B/r/1 at 37 C (Pierucci, 1969). As detected by the appearance of 1.80 density labelled ^{14}C-5-BrUra, a round of replication takes approximately 240 minutes at 30 C for E. coli 15 TAU or T83. For the experiments described above, a 50 minute BrdUrd pulse was used for the density labelling of the starts. Thus 20 percent of the DNA would be the maximum density labelled region.

The results from CsCl density gradient centrifugation of DNA replicated during recovery from a 42 C pulse or growth under 150 µg per ml CAM at 30 C are presented in Figures 30 and 31, respectively. The density gradient profiles have two main bands of hybrid and light density corresponding to 1.76 and 1.70 gm per cc buoyant densities. The characteristics of the labelled DNA found under these bands were analysed and the results are given in Table II. In either case, it was clear that 5-BrUra became preferentially associated with ^{14}C-labelled DNA and not ^{3}H-labelled DNA. The bulk of the ^{3}H-labelled

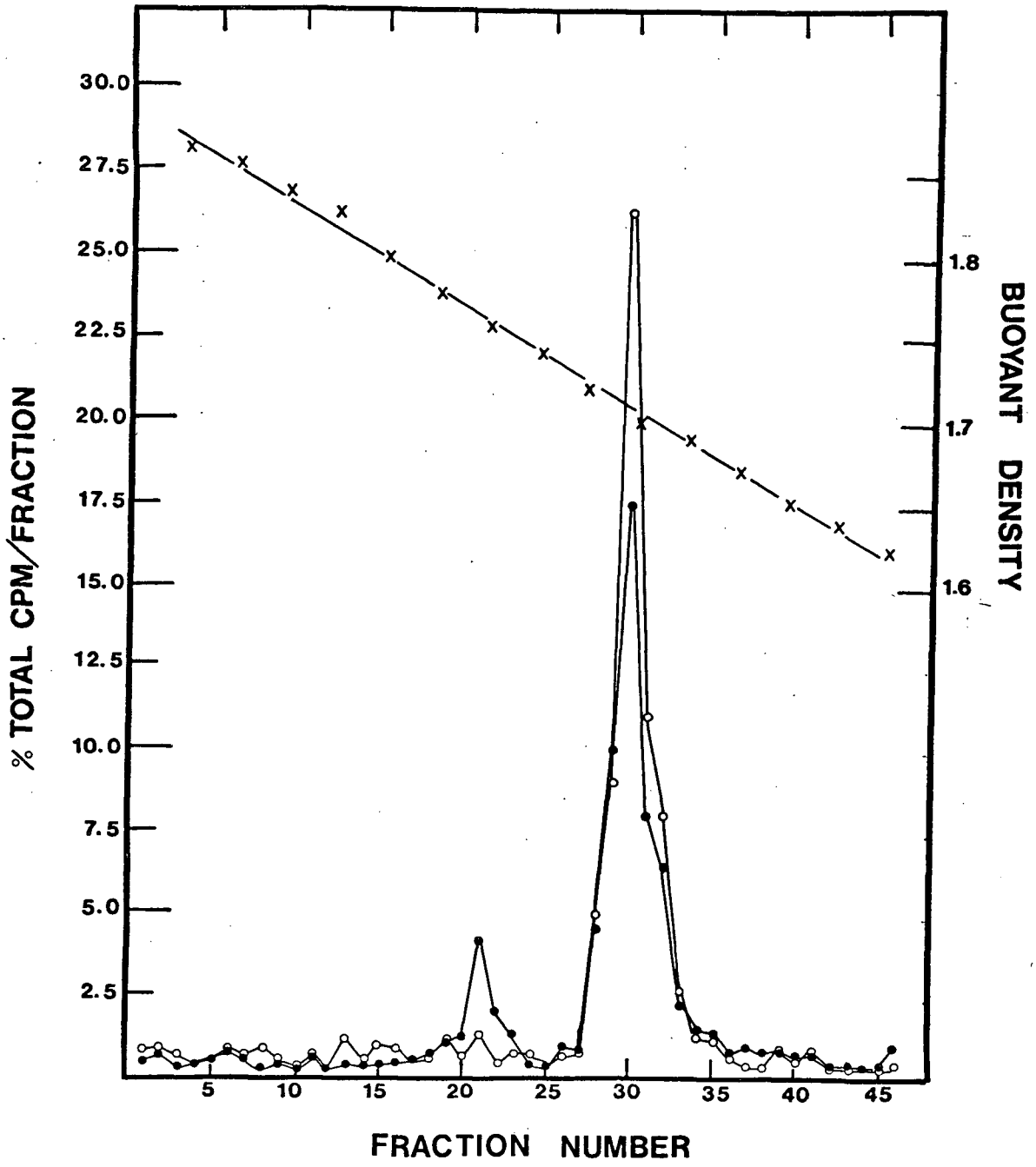

30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
% TOTAL CPM/FRACTION
1.8
1.7
1.6
BUOYANT DENSITY
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

Figure 29. Protocol for the experiments in Figures 30 and 31.

TREATMENT	PURPOSE
A. 42 C + ^{3}H-TdR	Label chromosome completions
B. 30 C - ^{3}H-TdR + ^{14}C-TdR	Label chromosome origins
C. 30 C - ^{14}C-TdR + cold TdR	Replicate chromosomes (randomization)
D. 1) 30 C + 150 µg per ml CAM + cold TdR or 2) 42 C - CAM + cold TdR	Complete chromosomes
E. 1) 30 C - CAM - TdR + 5-BrdUrd 2) 30 C - TdR + 5-BrdUrd	Reinitiate replication

TEST REPLICATION OF ^{3}H- OR ^{14}C-DNA BY INCORPORATION OF 5-BROMO-2-DEOXYURIDINE

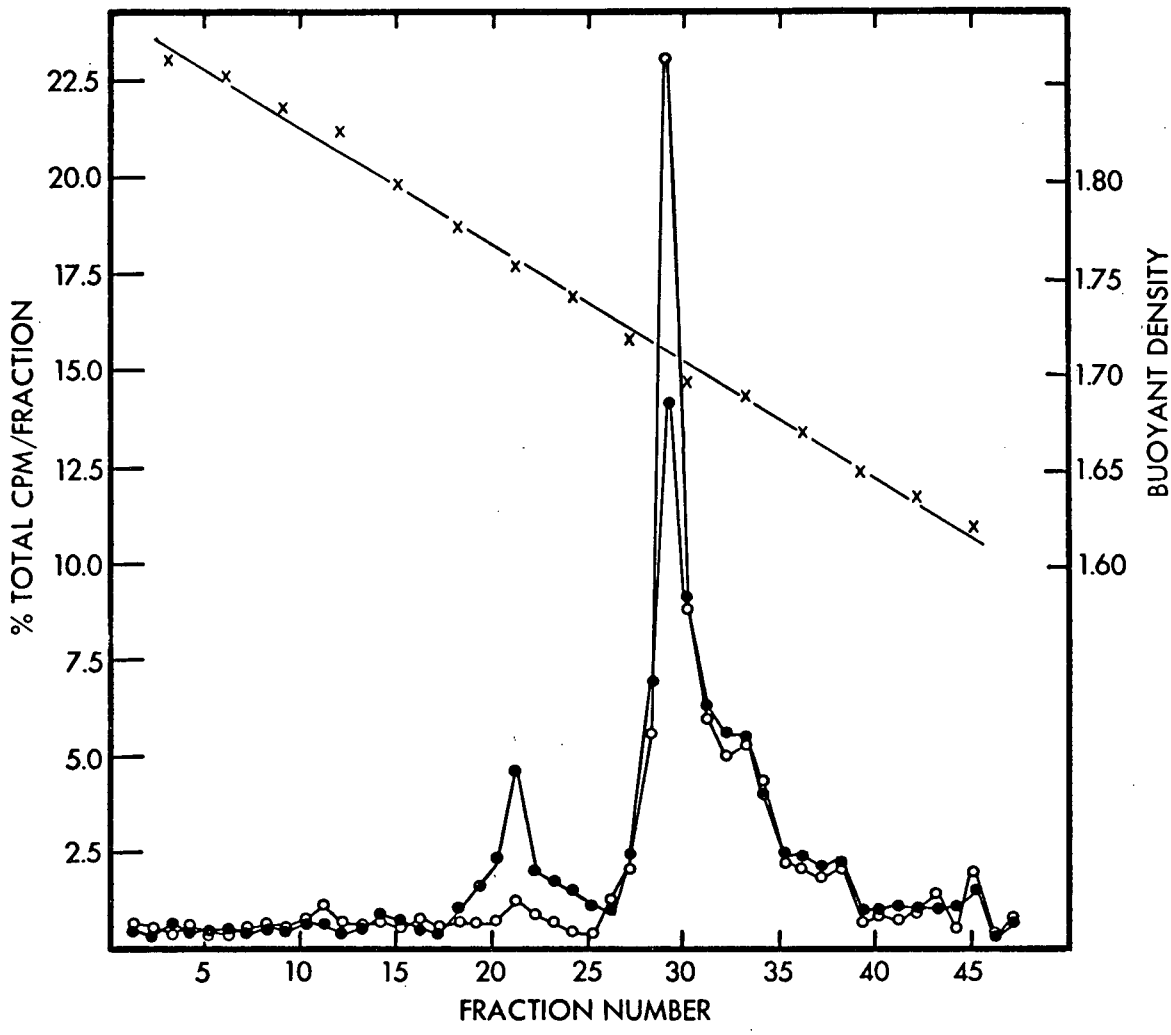


Figure 31. CaCl gradient analysis of the place of the reinitiation at recovery from growth in the presence of CAM.

The detailed experimental procedure is described under Materials and Methods, and in the text. The abscissa represents the fractions collected. The ordinate is CPM per fraction represented as the percent of the total radioactivity (10,420 CPM ^{14}C- and 6, 140 CPM ^{3}H) for ^{14}C-(●) and ^{3}H- (○) thymine. X———X represents buoyant density.

Figure 30. CsCl gradient analysis of the place of the reinitiation at recovery from growth at 42 C.

The detailed experimental procedure is described under Materials and Methods, and in the text. DNA was labelled with ^{3}H-TdR (0.045 µc per ml) with 2 µg per ml unlabelled TdR and 50 µg per ml AdR, during residual replication at 42 C. After 60 minutes, ^{3}H-TdR was removed. Cells were returned to 30 C and given ^{14}C-TdR (0.5 µc per ml) in the presence of 2 µg per ml unlabelled TdR and 50 µg per ml AdR, for 24 minutes. ^{14}C-TdR was removed and cells were grown for 5 generations in cold TdR medium, for randomization. Cells were then pulsed with a second 42 C incubation for 60 minutes. Subsequently, cells were filtered free of TdR and resuspended in fresh medium containing 10 µg per ml BrdUrd, for 50 minutes. DNA was extracted and run for 41 hours at 35,000 rpm in a CsCl density gradient. The abscissa represents fractions collected. The ordinate is CPM per fraction represented as a percent of the total radioactivity (9,800 CPM ^{14}C- and 5,900 CPM ^{3}H-thymine) for ^{14}C- (●) and ^{3}H- (O) thymine. X_____X represented buoyant density.

Table II. Characteristics of labelled DNA in T83: Buoyant densities and percentage of total DNA.

Conditions for re-initiation following chromosome alignment at 42 C	DNA labelled as ^{3}H or ^{14}C	% total label between fractions 18 to 35	Sum of radioactivity* in density band	
			Hybrid**	Light***
A. Recovery from 42 C.	^{3}H-thymine	76.66 (100%)	6.08 (8.0%)	70.58 (92.0%)
	^{14}C-thymine	67.45 (100%)	12.60 (18.6%)	54.85 (81.4%)
B. Recovery from CAM at 30 C.	^{3}H-thymine	67.95 (100%)	5.05 (7.5%)	62.90 (92.5%)
	^{14}C-thymine	71.43 (100%)	17.43 (24.4%)	54.00 (75.6%)

* Numbers represent percent of total CPM per fraction in the density band.

** Hybrid band = fractions between 1.780 and 1.7344 density, peak at 1.760.

*** Light band = fractions between 1.7344 and 1.670 density, peak at 1.706.

DNA, on the contrary, remained at the light density region. It must be borne in mind that, during 5-BrdUrd labelling of the DNA only $\frac{50}{240}$ minutes worth, or approximately 20 percent of the origin were density labelled. The ^{14}C-labelling of the initiation region was approximately $\frac{20}{60}$ minutes, or 33 percent, of the DNA. Thus 20 percent of the total ^{14}C-label would be present in the hybrid density band. It was evident that 17.5 percent from the 42 C initiation, and 12.6 percent from the CAM initiation banded in this region.

It was concluded that at recovery from 42 C, T83 reinitiated new rounds of chromosome replication at a specific region of the DNA. Furthermore, the place of the reinitiation subsequent to completion of rounds from 150 µg per ml CAM (Lark et al. 1963) was the same as that of the temperature sensitive step.

B. Comparison of the effect of inhibition of protein synthesis and the T83 mutation, on the initiation of DNA replication.

Studies on DNA replication under conditions in which gross protein synthesis is inhibited has indicated that initiator proteins are present in a cell in stoichiometric amounts, and additional protein synthesis is needed for normal reinitiation of new rounds (Lark, 1969b).

The results presented in the previous section pointed out that the temperature sensitive mutation affected the properties of a protein, the requirements of which were evidenced by 42 C incubation. Since CAM duplicated the effect of the 42 C, it was concluded that the temperature sensitive mutation involved initiator protein(s). Thus,

it was desirable to examine the separate effects of high and low levels of CAM on the process of replication in T83 and compare that to the 42 C effect.

The incorporation of ^{3}H-TdR into DNA at 30 C and 42 C in the presence or absence of CAM was examined and the results are presented in Figure 32. Cells at 30 C treated with 150 µg per ml CAM continued DNA replication at 4/10 the rate of the 30 C (Figure 32A). The rate is linear for 40 minutes after addition of CAM. When compared with the 42 C effect (Figure 32B), the rate of replication was accelerated, most probably due to the change in temperature, and the plateau was reached in approximately 50 minutes. However, the amount of DNA replicated was 40 to 50 percent of the residual level, and was comparable to the results of Maaløe and Hanawalt (1961). In the presence of CAM, this level was slightly less.

When T83 was treated with 25 µg per ml CAM at 30 C, DNA replication continued for at least two hours but at about 25 percent of the rate observed for the control cells. A plateau was reached by 110 minutes post treatment, which was equal to a 42 percent increase in the residual DNA synthesis (Figure 33). When the cells were shifted to 42 C and treated with CAM, DNA replication came to a halt after 20 minutes. This plateau lasted for approximately 50 minutes, after which there was a second burst in residual synthesis amounting to 19 percent. Final plateau value reached at 42 C thus added up to 46 percent residual DNA synthesis.

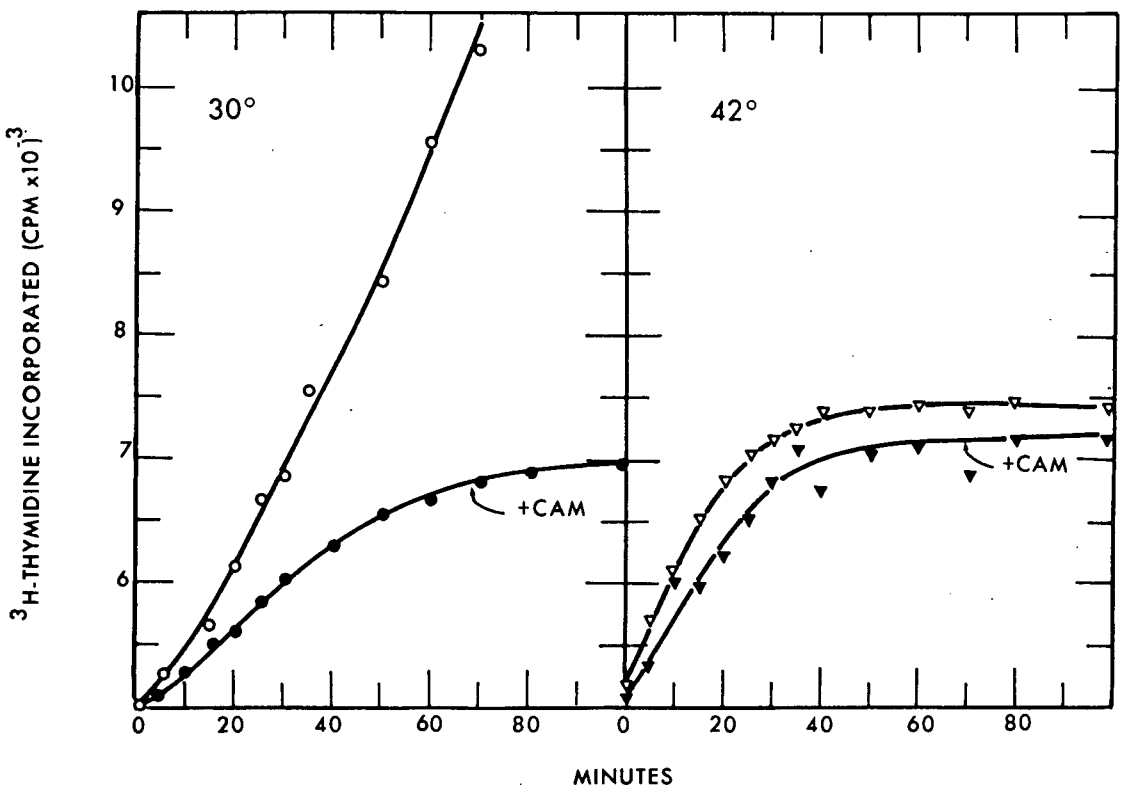


Figure 32. Comparison of the 42 C effect to that of addition of 150 µg per ml CAM.

Uptake of ^{3}H-methyl-TdR at 1 µc per ml, 20 µg per ml unlabelled TdR, and 50 µg per ml AdR, into the cold TCA insoluble fraction in T83, in the presence of 150 µg per ml CAM (filled symbols) and absence of CAM (empty symbols), at 30 C is shown in panel A and at 42 C in panel B.

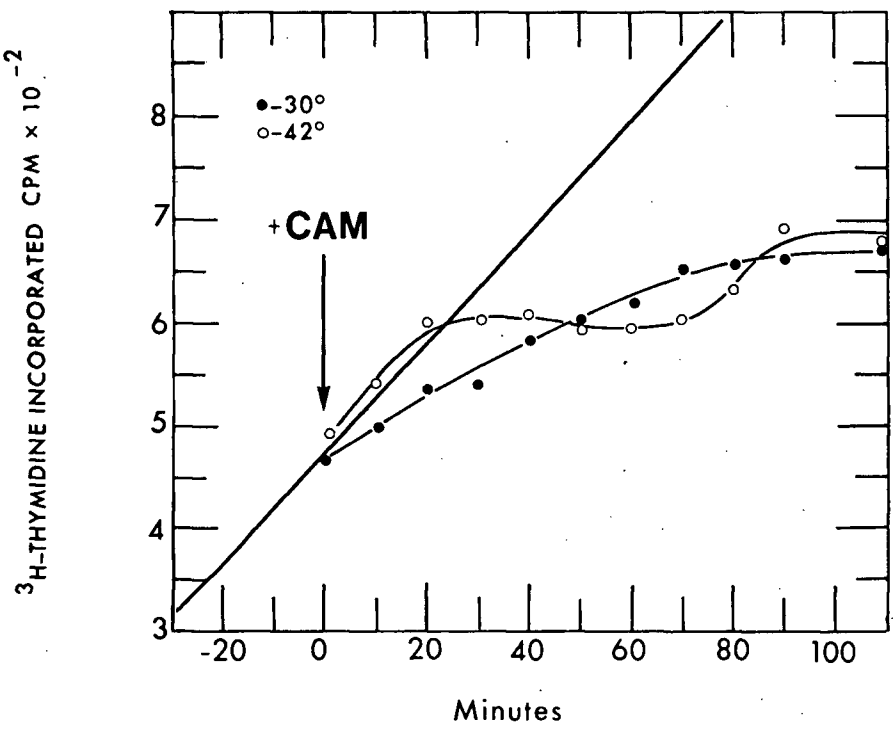


Figure 33. Effect of a low level of CAM on DNA replication in T83.

A culture of T83 grown in A and E medium in the presence of 0.5 µc per ml of ^{3}H-TdR, 20 µg per ml cold TdR, and 50 µg per ml AdR, was divided three ways. CAM (25 µg per ml) was added to subcultures at 30 C (○) and 42 C (●). Uptake of labelled TdR into cold TCA precipitable material was followed in the presence of CAM for 30 C (○) and 42 C (●). The diagonal solid line represents the 30 C control without CAM. CPM are plotted versus time.

C. Studies on stability of the growing point and the replication complex at the non-permissive condition.

In E. coli, chromosome replication could be inhibited by thymine starvation (Pritchard and Lark, 1964) or Nalidixic acid (Goss et al. 1965). In the case of thymine starvation, addition of thymine caused cells to induce new rounds of replication (premature initiations). Pritchard and Lark (1964) provided evidence that previously initiated chromosomes continued replication to completion.

Premature initiations were also induced following reversal of Nalidixic acid-induced inhibition of chromosome replication (Ward et al. 1970; Pritchard et al. 1969). It has also been observed that, when a pulse of Nalidixic acid was applied during conjugation in E. coli, chromosome transfer stopped (Hollom and Pritchard, 1965; Barbour, 1967). As to the effect of Nalidixic acid on transfer, two conclusions have been reached which disagree with one another. The results of Pritchard have been interpreted as a reversible inhibition of transfer, whereas results and conclusions of Bouck and Adelberg (1970) and Hane (1971) indicated commencement of a new round of transfer from the origin. The Ward and Glaser (1970) observations were similar to those of bacterial mating (Hane, 1971; Bouck and Adelberg, 1970), since they concluded that the replicating fork was destroyed by treatment of E. coli with Nalidixic acid for 30 minutes.

The Ward and Glaser model predicts that, when Nalidixic acid is added to a culture of T83 at 42 C for sufficient time to allow

complete inactivation of the fork, one should not observe any residual DNA synthesis subsequent to its removal. The result of such experiments are shown in Figure 34. NAL, when added, effectively blocked DNA replication. However, upon removal, the residual replication went to the same saturation level as the controls. Cells with NAL added at times shortly after shift up yielded much more residual DNA than those treated after longer incubation at 42 C. For example, 60% residual replication resulted for cells treated 5 minutes after shift up as compared to 10 to 14% for cells treated after 45 minutes at the non-permissive temperature. This indicated that cells held at 42 C approached completion of their rounds with time which resulted in very little residual division at the shift down. The possibility that T83 reinitiated prematurely subsequent to the removal of NAL has also been examined. Cells were incubated at 42 C in the presence of NAL and CAM to prevent initiator and general protein synthesis. Subsequently, after 30 minutes, cells were released into fresh medium lacking NAL but with or without CAM. The final residual DNA synthesis level was the same in either case. In B. subtilis strains, a degradation of 20 to 30 percent of the cell DNA in the replicating point region occured when cells were treated for 3.5 hours with NAL (Ramareddy and Reiter, 1969, 1970). In these experiments, however, not more than 35 minutes incubation was used and NAL at the level used, no detectable degradation of DNA was observed (Cook et al. 1966). Thus residual DNA synthesis in a total uptake of ^{3}H-TdR into prelabelled DNA

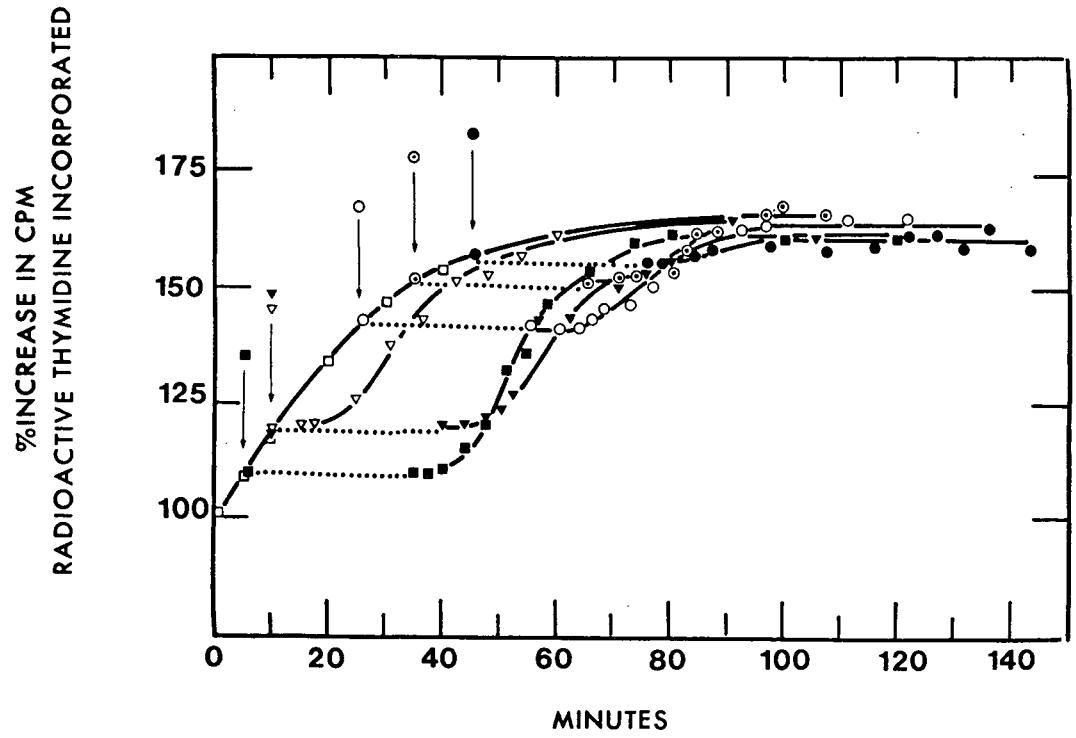


Figure 34. DNA synthesis in T83 at 42 C following an exposure to Nalidixic acid.

A culture of T83 grown at 30 C in A and E medium with 5 µc per ml ^{3}H-TdR, 20 µg per ml unlabelled thymidine (final concentration), and 50 µg per ml AdR, were shifted to 42 C at zero time (□). At 5 (■), 10 (▼), 25 (0), 35 (0), and 45 (●) minutes, as indicated by vertical arrows, portions from the control flask were removed and the cells were pulsed in NAL (10 µg per ml). After 30 minutes (dotted line) they were washed free of NAL with 42 C prewarmed medium and immediately resuspended in fresh NAL-free medium. Residual incorporation of radioactive label into the TCA insoluble fraction was followed during incubation at 42 C, and is represented as the percent increase in radioactivity (CPM). Open triangles (∇) represent results from a similar experiment, with a 5 minute pulse in NAL 10 minutes after the shift to 42 C.

could not be due to turnover of degraded label.

The Ward and Glaser model predicts that the initiator mutant should undergo 100 percent residual DNA synthesis when treated with NAL for 30 minutes at 30 C and then shifted to 42 C without NAL. In such an event, T83 would be aligned for only one round of DNA replication under the restrictive conditions. The results from such experiments are shown in Figure 35. Clearly, the Ward and Glaser model is in error, since the residual DNA synthesized remains at a level compatible with the notion of resumption of temprarily blocked cycles at 42 C and which approximates the control.

IV. Synthesis and decay of the division potential in CR34T83 studied by *in vivo* kinetics.

A. Time course of appearance of the division potential.

A closer examination of results from physiological requirements for recovery showed that, for cells kept at 42 C, there existed a potential for division which accumulated coincident with the rounds of DNA completed.

It was assumed that the expression of division potential already available at recovery, when net protein synthesis was inhibited, must be proportional to the amount of completed DNA cycles, cellular growth, and other rules governing the replication-segregation complex. Experiments were set to measure the amounts and kinetics of decay and synthesis of this division potential. In connection with this question,

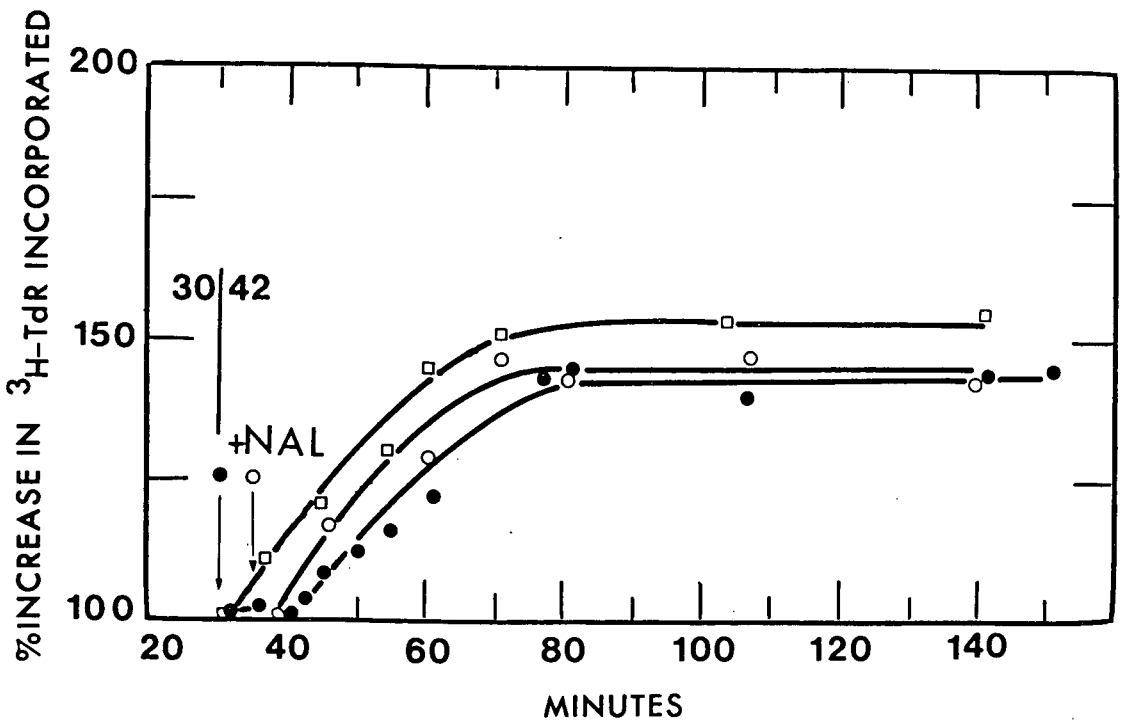


Figure 35. DNA replication in T83 at 42 C subsequent to a 30 minute Nalidixic acid treatment at 30 C.

A culture of T83, grown at 30 C in the presence of 0.8 µc per ml ^{3}H-TdR, 20 µg per ml unlabelled TdR, and 50 µg per ml AdR, was split into three parts. At zero time, one portion (●) was treated with NAL for 30 minutes at 30 C, washed free of NAL, and shifted to 42 C. A second portion (○) was treated with NAL at 5 minutes (25 minutes at 30 C), shifted to 42 C, and left in NAL for 5 minutes at 42 C. The NAL was removed and the incubation at 42 C was continued. A third portion (□) which served as control, was shifted to 42 C at the same time as the others but was not treated with NAL. Residual incorporation of radioactive label into the cold TCA insoluble fraction was followed during incubation at 42 C and is represented as the percent increase in radioactivity (CPM).

experiments were designed to determine: (a)whether the synthesis of the division potential was a continuous or discontinuous one; (b) the rate of the appearance and decay of the division potential and regulation of its synthesis during exponential growth at 30 C, at the non-permissive temperature (42 C), and during recovery from a 42 C pulse; (c) the measurement of the division potential messenger decay by the study of the kinetics of transcription using Rifampicin inhibition of RNA synthesis; (d) the comparison of the two effects; and (e) the measurement of the survival of mRNA in the temperature sensitive condition. These questions could be studied by means of pulses at 42 C in concert with a number of inhibitors acting on well-defined steps of the process of gene expression. Thus, not only could one measure the time needed for the elongation process of mRNA, but also the formation of poly-peptide chains and the half life of the coding capacity of the preformed mRNA.

B. The wave of expression of cell division at recovery in the absence of translation or transcription.

Figure 36 describes the results from experiments where the net protein synthesis of a culture of T83, pulsed for 15 to 60 minutes at 42 C, was inhibited at shift up and recovery. If the appearance of division potential was proportional to the length of the 42 C block, then one should see a gradual build-up. To have an accurate estimate of the time course of the appearance of the division potential before shift-up, each culture was divided into 30 subcultures which were treated

at one minute intervals at 42 C, for 15 minutes before and after shift down, with CAM at 150 µg per ml. During the next 200 minutes at 30 C, the cells went through some residual division, proportional to the potential they had for such division, and came to a halt. The plateau values for each culture were then plotted against the time when CAM was added. It must be remembered that, during the CAM inhibition of protein synthesis, the cells could express division only if they had the necessary proteins and other substrates for such an event. Obviously at 42 C, as longer intervals of protein synthesis, coincident with the residual DNA synthesis, was allowed, greater numbers of cell equivalents were generated. In fact, such was the case since the place where cell numbers intercept the temperature shift lines closely followed the generation time of the cell. However, after 45 minutes of incubation, despite the increase in cell mass, no more increase in cell numbers occured. Clearly, there must have been a stringent coupling between cell division and DNA replication which determined the number of cells generated under such conditions. The experiment represented in Figure 36 shows clearly that virtually in every case, upon incubation of cells at 42 C longer than 10 minutes, in the absence of protein synthesis, one obtained a complete loss of division potential of the cells.

The loss in the division potential at 42 C was clearly not due to a decreasing amount of messengers which remained translatable. In fact, when mRNA synthesis was inhibited by Rifampicin block, (see the

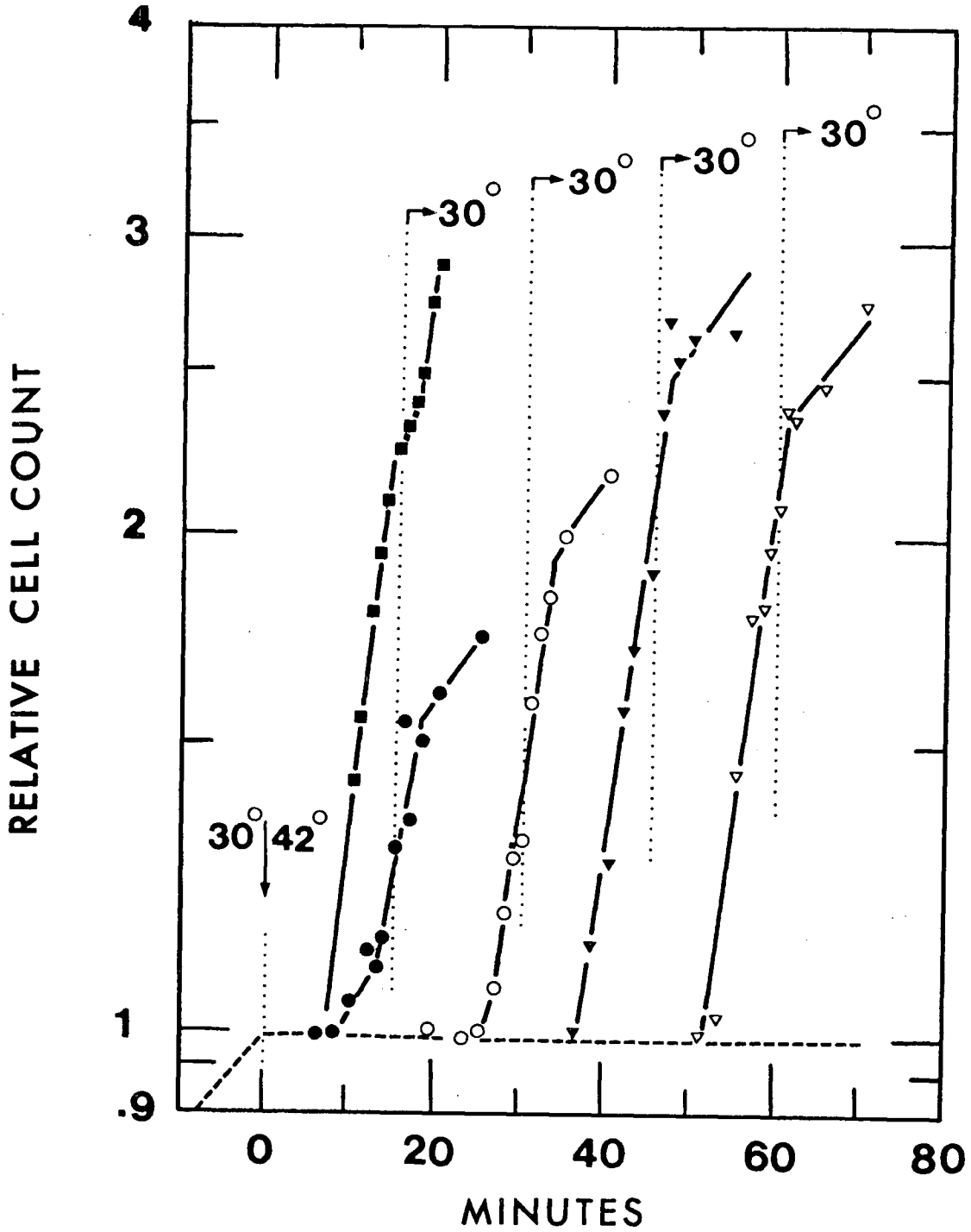


Figure 36. The wave of cell division during a transition from 42 to 30 C.

CR34T83 grown at 30 C to a density of 3.0×10^7 cells per ml was shifted to 42 C. After 15 (●), 30 (○), 45 (▼), and 60 (▽) minutes, cells were returned to 30 C. Fifteen minutes before and after the shift down to 30 C, aliquots were removed from the main flask treated with CAM (150 µg per ml) and were dispensed into new flasks. The final cell numbers arrived at after 200 minutes following the shift down are plotted against the time of addition of CAM. The vertical dotted lines represent the zero time for shift down to 30 C for each culture. The effect of Rifampicin at 10 µg per ml under identical conditions for the 15 minute pulse is represented by squares.

results of final cell numbers in the absence of transcription) (Figure 36), the number of cells generated was greater from that of CAM alone. The two antibiotics, though affecting macromolecular synthesis in a unique fashion, could have had different effects on cell division in this case. When E. coli were incubated in the presence of CAM, protein synthesis was arrested (Nomura and Watson, 1959) while RNA synthesis was continuous (Sells and Sayler, 1971). Rifampicin, by binding to the RNA polymerase, blocks the initiation of new mRNA chains. Currently, the data supporting the existence in E. coli of mRNA with widespread half-lives is meager. However, this possibility, although not precluded here, seems less likely to be the case. If, in fact, messenger RNA was inactivated at random, mRNA survival curves could be exponential functions and the rate of translation of any surviving mRNA, assumed to be constant, should also have been an exponential function. The semilogarithmic representation of percent residual division as a function of pulse of incubation at 42 C, in the absence of protein synthesis used in Figure 36, shows that this was the case indeed. The half life of the division potential calculated from this was very close to 1.4 minutes. In fact, the measurement of the residual division in the presence of Rifampicin in experiments similar to those of Figure 37, showed mRNA half life of 1.3 minutes. The Rifampicin concentration used inhibited well over 90 percent of radioactive uracil incorporation into RNA.

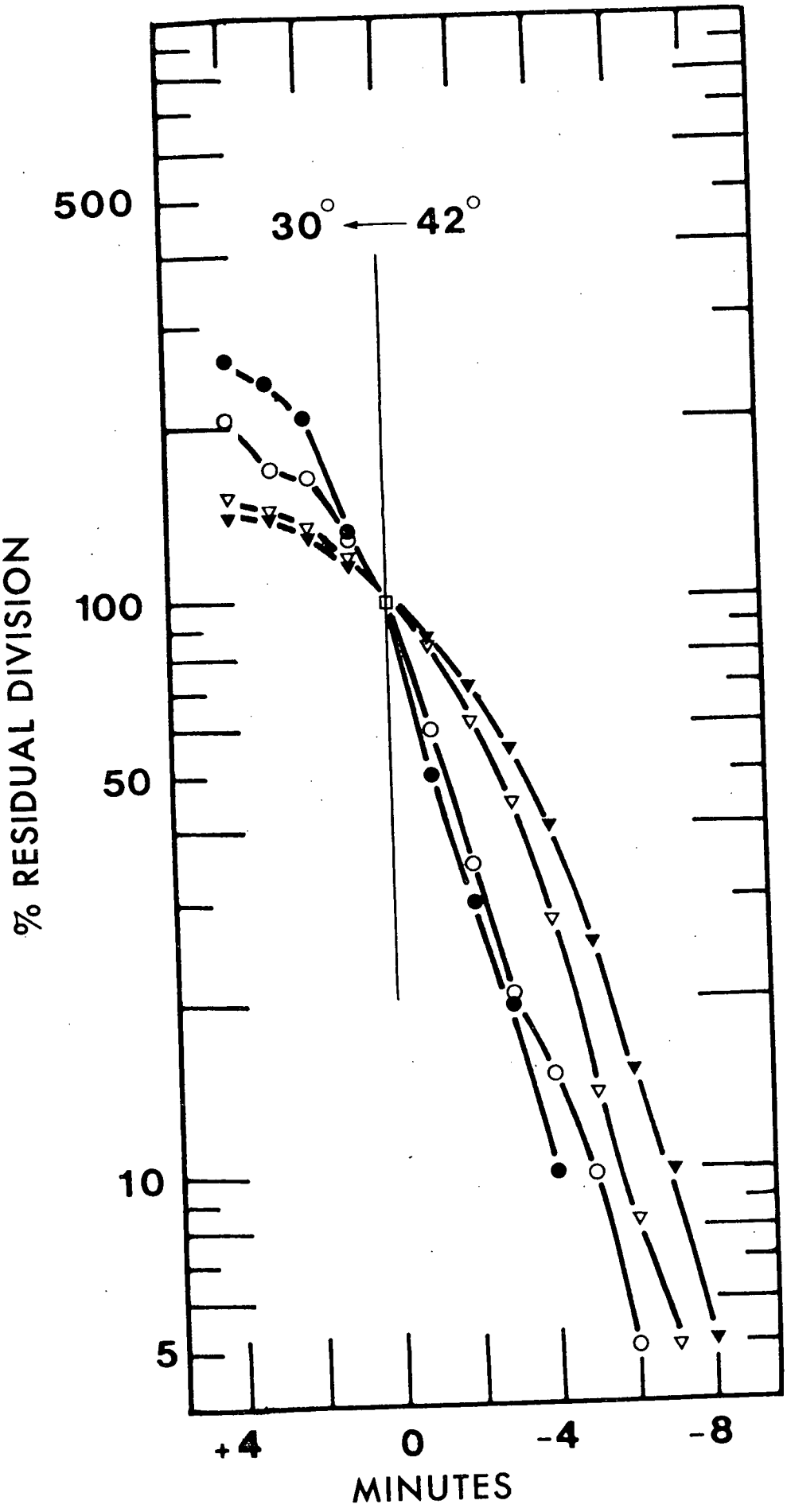

500
100
50
10
5
% RESIDUAL DIVISION
30° ← 42°
+4
0
-4
-8
MINUTES

Figure 37. Decay of cell division potential of CR34T83 at 42 C.

This graph represents the semilogarithmic plot of the data from the experiment represented in Figure 36 before zero time. The place of the intercept of wave curves with the shift axis was taken to be 100 percent residual division. The percent increase in cell number in the CAM treated samples from the intercept value is plotted against the time of addition of CAM prior to recovery

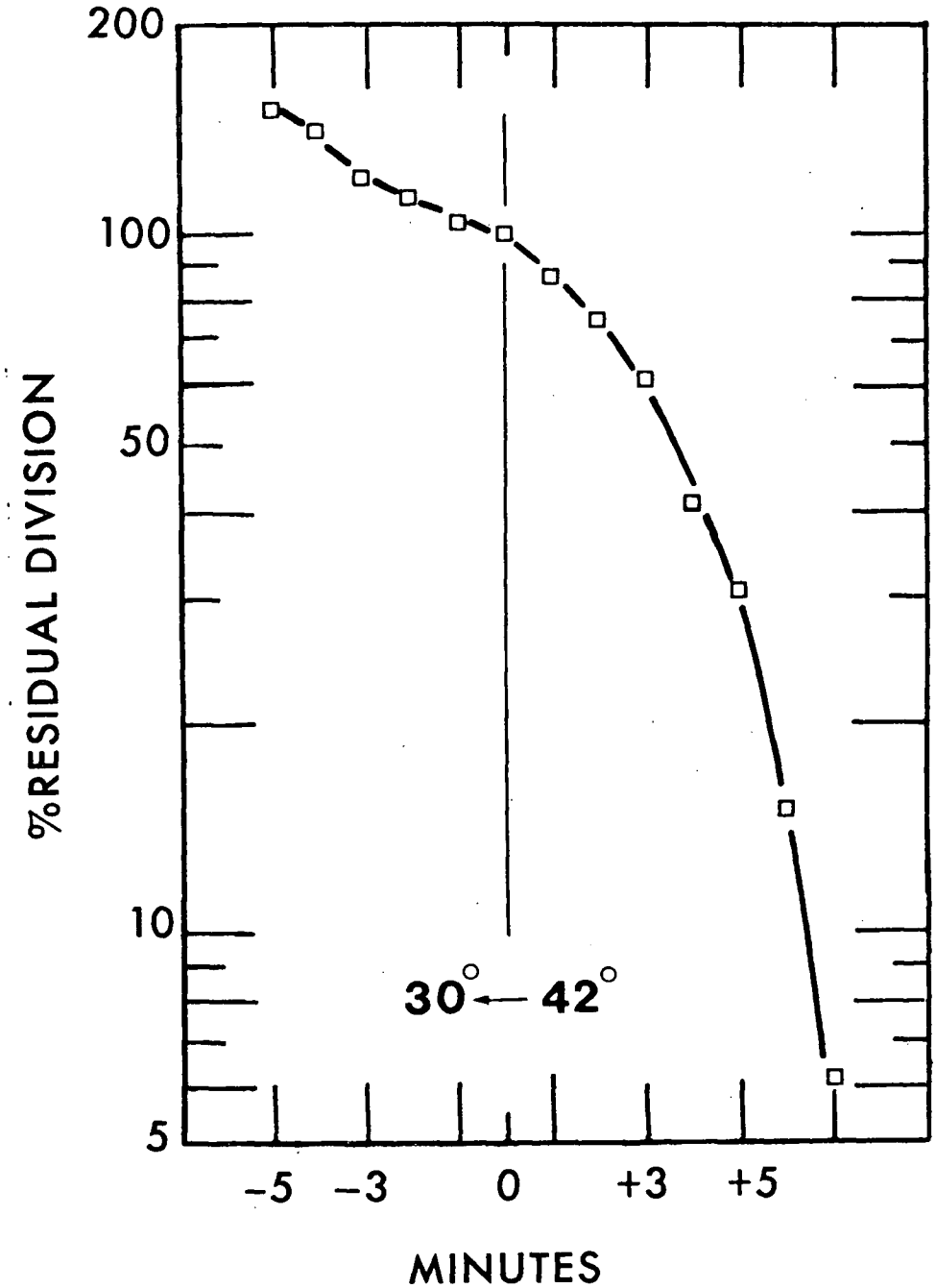


Figure 38. Decay of cell division potential of CR34T83 at 42 C.

This graph represents the semilogarithmic plot of the data from the experiment represented in Figure 36. The place of the intercept of wave curves with the shift axis was taken to be 100 percent residual division. The percent increase in cell number in the Rifampicin treated samples from the intercept value is plotted against the time of addition of Rifampicin prior to recovery.

V. Genetic analysis of the mutant CR34T83.

Pleiotropic mutants might, in theory, arise in two ways: (1) as a result of a single mutation in a structural gene whose product is involved in a complex; or (2) from two independent mutations. Several examples of the former type are known (Jones-Mortimer, 1968; Holland and Threfall, 1969; Demoss and Wagmar, 1965). It was, therefore, desirable to establish the genetic basis for the temperature sensitive mutation and the pleiotropy.

Preliminary mapping of T83 by conjugation had located the mutation in the Ilv region (Hirota et al. 1968). However, the correlation observed between temperature sensitive initiation and cell division led to a closer examination of the genetics of T83 and a more precise mapping by transduction. It was quite possible that there existed two mutations, one affecting DNA initiation and the other, membrane properties involved in division. The existence of multiple mutation was possible since NTG, which was known to specifically produce several mutations at the fork region (Ceredá-Olmedo et al. 1968), had been used in preparing this strain (Kohiyama et al. 1966). Three types of experiments were carried out: (1) the study of temperature resistant revertants (tr DnaA); (2) the study of T83 strains with ts DnaA Ilv^+ genotype; and (3) the construction of other strains of E. coli K12 which were isogenic for more than 98% of their chromosome, except for the one minute transduced portion of their chromosome, the Ilv DnaA segment.

A. Study of temperature resistant (tr) revertants.

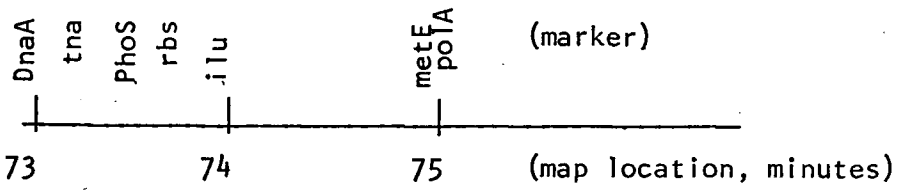
If the T83 mutation was to be a point mutation, then a reversion frequency to temperature resistance in the range of 10^{-5} to 10^{-7} should have been observed. On the other hand, if the pleiotropy was due to a double mutation, the frequency of the back mutation for two markers would have been much lower. The nature of the coupling of DNA to cellular growth needed for colony formation allowed the examination of these two possibilities.

T83 was grown overnight in A and E medium, diluted 1:100 and then 0.1 ml of this was inoculated into a number of test tubes or onto agar plates. After 24 hours of incubation at 42 C, growth was observed in the tubes and 20 to 24 colonies were formed on the plates. Each of these colonies were inoculated into liquid medium and were tested further for cell division and DNA replication. In general, the DNA replication was found to be normal and cell division continued at 42 C. The doubling time for tr strains, under 42 C growth conditions, was retarded to approximately 50 minutes. The reversion frequency calculated was approximately 2×10^{-6}, which is in good agreement with a single point mutation (Hayes, 1968).

B. Construction and analysis of CR34T83 Ilv^+ ts DnaA strains.

E. coli CR34T83 requires isoleucine and valine for growth. The order of the markers that are known to be in this region of the map are tna, the regulatory gene for tryptophanase production (Gartner and Riley, 1965), phoS, the regulatory gene for phosphatase (Echols et al. 1961),

Ilv, the isoleucine-valine gene cluster (Ramakrichnan and Adelberg, 1965), and DnaA (Hirota *et al.* 1968; 1970). Their order is shown below.



The linkage between Ilv^- and ts DnaA allowed for introduction of the Ilv^+ marker by transduction, and analysis of such transductants for the proximal ts DnaA marker.

In a transduction experiment, a lysate of generalized transducing phage P1kc (L4) was made on *E. coli* K12 AB 1157, which was Ilv^+. T83 was infected with the donor phage and the Ilv^+ transductants were tested under two selective conditions: (a) growth of T83 Ilv^+ transductants at 30 C on minimal agar plates lacking Ilv; and (b) growth of T83 Ilv^+ transductants on fully supplemented agar plates at 42 C. Table IV shows typical results. Fifty-six percent of the Ilv^+ T83 recipients had lost the temperature sensitivity. This could have resulted if the donor phage lysate carried a wild type allele for $DnaA^+$ and Ilv^+. The L4 strain of phage P1kc that was used here could transduce approximately 3 minutes worth of *E. coli* chromosome (Caro and Schnös, 1970). The longest distance for cotransduction of two markers was about 2.2 minutes of the map (Taylor and Trotter, 1967). The results agreed with the possibility for cotransduction of two markers, DnaA and Ilv, which were adjacent to one another.

Table III. The frequency of joint transductions of the Ilv and DnaA loci.

Donor	Recipient	Genotype Selected	Numbers of Transductants scored	Unselected Marker	
				Type	Number
AB 1157 ($DnaA^+$ Ilv^+)	CR34T83 (ts DnaA Ilv^-)	Ilv^+	70 (100%)	$DnaA^+$	39 (56%)
		$DnaA^+$	32 (100%)	Ilv^+	32 (100%)

Transductants were scored on minimal medium supplemented with all the requirements but for isoleucine and valine (selection for Ilv^+) at 30 C, or with full supplements (selection for $DnaA^+$) at 42 C. Experiments were performed as described in Materials and Methods.

C. Introduction of the T83 gene into *E. coli* K12 strains and the analysis of the pleiotropy.

One of the parental strains isolated in the previous section was used as donor in the experiments listed below. This strain, KG 776, was ts DnaA and had received the Ilv$^+$ marker from *E. coli* K12 AB 1175. Pi phage lysates were made on this strain and cells of other K12 strains were infected with this donor phage. Table IV shows the results of these experiments. The results clearly indicated that the ts DnaA was linked with the Ilv and tna markers. Of the ts DnaA transductants that were isolated, analysis of growth at 30 C and 42 C was performed. In 15 of the 42 ts DnaA strains checked, a halt in cellular division resulted upon incubation at 42 C from growth at 30 C. DNA replication patterns as examined by measurement of the rate of incorporation of ^{3}H-thymidine (since the strains were thy$^+$) into the cold TCA insoluble fraction, resembled that of T83.

Thus, in the *E. coli* K12 Ilv$^+$ transductants constructed, which had inherited 1 to 2 minutes of the T83 chromosome from the 73 to 74 minutes region of the map, the pleiotropy displayed must have been acquired via the transducing phage particle. With the controls performed for these experiments, the possibility of the existence of two separate mutations responsible for the pleiotropy, were ruled out. It remains to be seen whether only one or more cistrons in this segment of the chromosome are involved in the regulation of DNA initiation and in the other membrane properties. However, recent studies of

Table IV. Frequency of joint transductions of the Ilv and DnaA loci from *E. coli* T83.

Donor	Recipient	Genotype Selected	Number of Transductants scored	Unselected Marker	
				Type	Numbers
KG 776 (ilv^+), tna^+ $DnaA^{ts}$)	KG 146 (Ilv^-)	Ilv^+	60 (100%)	$DnaA^{ts}$	23 (40%)
				$DnaA^+$	34 (60%)
	KG 163 (Ilv^-, tna^-)	Ilv^+	67 (100%)	tna^+DnaA^+	39 (58%)
				tna^-DnaA^+	18 (27%)
				tna^+DnaA^{ts}	10 (15%)
				tna^-DnaA^{ts}	0 (0%)
	KG 166 (Ilv^-, tna^-, $phoS^-$)	Ilv^+	80 (100%)	tna^+DnaA^+	52 (65%)
				tna^-DnaA^+	19 (24%)
				tna^+DnaA^{ts}	9 (11%)
				tna^-DnaA^{ts}	0 (0%)

Transductants were scored on minimal medium supplemented with all the requirements but for isoleucine and valine (selection for Ilv^+) at 30 C or with full supplements (selection for $DnaA^+$) at 42 C. The *tna* marker was tested by the tryptophanase assay. Experiments were performed as described in Materials and Methods.

Hirota et al. (1970) on the genetics of both T46 and T83 have indicated that such possibilities are very unlikely.

DISCUSSION

The ts DnaA gene was shown to affect cellular division and continuation of DNA replication in E. coli CR34T83 at the non-permissive temperature. Support for this pleiotropic relationship was obtained by the genetic studies. The lesion was shown to be a point mutation, since wild type revertants which could grow normally at 42 C were isolated at a frequency of 2×10^{-6}, compatible with the reversion rate of a point mutation. The gene was co-transducible with the isoleucine-valine cluster and was linked to the structural gene for the enzyme tryptophanase. These results confirmed and extended the analysis of Hirota and co-workers (Hirota et al. 1968; 1970). Transductional analysis (Table IV) indicated that there was nothing but the T83 mutation which could be responsible for the pleiotropy, since when the 1 min segment of the chromosome carrying the DnaA gene was transduced to wild type E. coli K12 strains, a similar response was obtained.

The DnaA mutation specifically affected continuation of DNA replication at 42 C. Uptake of exogenous thymidine into the cell was normal (results not shown), yet DNA synthesis ceased after a short time at 42 C. These results agree with Kohiyama's original observations (Kohiyama et al. 1966). The amount of the residual DNA synthesized at 42 C was compatible with completion of rounds of replication in an exponential population, according to the Helmstetter and Cooper model.

The gradual decline in the DNA synthesis was not due to low nucleoside triphosphate pools. In fact, most of the nucleoside triphosphate pools accumulated during the incubation at 42 C (Figure 10 and 11). However, the dTTP pool did not expand significantly. Beacham and Pritchard's (1971) finding that dTTP pool was normally low in thy⁻ strains of E. coli, supports this observation. The presence of a low dTTP level in T83 at 42 C, could also be taken as supportive evidence for Werner's arguments (Werner, 1971) that dTTP is not the precursor for thymine, in DNA replication. This would explain why only dATP, dCTP and dGTP pools accumulated with the cessation of DNA synthesis; they were the true precursors of DNA synthesis. However, no other thymine containing precursors of DNA synthesis was measured in this work to further resolve this question. Since the replication of bacteriophages T4 and lambda were unaffected in T83 grown at 42 C (Kohiyama, 1968), it could be argued that there was no limitation in all four precursors.

Under exponential growth conditions at 30 C, addition of CAM at a concentration known to block reinitiation of new rounds of DNA replication (Lark and Ranger, 1969), resulted in residual DNA replication compatible with that of growth at 42 C. Addition of CAM at 150 µg per ml, at 42 C, did not alter the mode of residual DNA synthesis (Figure 32). However, at a level of 25 µg per ml of CAM, a biphasic residual DNA synthesis curve resulted (Figure 33). The reason for this deviation is not clear.

Initiated DNA replication forks in T83 were shown to be stable, at 42 C, to the effects of Nalidixic acid. Ward and Glaser (1970) have shown that treatment of E. coli B/r with Nalidixic acid for 30 minutes prevents the resumption of the replication of the affected forks. Accordingly, in T83, residual DNA synthesis after release from a Nalidixic acid block could occur only if old forks were stable. As shown in Figure 34, residual DNA synthesis at 42 C did occur subsequent to removal of the Nalidixic acid block. Lark (J. Urban, pers. commun.) has used a density labelling technique to follow DNA replication after Nalidixic acid treatment for 50 minutes. Their results agreed with this work indicating completion of previously initiated cycles. The evidence of Ward and Glaser, which is based on genetic approaches, cannot be refuted, yet it is not compatible with these findings.

In the study of an initiator type mutant, it is critical to establish the place of reinitiation of rounds of DNA replication at recovery at the permissive temperature after inhibition of DNA synthesis at the non-permissive temperature. Ideally, one should also demonstrate the relationship between the place for reinitiation and the origin of DNA replication. In the characterization of T83, Kohiyama (1968) examined the place for the termination of chromosome replication at 42 C. This was shown by ^{14}C-TdR labelling of the DNA in amino acid starved cells at 30 C. By returning the cells to amino acid supplemented media at 30 C and subsequently pulsing with ^{3}H-TdR, the

starts of rounds of replication were labelled. The pulse of ^{3}H-TdR, was chased with 600 µg per ml cold thymidine, and the cells were allowed to randomize their DNA cycles. Cells were shifted to 41 C and given 5-BrdUrd in the place of thymidine. The density gradient analysis of the extracted DNA showed that the hybrid density band contained 50% of the ^{14}C- and 25% of the ^{3}H-labelled DNA. Thus, it was concluded that T83 was blocked in the initiation of DNA cycles at 41 C, and the shift to 41 C with 5-BrdUrd allowed preferential replication at the terminus of rounds as defined by amino acid starvation and alignment of chromosomes. However, if this argument were to be true, no ^{3}H-TdR should have banded in the hybrid density region. There are several other objections to this experiment:

(1) The use of amino acid starvation to align chromosomes although widespread, is not very satisfactory. One problem associated with this method is that the capacity of the cells to replicate DNA declines during amino acid starvation (Doudney, 1966) and therefore, not all chromosomes complete rounds of replication (Caro and Berg, 1969).

(2) When labelling the starts of rounds of DNA replication, Kohiyama (1968) used low concentrations (0.5 µg per ml) of thymidine. At this level, the chromosome replication time at 37 C is equal to 97 minutes (Pritchard and Zaritsky, 1970). Thus, very little of the chromosome at initiation would have been labelled during the 8 minute pulse of ^{3}H-thymidine employed.

(3) In order to chase the ^{3}H-TdR, 600 µg per ml of unlabelled

TdR was used for 90 minutes. Addition of large quantities of thymidine to cells expands the deoxyribose-5-phosphate pools and retards the cellular respiration (Lomax and Greenberg, 1968). This could affect DNA replication, since the two processes are interdependent (Cairns and Denhardt, 1968).

(4) The 90 minutes of incubation at 30 C was not long enough to permit randomization of the population of chromosomes after their alignment. Thus, fortuitously, the ends of rounds could have been picked up after the shift into 5-BrdUrd medium.

In the experiments described in Figure 31, the place of resumption of DNA replication during recovery was established. The chromosomes were labelled at ends with ^{3}H-TdR and the starts with ^{14}C-TdR. The density-labelled DNA synthesized at recovery was always associated with the ^{14}C-label. Similarly, when chromosomes were aligned by chloramphenicol treatment (Figure 32), the density label was always found to be associated with the ^{14}C-label, that is, with the starts of rounds, as defined by the temperature block. Whether the place of resumption of DNA replication in T83 during recovery is the same as the origin of DNA replication (7 - 8 o'clock) on the E. coli genetic map, remains to be tested.

Inhibition of protein synthesis during the first 10 minutes of recovery of the permissive temperature, was shown to interfere with reinitiation of rounds of replication (Figures 22, 23, 24). If protein

synthesis was permitted, then cells returned to 30 C from a 42 C pulse resumed DNA replication (Figure 9). Cells returned to the permissive temperature, after a pulse at 42 C, showed a drop in the rates of DNA replication for some time. The reason for this behaviour remains unknown. One interpretation of the residual decline in the rate of replication at 30 C could be a need for the cells to recover from temperature inactivated initiator molecules by a "flush out" or dilution mechansim. Such a mechanism would predict a variable lag for the cells to acquire all the functional and active molecules needed for initiation replication of their chromosomes. In fact, the DNA replication resumed with a variable lag which was dependent upon the length of pulse at 42 C (Figure 9). However, with incubation at 42 C, longer than 15 minutes, the length of lag decreased, a result contrary to the predictions of the "flush out" mechanism. It is possible that complete inactivation of the initiator molecules in an exponential culture requires treatment longer than 10 minutes at 42 C; for pulses of 10 minutes or less, only a fraction of the population is affected, whereas with longer pulses, the whole population is hit.

Figure 9 could be interpreted according to the model of DNA replication proposed by Rosenberg et al. (1969). The enzyme needed for initiation of a round are always present in the cell, but a repressor of initiation interferes with their inauguration. Rosenberg et al. (1969) suggested that an antirepressor triggers initiation by its interaction with the repressor. The antirepressor, synthesized

at the end of every replication cycle, would trigger new cycles of replication. It could be assumed that in T83, the temperature sensitive element is the antirepressor. Thus, in cells kept at 42 C for a long time, all chromosomes would be at the end of their rounds, and would initiate at 30 C immediately upon the acquisition of the antirepressor. With shorter pulses at 42 C, fewer ends of rounds are produced and hence, longer delays at recovery would be required. While this explains the results for temperature pulses for longer than 10 minutes, there is no obvious explanation for the short lag observed with the 10 minute pulse (Figure 9).

It was shown (Figure 2) that, once shifted to 42 C, cell division ceased and filamentous growth resulted in E. coli CR34T83. Pantoyl lactone induces other filamentous strains of E. coli to divide (Adler and Hardigree, 1965), but had no effect on T83 growing as filaments at 42 C (results not shown). Similarly, division induction of temperature sensitive filaments by addition of salt (Richard and Hirota, 1969) was ineffective in T83 at 42 C (results not shown).

During filamentous growth, the replicated chromosomes segregated normally (results not shown) and, upon a return to 30 C, filaments divided into normal sized cells (Figure 8). Cell division during recovery at 30 C in cultures that were blocked in chromosome replication by Nalidixic acid (Figure 14) at 42 C, substantiated that division was dependent upon completed chromosome cycles. An appreciable change in residual cell division was observed when Nalidixic acid was added

after 40 to 50 minutes recovery at 30 C (Figure 14B). Thus, at some time after shift from 42 C to 30 C, new cycles of replication were initiated which went to completion approximately 50 minutes after the shift.

If synthesis of RNA and protein were blocked at the time of a shift from 42 C to 30 C, residual cell division at recovery was observed. Inhibition of total RNA synthesis at the shift to 30 C after 45 minute incubation at 42 C, resulted in a four-fold increase in cell numbers whereas a doubling in cell number occurred upon addition of chloramphenicol or Nalidixic acid (Figures 14, 19, 20). Under the same conditions (Figures 15, 16), the ability to complete two doublings probably was due to translation of all available mRNAs in the cells. Cell division at recovery was proportional to the number of "unit cells" (Donachie and Begg, 1970) generated at 42 C. Since the maximum number of normal cell equivalents generated at 42 C, would be limited by the numbers of chromosomal copies available after completion of rounds of replication, final cell number produced by residual cell division should plateau for pulses at 42 C longer than 45 minutes. This was demonstrated in Figure 21.

The activity of the initiator molecule was not regained at recovery in the presence of chloramphenicol. Results presented (see section II. C) classify the behaviour of the initiator molecule at 42 C as denatured and inactive (type a transition). Thus new protein synthesis is obligatory for new activity during the 30 C recovery period.

However, the transition for the division element in the complex was that of native and active at 30 C to native but inactive at 42 C (type b transition). The expression of cellular division during recovery at 30 C based on "potential cell equivalents", ie., DNA to cell mass, did not require active protein synthesis as was shown by the addition of chloramphenicol at the time of the shift from 42 C to 30 C. This indicated that the transition for the division element is reversible once returned to the permissive temperature, and does not require synthesis of new proteins.

The occurence of pleiotropic effects in conditional lethal mutants is not without precedent. Chiu and Greenberg (1968) described a mutation in the gene for dCMP-hydroxymethylase that rendered bacteriophage T4 DNA synthesis inoperative at 42 C but not at 28 C. dCMP-HMase is one of the early enzymes in T4-infected E. coli and controls the tetrahydrofolate-dependent conversion of dCMP to 5-hydroxymethyl-dCMP, and is coded by the T4 gene 42. T4 DNA polymerase (gene 43) was shown to interact with the dCMP-HMase and a third component before viral DNA synthesis could commence. Gene 42 and 43 proteins were unable to operate in a double shift experiment (28-42-28-42) when the synthesis of the third component, which was made between 5 and 8 minutes after recovery, was blocked by chloramphenicol. The integrity of the new complex was an obligatory requirement for DNA replication.

It is quite likely that the complex events that are known to be associated with DNA-membrane attachment and division at the septum region, involve protein-DNA and protein-protein binding reactions of

a cooperative nature. In any event, the pleiotropic relation observed can best be explained in terms of a multi-enzyme complex localized at the cell membrane, involved in DNA replication and cell division.

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