GENETIC AND BIOCHEMICAL STUDIES ON THE CONTROL OF DNA REPLICATION IN BACTERIA

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C O N T E N T S

I N T R O D U C T I O N

A Review of Chromosome Structure and Replication in Bacteria

The synthesis of bacterial DNA *in vivo* has been studied intensively in recent years. Although our understanding of this complex process is far from complete, it can be summarised as follows:

(1) Structure of the bacterial chromosome

The genome of *Escherichia coli* has been shown to consist of a single, looped linkage group (Jacob & Wollman, 1961; Taylor & Adelberg, 1961). Reports on the genetic maps of *E.coli* and *Salmonella typhimurium* have been recently published by Taylor & Trotter (1967) and Sanderson (1967) respectively. Evidence suggests that the chromosome of these and other bacteria is a continuous closed loop, consisting largely, if not entirely, of double-stranded DNA. Thus autoradiography of labelled chromosomes from *E.coli* has demonstrated such a structure, with a measured length corresponding closely to the length of DNA calculated from the estimated weight of DNA present in a single non-replicating nucleoid (Cairns, 1963a,b). Electron micrographs of DNA released from *Micrococcus lysodeikticus* protoplasts (Kleinschmidt *et al*, 1961) show a tangled skein with no free ends, the diameter of the threads being equal to that proposed for double-stranded DNA (Watson & Crick, 1953).

(2) The mode of chromosome replication

The data of Meselson & Stahl (1958) show that the mode of DNA replication is semi-conservative, as proposed by Watson & Crick (1953). Furthermore, the data demonstrated an orderly sequence of replication, in that isolated DNA fragments which had replicated twice were not found until all the fragments had replicated once. Using a different technique Lark, Repko & Hoffman (1963) also demonstrated sequential replication of the chromosome. They pulse-labelled an exponentially-growing culture of E.coli $15T^-$ with ^{3}H-thymine and then transferred it to medium containing 5-bromouracil (BU) in place of thymine. Little of the ^{3}H-labelled DNA became hybrid in density (ie. was replicated) until most of the DNA had replicated. Some of the ^{3}H-labelled DNA was, however, replicated earlier than would be expected by sequential replication. Lark et al (1963) suggested that this was due to some heterogeneity in the rate of DNA replication in different cells. Using ^{13}C- and ^{15}N-labels, Eberle (1968) showed that the pattern of replication observed by Lark et al (1963) was not an artifact of the use of BU.

Autoradiographs of replicating E.coli chromosomes (Cairns, 1963a,b) revealing only one replication fork per chromosome, suggested that replication is sequential because it is carried out by means of a single replication fork traversing the entire length of the chromosome. They also revealed that both newly-replicated DNA strands remain attached to the position where replication

started, thus forming a loop within the chromosomal loop. Such evidence for a single replication fork per chromosome may be misleading, however, because it is open to subjective or experimental bias in the selection of labelled chromosomes for examination. Bonhoeffer & Gierer (1963) came to the same conclusion, however, by an entirely different method which avoided this disadvantage. They argued that the average density of sheared, BU-pulsed-labelled DNA should lie half way between that of light and hybrid DNA, when the length of DNA synthesized during the BU-pulse is equal to the average length of the sheared DNA. From the density distribution of a BU-pulse-labelled section of DNA they were thus able to calculate its length (knowing the length of the sheared fragments from their sedimentation coefficient). This corresponded closely to the length of DNA synthesized during the time of the pulse only if one assumes that a single replication fork was active throughout this period.

(3) The origin and polarity of replication

The experiments of Yoshikawa & Sueoka (1963a,b) on transformation in Bacillus subtilis indicate that replication, in this species, starts from a unique genetic site on the chromosome (the "origin"), and proceeds in a unique direction. Such a scheme predicts that during the exponential growth of a culture (if replication is proceeding by means of a single replication fork per chromosome) a marker just past the origin will be present with

twice the frequency of one just before it, while markers in between will occur with an intermediate frequency which is an exponential function of their distance from the origin (Sueoka & Yoshikawa, 1965). They used the transformation system of B.subtilis to determine the relative titre of ten different markers. They calculated, for each marker, the ratio of the transformation frequency obtained with DNA extracted from an exponentially-growing culture, to the frequency obtained with DNA from a stationary culture. This ratio was used to correct for any intrinsic differences in the efficiency of transformation of different markers. This correction is valid if their assumption is valid, that in stationary-phase cultures, most chromosomes have no replication fork, all markers being present with equal frequency. As predicted by them, these "transformation ratios" varied between 2 and 1. Using these results, they constructed a map of the ten markers. The validity of this approach was strengthened by their finding that in two cases, markers close together on their map were linked in transformation.

Nagata (1963) concluded that replication starts from a unique origin and proceeds in a fixed direction also in two Hfr strains of E.coli K12. His experimental approach depended on the assumption that the relative number of copies of prophage λ and 424 in a doubly-lysogenic strain at the time of induction, affects the λ/424 progeny ratio. Using synchronized cultures, he found a doubling or halving of this ratio at times consistent with the

origin being at the position of F integration, and replication proceeding sequentially in the opposite direction to that of gene transfer during conjugation. Finding no such changes using F^- strains, he concluded that they do not have a unique site for the origin, or they switch their polarity of replication from an origin, at random. These conclusions are not accepted by many workers, however, as his starting assumption has not been established.

In contrast to Nagata's model, Jacob, Brenner & Cuzin (1963) have proposed that all strains, whether Hfr or F^-, have a unique origin during vegetative growth, and that only during conjugation is replication initiated at the position of F attachment in Hfr strains. Recent work has unfolded a more complicated situation.

The approach of Berg & Caro (1967) to this problem in E.coli was analogous to that of Yoshikawa & Sueoka (1963a,b) described above. As E.coli is not transformable, they used generalised P1 transduction to assay relative gene frequencies in exponentially-growing cultures. P1 will not grow in stationary cultures, so they compared the frequencies obtained from three isogenic Hfr strains. They found no significant differences, and thus concluded that the site of integration of the F factor had no effect on the origin or direction of chromosome replication.

Wolf, Newman & Glaser (1968) also used this assay technique, but after selecting, by density-gradient ultra-centrifugation, DNA regions that had been labelled with BU during, or

immediately after, amino acid starvation (which was thought to permit the completion of cycles of replication to the "terminus", see next section). Thus they assayed the markers in chromosome "ends" and "beginnings" respectively. From relative gene frequencies, they concluded that one Hfr and two F^- strains of E.coli K12 have their terminus and origin in the same map position, viz, in the region between 54 and 70 minutes (Taylor & Trotter, 1967), and a clockwise direction of replication. Similar experiments performed by Abe & Tomizawa (1967) on two Hfr strains, suggest that their origins are at a similar map position (between 40 and 55 minutes), and that they have the same orientation of replication. On the other hand, Wolf et al (1968) found that one Hfr strain had a different location for its terminus-origin, viz, in the same region as the F factor. In contrast to the findings of Nagata (1963) however, its direction of replication was the same as its direction of gene transfer during conjugation. Some unpublished evidence of of Vielmetter & Messer (1964) was quoted by Berg & Caro (1967) as also showing the origin of Hfr strains to be located in the region of the F factor.

The polarity of chromosome replication has also been investigated by measuring the induction of mutations, during synchronised cell growth, by 2-aminopurine (Ryan & Cetrulo, 1963), nitrosoguanidine (Altenberg, 1966; Cerda-Olmedo, Hanawalt & Guerola, 1968) or ultraviolet light (Stonehill & Hutchison, 1966). The patterns of mutagenesis observed are consistent with the

hypothesis that the replication fork region is much more sensitive to mutagenesis than the rest of the chromosome (Cerda-Olmedo et al, 1968). An increase in mutation frequency is observed for a particular marker at a particular time after the onset of a DNA replication cycle, the time being different for different markers. This allows the construction of a marker "mutation" map. From a comparison between mutation and genetic maps, Cerda-Olmedo et al (1968) concluded that the origin of E.coli 15T$^-$, a K12 Hfr and a K12 F$^-$ strain, was situated at a map position of about 50 minutes, and replication travelled clockwise in all three strains.

The rate of synthesis of an enzyme on induction has been reported to be related to the number of copies of the corresponding structural gene which the cell carries (Jacob, Schaeffer & Wollman, 1960; Garen & Garen, 1963; Pittard & Ramakrishnan, 1964; Donachie, 1964). This has been suggested to be the reason for the abrupt changes of enzyme inducibility noted in cultures growing synchronously (Masters, Kuempel & Pardee, 1964; Kuempel, Masters & Pardee, 1965; Donachie & Masters, 1966; Helmstetter, 1968). The sequential nature of these changes is consistent with replication being initiated from a fixed origin and proceeding sequentially, at least in the majority of cells. This conclusion was drawn for three E.coli F$^-$ strains (Donachie & Masters, 1966) and for E.coli B/r by Helmstetter (1968). Helmstetter estimated the origin to be at a map position of about 60 minutes, and concluded that

replication proceeds clockwise, as did Cutler & Evans (1967) from their experiments.

The conclusions that emerge from the data in this section is that F^- and most Hfr strains of E.coli, so far studied, have a fixed origin in the 50-60 minutes region of the standard conjugation map (Taylor & Trotter, 1967) and a clockwise direction of replication, while some Hfr strains may initiate replication from the position of F integration.

(4) The effect of amino acid starvation on replication

Maaløe & Hanawalt (1961) proposed that in the absence of amino acids, cycles of DNA replication in progress are completed, but the initiation of new ones is prevented (because the starved cells are unable to produce a protein thought to be necessary for the initiation process). DNA synthesis is apparently continuous throughout the division cycle of E.coli 15TAU$^-$ growing with glucose as a carbon source. If one assumes that this is mediated by a single replication fork moving with a constant velocity along each chromosome, then their postulate predicts a 39% increment of DNA synthesized during amino acid starvation. In agreement with the earlier observations of Pardee & Prestidge (1956), they obtained increments of 40-55% under these conditions. Their minimum is close to the theoretical value, and higher increments they ascribed to a fraction of those cells first completing a cycle of replication, entering and completing a second cycle. Their observations on the

acquisition of immunity to thymineless death throughout amino acid starvation (Maaløe & Hanawalt, 1961; Hanawalt et al, 1961) are also consistent with their hypothesis, if one assumes that an immune cell is one that has completed a cycle of replication.

The biophysical experiments of Lark, Repko & Hoffman (1963) support Maaløe & Hanawalt's hypothesis. They starved an auxotrophic 15T⁻ culture of amino acids for 60 minutes, then replaced them and gave a pulse of radioactive thymine. Normal growth was allowed for several generations, then a second period of amino acid starvation was imposed. The culture was now transferred to growth medium containing BU in place of thymine. Ultracentrifuge analysis of the hybrid density DNA showed that the radioactive section of the DNA was replicated preferentially rather than at random. This shows there is a point on the chromosome - the terminus - at which replication ceases after a period of amino acid starvation, and which is "remembered" by the cell with a high probability throughout several generations of growth.

The data of Wolf, Newman & Glaser described in section (3) strongly support this conclusion. They showed further that the terminus reached during amino acid starvation is a specific genetic site on the chromosome, rather than a locus specific only to a particular cell line over several generations. This site is probably identical to the terminus reached during phenethyl alcohol treatment (Abe & Tomizawa, 1967). The synchronization technique used by Cerda-Olmedo et al (1968) involved amino acid starvation, thus

their finding of a specific genetic origin also supports Maaløe & Hanawalt's hypothesis.

It was shown by Friesen & Maaløe (1965) that the effect of amino acid starvation on DNA synthesis is the same in strains with stringent or relaxed control of RNA synthesis (the "relaxed" mutation permits RNA synthesis in the absence of amino acids, Stent & Brenner, 1961). Also they noted that the addition of chloramphenicol to a culture during starvation had no effect on the increment of DNA synthesised, although this permits RNA synthesis and, it is thought, amino acid accumulation from normal protein turnover. Thus they concluded that amino acids do not control DNA synthesis in an analogous way to stringent RNA control, but that the inhibition of protein, rather than RNA, synthesis is responsible for the amino acid starvation effect. Consistent with this hypothesis is the isolation o f a temperature-sensitive mutant which apparently cannot initiate new cycles of replication at high temperatures, ie. a shifted culture formed filamentous cells and synthesized about a 40% increment of DNA, although both protein and RNA synthesis continued for a considerable period (Kohiyama, Lanfrom, Brenner & Jacob, 1963).

(5) The rate of DNA synthesis at a replication fork

Evidence has accumulated which suggests that the rate of DNA synthesis at each replication fork is constant throughout the replication cycle, and is more or less independent of growth rate

in all normal balanced cultures (Maaløe & Kjeldgaard, 1966). Consistent with the first part of this hypothesis, Cairns (1963a,b) noted that the length of the DNA strands labelled, was proportional to the period of growth in ^{3}H-thymidine medium. Also, the kinetics of replication observed by Lark et al (1963) are more consistent with linear replication per fork than with an accelerating rate throughout the cycle, as proposed by Abbo & Pardee (1960). This latter conclusion was possibly due to inadequate synchronization of the culture.

The introduction of good synchronization techniques has largely overcome this problem. Thus Clark & Maaløe (1967) measured the rate of radioactive thymidine incorporation into DNA during short periods (less than 5 minutes), in a culture synchronized by the method of Helmstetter & Cummings (1963, 1964). The rate of DNA synthesis, in cultures growing at several rates, was substantially constant throughout a large part of the cycle, in agreement with their hypothesis. Increases of this rate occurred discontinuously at a specific time in the division cycle. Clark & Maaløe (1967) provided evidence that this was due to the initiation of a new cycle of replication at this time, ie. an increase in the number of replication forks took place, rather than an increase in the rate of DNA synthesis at the same number of forks. They did this by observing that the increment of DNA synthesized after the addition of chloramphenicol to the synchronized culture was also suddenly increased at this time. Chloramphenicol acts in the same

way as amino acid starvation (Friesen & Maaløe, 1965), thus the increment observed is a measure of the number of replication forks in existence at the time of treatment.

Strong support for Maaløe & Kjeldgaard's hypothesis has come from similar experiments to the above, performed by Helmstetter & Cooper (1968). They pulse-labelled the DNA of an exponentially-growing culture of E.coli B/r with ^{14}C-thymidine (for about one minute), and immediately bound the cells onto a membrane filter. By continuously eluting newly born cells with conditioned medium, cells of different ages are separated - the first cells to be eluted are the daughters of the oldest cells at the time of the pulse (ie. those just about to divide), while after one generation period, the daughters of the youngest cells will be eluted. Thus they were able to measure the rate of DNA synthesis in cells of different ages with respect to the division cycle. Their results also showed that the rate of DNA synthesis was constant throughout most of the cycle. They measured the times between the abrupt changes of rate, assuming, from the work of Clark & Maaløe (1967), that an increase represented initiation and, therefore, a decrease represented completion of a cycle of replication. From this they concluded that the time taken to replicate the chromosome is constant, ie. independent of the growth rate throughout the range they studied (generation times of 22-53 minutes).

(6) The number of replication forks per chromosome

A consequence of the hypothesis that the chromosome replication time is independent of growth rate is that, in order to replicate the whole chromosome in the cell generation time, the number of replication forks engaged in this task should vary with the growth rate. Thus slow-growing cells should have a period devoid of DNA synthesis in their division cycle. This has been reported by C. Lark (1966); Helmstetter (1967) and Kubitschek, Bendigkeit & Loken (1967). On the other hand almost continuous DNA synthesis in slow-growing cells has also been reported (Sud, 1967).

Secondly, cells growing with a generation time less than the chromosome replication time, should have a multiple fork replication pattern. Evidence was described in section (2) suggesting that there is only one replication fork on each chromosome. It now seems that this result was a fortuitous consequence of the growth rate used. Thus multifork replication has been observed in B.subtilis by Yoshikawa, O'Sullivan & Sueoka (1964). The "transformation ratio" (explained in section (3)) of a marker just past the origin, ie. with a ratio of 2 in glucose medium, rose to 4 when the culture was growing rapidly in rich medium. This ratio is consistent with there being three replication forks on each chromosome during rapid growth, two of which were initiated when the first had proceeded about half way along the chromosome. This would give 4 origins for each terminus. Consistent

with this pattern were the results of Oishi, Yoshikawa & Sueoka (1964) on an analysis (by transformation) of markers in light, hybrid and heavy DNA, after the germination, in rich medium, of B.subtilis spores uniformly labelled with D_2O.

The increment of DNA synthesized during amino acid starvation is 80% for a broth-grown culture of 15TAU$^-$ (Schaechter, 1961) rather than 40% which is found for a glucose-grown culture (Maaløe & Hanawalt, 1961). This result is consistent with a culture growing in broth having a multifork replication pattern (Sueoka & Yoshikawa, 1965).

Finally, the over-all pattern of DNA synthesis observed by Helmstetter & Cooper (1968) in their experiments, described above, is consistent with the concept of multiple forks existing for a part of the division cycle, when this is shorter than the chromosome time.

(7) The relationship between the cell division, and DNA replication cycles

Since the discovery that DNA synthesis is continuous throughout most of the division cycle of a culture growing in glucose-salts medium (Schaechter, Bentzon & Maaløe, 1959), it has most often been assumed that replication is initiated immediately following cell division (see for example Nagata, 1963; Kuempel, Masters & Pardee, 1965; K.G. Lark, 1966; Lark et al, 1968). Evidence is accumulating, however, that replication can start in

mid-cycle. Thus Forro & Wertheimer (1960) analysed the progeny from ^{3}H-thymidine labelled cells of E.coli 15T^{-} autoradiographically, after separating the cells of different sizes by micromanipulation. The smaller, and therefore younger, cells were found to contain one, and the larger (older) cells, two, replicating chromosomes. Forro (1965) concluded that in 15T^{-}, division did not occur until each chromosome was substantially advanced on a cycle of synthesis.

A similar conclusion was reached by Clark & Maaløe (1967) for the strain B/r, using their experimental technique described above. They noted that in a culture growing with a mean generation time of 45 minutes, the sharp increase in the rate of DNA synthesis occurred near the middle of the division cycle. Also at the other growth rates they used, this increase did not coincide with division.

Schaechter, Maaløe & Kjeldgaard (1958) observed that in cultures of S.typhimurium growing with generation times between 20 and 95 minutes, the mean DNA content and mass of the cells both increased as a logarithmic function of an increase of growth rate. The value of the function is greater for mass than for DNA content. These changes are presumably a reflexion of the relationship between the DNA replication, and cell division, cycles. Until recently they have been only loosely understood.

The first model describing this relationship which accounts quantitatively for these variations in DNA content, is the one proposed by Cooper & Helmstetter (1968). It is based mainly on

their measurements of the rate of DNA synthesis occurring throughout the division cycle of B/r, growing at various rates. Their technique was described in section (5). Their model specifies the relationship between cell division and DNA replication, throughout these growth rates, in terms of two constants: C, the time taken for a replication fork to travel along the entire length of the chromosome, and D, the time between the completion of a cycle of replication and cell division. Their measured estimates of C and D are 41 and 22 minutes respectively. Their model means that the time between the initiation of a cycle of replication, and the subsequent cell division that separates the daughters of that replication, is constant and equals about 60 minutes. For a generation time equal to C+D, initiation occurs at the beginning of the division cycle, replication is completed C minutes later and there is no DNA synthesis for the rest of the cycle. For generation times less than C, initiation occurs before the previous replication cycle has completed, thus producing multifork replication patterns.

From the replication patterns predicted by their model, they were able to calculate the mean DNA content of cells as a function of their growth rate. The predicted relationship between these, closely corresponded to that obtained by Schaechter, Maaløe & Kjeldgaard (1958) for S.typhimurium (as mentioned above), and with two values of DNA content in B/r measured by Cooper & Helmstetter (1968). Their model predicts that the time of

initiation will vary in the division cycle with the growth rate, as they found. Their predicted time agrees with estimates of Forro (1965) and Clark & Maaløe (1967) for a generation time of 45 minutes.

The model is consistent with most of the experimental findings that have been described in the sections above. There are indications, however, that C and D are not strictly constant, but are greater at slow growth rates (C. Lark, 1966; Cooper & Helmstetter, 1968). Also, it may not be stricly applicable to other strains of E.coli. Thus the cells of $15T^-$ have a greater DNA content than those of B/r at the same growth rate (C. Lark, 1966; Cooper & Helmstetter, 1968). This could be due to $15T^-$ either having a larger value of C+D*, but otherwise replicating as predicted by the model, or else replicating according to different principles. K.G. Lark (1966) has postulated replication patterns for $15T^-$ that are different to those predicted by Cooper & Helmstetter's model. However the data on which his postulates are based (Lark & Lark, 1965; Lark & Bird, 1965b; C. Lark, 1966; Lark et al, 1968) are generally compatible with

*For example, the DNA content of $15T^-$ cells growing with a generation time of 40 minutes (C. Lark, 1966), keeping C = 41 minutes would give D a value of 43 minutes, ie. about double the estimate for B/r. (This calculation was made with equation (5) of Cooper & Helmstetter, 1968).

Cooper & Helmstetter's model while some actually favour the latter (see Cooper & Helmstetter (1968) for a discussion of this point).

(8) The concern of this thesis: the control of the initiation of replication

Some of the studies on the relationship between the DNA replication, and cell division, cycles at various growth rates, have been described in the previous section. It has become clear that whatever this relationship may prove to be, a key event in the cell division cycle is the timing of the initiation of DNA replication. According to Cooper & Helmstetter (1968) and Helmstetter & Pierucci (1968) only this event need be controlled for both DNA replication and cell division to be determined as a consequence.

Basically, two models have been proposed for the control of initiation, a positive and a negative one. The positive one envisages control through the synthesis of chromosomal attachment sites on the bacterial membrane, initiation only being permitted when these are completed (Jacob, Brenner & Cuzin, 1963; K.G. Lark, 1966). Negative models postulate the existence of an inhibitor which prevents initiation, all the enxymes, attachment sites etc. necessary for initiation never being limiting throughout normal growth cycles. One version of this type of model (Pritchard, 1968; Pritchard, Barth & Collins, 1968) postulates that this inhibitor is diluted by cellular growth to a critical concentration at which

initiation is permitted. Immediately after, and as a consequence of, this act of initiation a pulse of inhibitor is synthesized which thus prevents further initiation. The difficulty in verifying either of these models, or any other, is due to the meagre data that have been produced on the control of initiation.

Some information on this topic is available in the data of Pritchard & Lark (1964). Using density-label experiments similar to those of Lark et al (1963) they showed that the thymine starvation of a thymine-requiring strain of E.coli 15 interferes with the initiation process; a new cycle of DNA replication proceeds from the origin when thymine is replaced. Recently, using entirely different techniques (described above) Wolf et al (1968) and Cerda-Olmedo et al (1968) have confirmed that thymine starvation causes reinitiation from the same origin as that used during replication. In the absence of amino acids, however, this effect of thymine starvation is abolished (Pritchard & Lark, 1964). Is this effect, therefore, due to stopping DNA synthesis while allowing mass increase to continue, or is it an effect specific to thymine starvation?

Pritchard & Lark (1964) favoured the view that thymine has a special role in the initiation control mechanism, mainly because of data then available, which suggested that inhibiting DNA synthesis (a) by deoxyriboside starvation in Lactobacillus acidophillus R26 (Okazaki & Okazaki, 1959), or (b) with

deoxyadenosine in Alcaligenes faecalis LB (Lark, 1960), did not lead to reinitiation of replication.

The question was still open, however. The data of Okazaki & Okazaki (1959) are ambiguous, they were quoted later by Soska & Lark (1966) as showing that deoxyriboside starvation does have this effect, which is what these latter authors themselves found. Deoxyadenosine is probably not a sufficiently specific inhibitor of DNA synthesis; it is now known that in sensitive strains of S.typhimurium, deoxyribosides inhibit RNA synthesis more than DNA (Beacham et al, 1968). Also specific experiments to observe reinitiation from the origin were not performed by Okazaki & Okazaki (1959), Lark (1960) or Soska & Lark (1966). On the other hand, such experiments were carried out by Lark & Lark (1964) with negative results, using inhibition by cytosine arabinoside in E.coli 15T$^-$. The specificity of this inhibitor has not been studied.

Further study was, therefore, necessary to resolve this question, and to throw light on the nature of the control of initiation. It was undertaken along two lines. Firstly, the agent nalidixic acid, which seemed to be a suitably specific inhibitor of DNA synthesis, was tested, and compared with thymine starvation, for its effects on reinitiation. These studies are described in Chapter I, while Chapter II is concerned with the properties of nalidixic acid that reflect on this use and on its mode of action. Secondly, because of the implication that thymine is specially

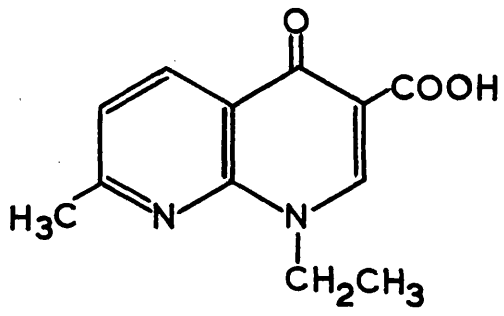
involved in the initiation of DNA replication, its apparent effect on the regulation of deoxynucleotide metabolism was investigated. This forms the subject of Chapter III.

C H A P T E R I

Alteration of the Control of DNA Replication Initiation

Introduction: Nalidixic Acid

The bacteriocidal drug nalidixic acid is 1-ethyl-1, 4-dihydro-7-methyl-4-oxo-1, 8-naphthyridine-3-carboxylic acid, and was first synthesized by Lesher et al (1962).



It is toxic to proliferating bacteria, especially gram-negative species (Deitz, Bailey & Froelich, 1964). Nalidixate appears to be a rapid and very specific inhibitor of DNA synthesis: Goss, Deitz & Cook (1964) noted little or no concomitant inhibition of respiration or of RNA, protein or lipid synthesis. A concentration of 50 µg/ml almost completely blocks the incorporation of labelled thymine into the acid-insoluble fraction of a culture of E.coli 15TAU$^-$, but has little or no effect on the incorporation of arginine or uracil, except after prolonged exposure (Goss, Deitz & Cook, 1965). Inhibition by nalidixate causes the development of long filamentous cells and loss of viability, (Goss et al, 1964) and in this respect it behaves similarly to thymine starvation (Cohen & Barner, 1954).

They are similar in another respect also: lethality can be eliminated by inhibiting the growth of a culture. Using nalidixate, it has been shown that low temperature incubation or

starvation of a required amino acid (Goss et al, 1964) or treatment with chloramphenicol or dinitrophenol (Deitz, Cook & Goss, 1966) restrict lethality. Chloramphenicol was shown not to prevent the inhibition of DNA synthesis by nalidixate (Deitz et al, 1966).

An important and useful property of nalidixate in inhibiting DNA synthesis is its rapid reversibility. Removal of the drug from treated cultures (by filtration and resuspension in fresh medium) results in immediate restoration of DNA synthesis, while retreatment of the same culture results in immediate inhibition again (Deitz et al, 1966). As noted by these authors, this indicates that nalidixate interacts rapidly and reversibly with the DNA replication system in some way. It eliminates the initiation function as a primary target for nalidixate.

Results

(1) Preliminary experiments on the effects of nalidixate

At the time this work was begun, careful kinetic studies of the effect of nalidixate on DNA synthesis had not been made. Preliminary experiments were therefore performed on the rate of DNA synthesis in E.coli before, during and after treatment with nalidixate.

Figure 1 shows the results of such an experiment performed on an E.coli K12 strain. DNA synthesis was measured as the incorporation of ^{14}C-thymine into acid-insoluble material. The addition of a deoxynucleoside to the culture was necessary to enhance this thymine incorporation, since the strain used

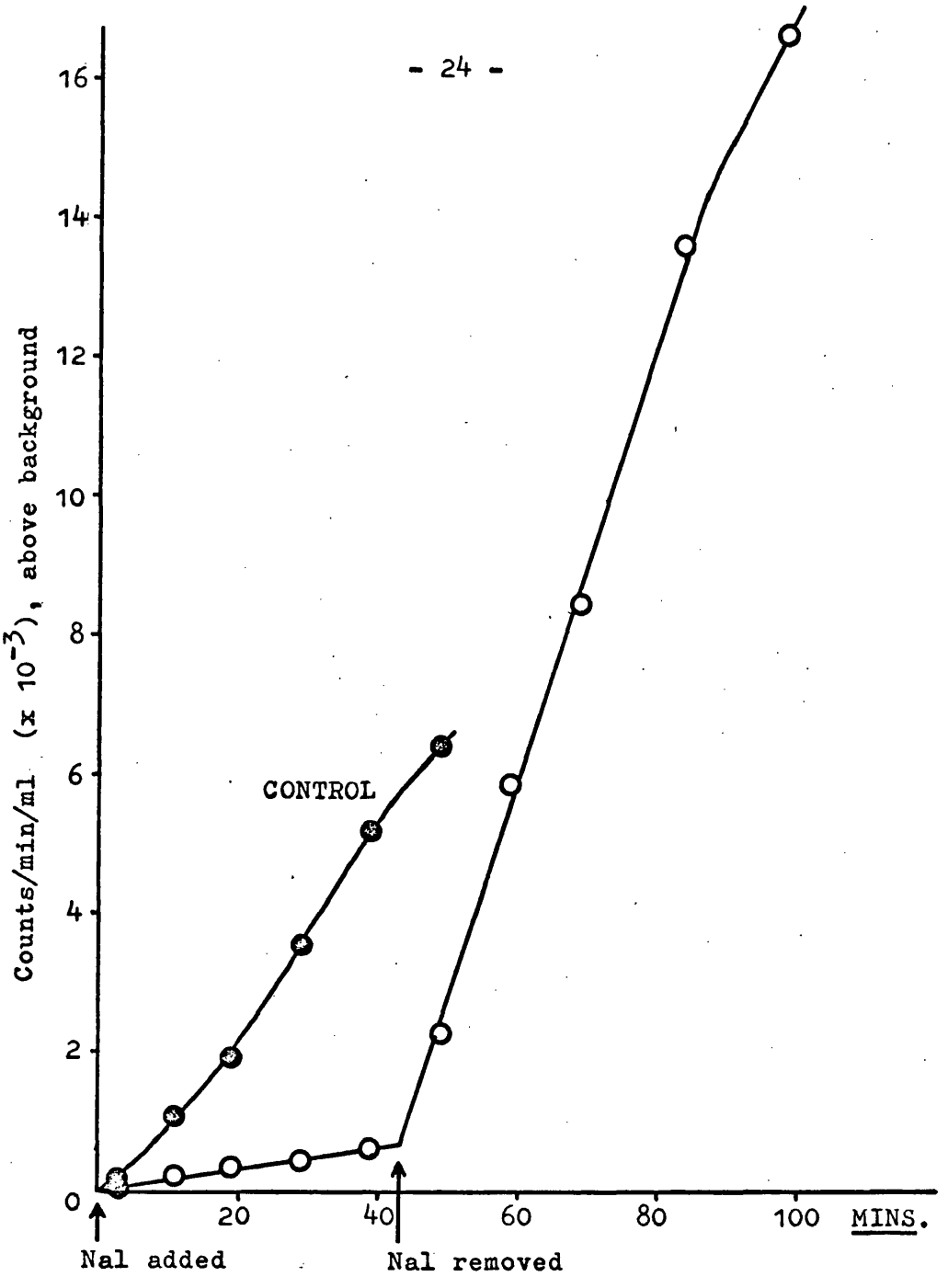


Figure 1 The rate of DNA synthesis in E.coli K12 before, during and after nalidixate treatment. A culture of P4 (K12 HfrH thi$^-$ thy$^-$ str^s) growing in minimal medium supplemented with 5 µM thiamine and 800 µM thymine, (generation time = 90 min) was resuspended at 2 x 10^8 cells/ml in this medium but containing ^{14}C-thymine (17.4 µM, 0.035 µC/ml) and 440 µM deoxyuridine. Half this culture was immediately supplemented with 50 µg nalidixate/ml, this being removed 43 minutes later. 1 ml samples were taken at intervals to determine ^{14}C-thymine incorporation. Media and techniques are described in Methods.

otherwise incorporates thymine poorly when given a low concentration (see Chapter III). DNA synthesis is immediately inhibited after addition of nalidixate. The time between its removal, and the resumption of DNA synthesis was too short to be measurable in this experiment. The most important feature of the results is that the rate of DNA synthesis after removal of nalidixate is enhanced, to about 2.3 times the control rate. This effect was noted in an analogous experiment involving thymine starvation (Pritchard & Lark, 1964), and is the result expected of a treatment that causes reinitiation of replication.

The effect of nalidixate on E.coli $15T^-$ (555-7) (the strain used by Pritchard & Lark, 1964) was also examined, and compared in the same experiment with the effect of thymine starvation. An exponentially growing culture of this strain was divided into four parts. One part (the control) was supplemented with labelled thymine, the incorporation of which, into acid-insoluble material, was measured. The other parts were either treated with nalidixate (at 50 µg/ml), starved of thymine, or had both treatments imposed simultaneously. After 44 minutes of treatment, these cultures were transferred into the labelled medium, and sampled and measured in the same way as the control.

The results are plotted in Figure 2 as the label incorporated versus the time function $2^{t/g}-1$ (where t = time after the addition of label, and g = mean generation time). The derivation of this function is given in Appendix 1, where it is shown that for a constant rate of incorporation of a substance into an

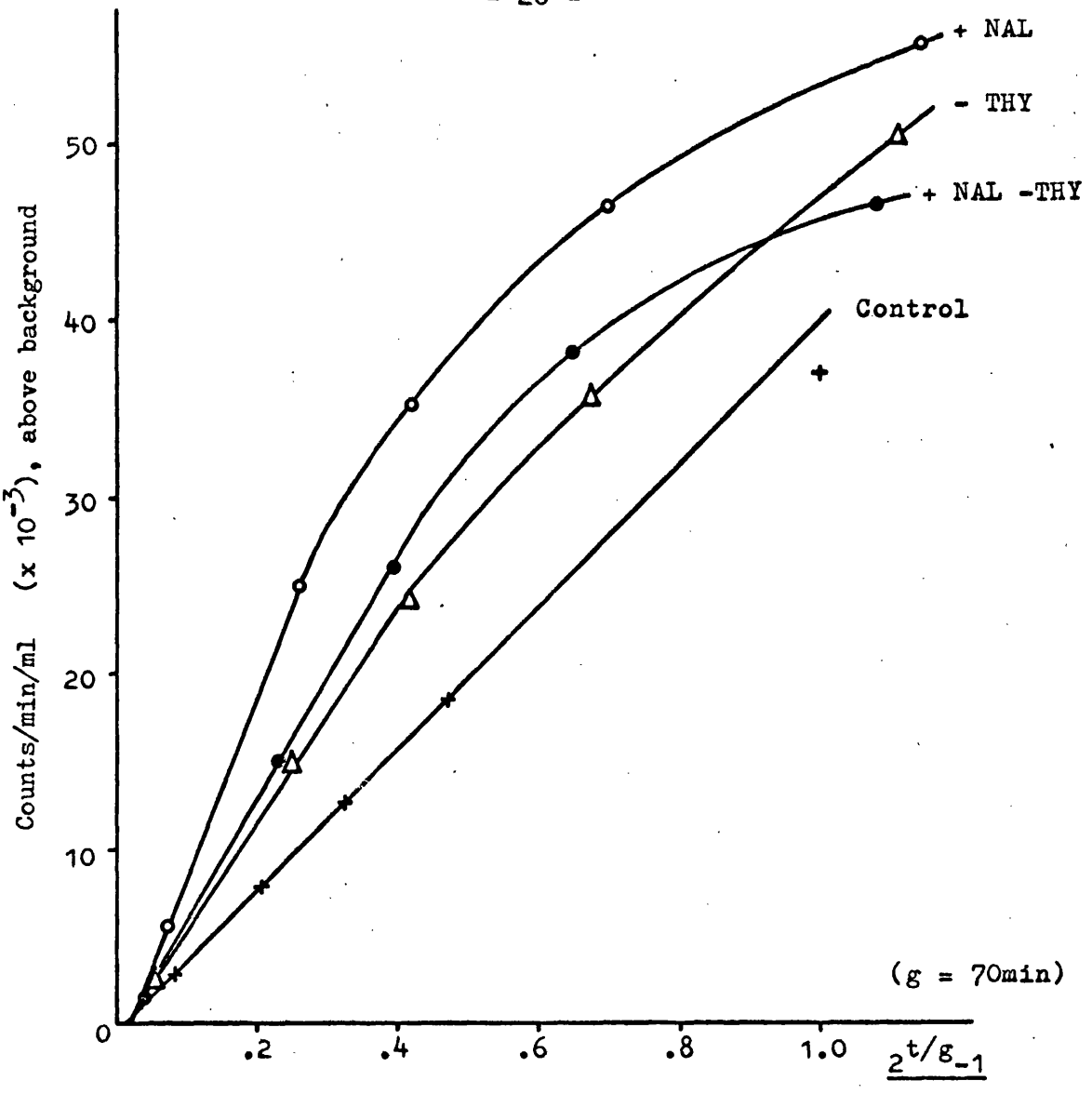


Figure 2 The effect of nalidixate and thymine starvation on the subsequent rate of DNA synthesis in $15T^-$(555-7). An exponentially growing culture of this strain, at 2 x 10^8 cells/ml, was divided and treated with 50 µg nalidixate/ml in the presence (+NAL) and absence (+NAL -THY) of thymine, and also thymine starved (-THY). An untreated (Control) culture was immediately supplemented with ^{14}C-thymine (0.03 µC/ml, 8 µM) and 44 minutes later, the treated cultures were transferred to growth medium with this concentration of ^{14}C-thymine. Samples were taken to measure the incorporation of thymine into acid-insoluble material.

exponentially increasing component (eg DNA), the amount incorporated is proportional to $2^{t/g}-1$. The slope of the line is proportional to the rate of incorporation in such a plot.

It can be seen in Figure 2 that a period of thymine starvation or nalidixate treatment enhances the subsequent rate of DNA synthesis. The initial rates correspond to a stimulation of the control rate of:

2.52 fold, after nalidixate treatment,

1.70 fold, after nalidixate treatment with simultaneous thymine starvation,

and 1.51 fold, after thymine starvation.

Apart from the control, these rates were only maintained for about half a generation time, when they decreased to control rate or less. It is interesting to note that simultaneous thymine starvation and nalidixate treatment gives a stimulation about the same as thymine starvation alone, rather than the considerably greater initial effect of nalidixate treatment alone. A model to account for these effects will be discussed later.

(2) Density-label analysis of replication initiation

The stimulation of DNA synthesis observed in the experiments described above, is not easily explained by the hypothesis that nalidixate treatment, like thymine starvation, causes reinitiation of DNA replication. This hypothesis was tested by the type of experiment performed by Pritchard & Lark (1964) using 5-Bromouracil (BU) as a DNA density-label.

Such experiments depend on the assumption that, if a culture is deprived of one or more required amino acids, DNA replication ceases at the chromosome terminus. The validity of the assumption has been discussed in detail in section (4) of the Introduction. If the resumption of DNA synthesis after restoration of the required amino acids occurs in the presence of ^{3}H-thymine, then this label will be incorporated exclusively into the region of DNA immediately following the origin. Reinitiation can now be detected by allowing this culture, firstly, to grow until it has become desynchronized (Hanawalt, Maaløe, Cummings & Schaechter, 1961), then applying the treatment thought to cause reinitiation, and finally, allowing DNA synthesis in BU medium. If reinitiation from the origin has been induced, the ^{3}H-labelled section of the chromosome will become hybrid in density early, ie. it will be preferentially replicated (Pritchard & Lark, 1964).

Experimental details

A 50 ml culture of 15T^{-} (555-7) was first grown for 6.5 generations in medium containing ^{14}C-thymine (8 µM, 0.0319 µC/ml). This uniformly labelled the DNA in order to facilitate its measurement later. At 2.3 x 10^{8} cells/ml, the bacteria were transferred to the same medium devoid of amino acids and incubated for 90 minutes. Then they were transferred to 26 ml of medium, complete except for thymine. 25 ml were immediately supplemented with ^{3}H-thymine (to give a final concentration of 0.8 µM and

2.94 μC/ml), and 1 ml was supplemented with ^{14}C-thymine to give ten times the previously used specific activity, (ie. 0.8 μM and 0.0319 μC/ml). Both were incubated for 29 minutes, the small volume being sampled throughout this time in order to measure the percentage of the total DNA replicated in this period. The 25 ml culture was filtered and transferred into 300 ml medium identical to the first growth medium, and incubated for about two hours, by which time the cell titre had reached 3 x 10^8/ml. The culture was then divided into three parts:

(1) was transferred to medium containing 100 μM BU substituted for thymine,

(2) was starved of thymine for 40 minutes after which BU was added, and

(3) was treated with nalidixate (50 μg/ml) for 40 minutes, and then transferred into BU-containing medium.

Samples were taken at approximately 5 minute intervals from the cultures growing in BU, the DNA was extracted from them and analysed by CsCl density-gradient centrifugation as described in Methods.

Before describing the results of this analysis, a brief description of measurements taken during the course of the above procedure is necessary. The Coulter counts showed that, initially, growth was exponential with a generation time of 40 minutes. During amino acid starvation the rate of growth decreased to zero after about 50-60 minutes. Acceleration back to the normal

rate of cell division took a similar period when amino acids were restored. Similar kinetics for the incorporation of ^{14}C-thymine into DNA were observed during these three periods. From the latter results, the percentage of the total DNA labelled during the 29 minutes immediately after amino acid starvation was estimated to be 4.2%.

The analysis of the CsCl gradient of one sample from each of the three BU-grown cultures is shown in Figure 3. Similar curves and separations of light and hybrid-density DNA bands were obtained for the other samples taken.

The counts in each band for each sample were summed. These were then plotted (Figure 4) as the percentage of ^{3}H counts found in the hybrid band against the percentage of ^{14}C counts in the same band. This gives a measure of the fraction of the ^{3}H-labelled region of the chromosome that has replicated (ie. the region following the origin) as a function of the fraction of total DNA that has replicated at the same time.

The curve for culture (1), which had no treatment after the second period of growth, closely followed the curve predicted from random replication of the ^{3}H pulse (derived in Appendix 2). It therefore provided a suitable control against which to compare the effects of the DNA inhibitors. Curves (2) and (3) show that both thymine starvation and nalidixate treatment cause preferential replication of the ^{3}H pulse. This is direct evidence in favour of the hypothesis that these treatments subsequently lead to reinitiation of DNA replication from the origin.

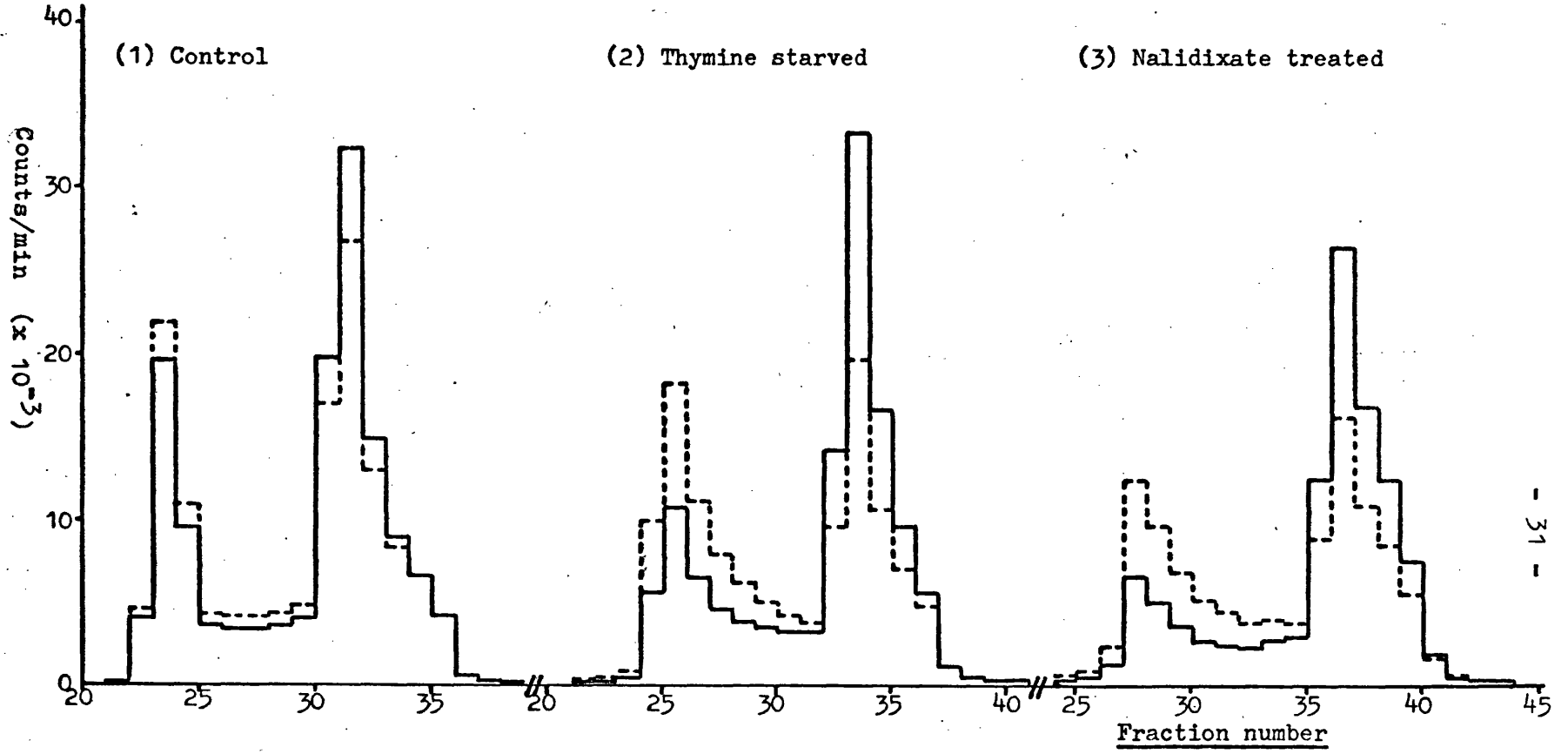


Figure 3 Examples of the CsCl density gradient analysis.
——— = ^{14}C (total DNA), ---- = ^{3}H (section following chromosome origin). The hybrid density DNA (BU-thymine) band is the left one of each pair. The samples shown were taken after 18-21 minutes incubation in the BU-medium.

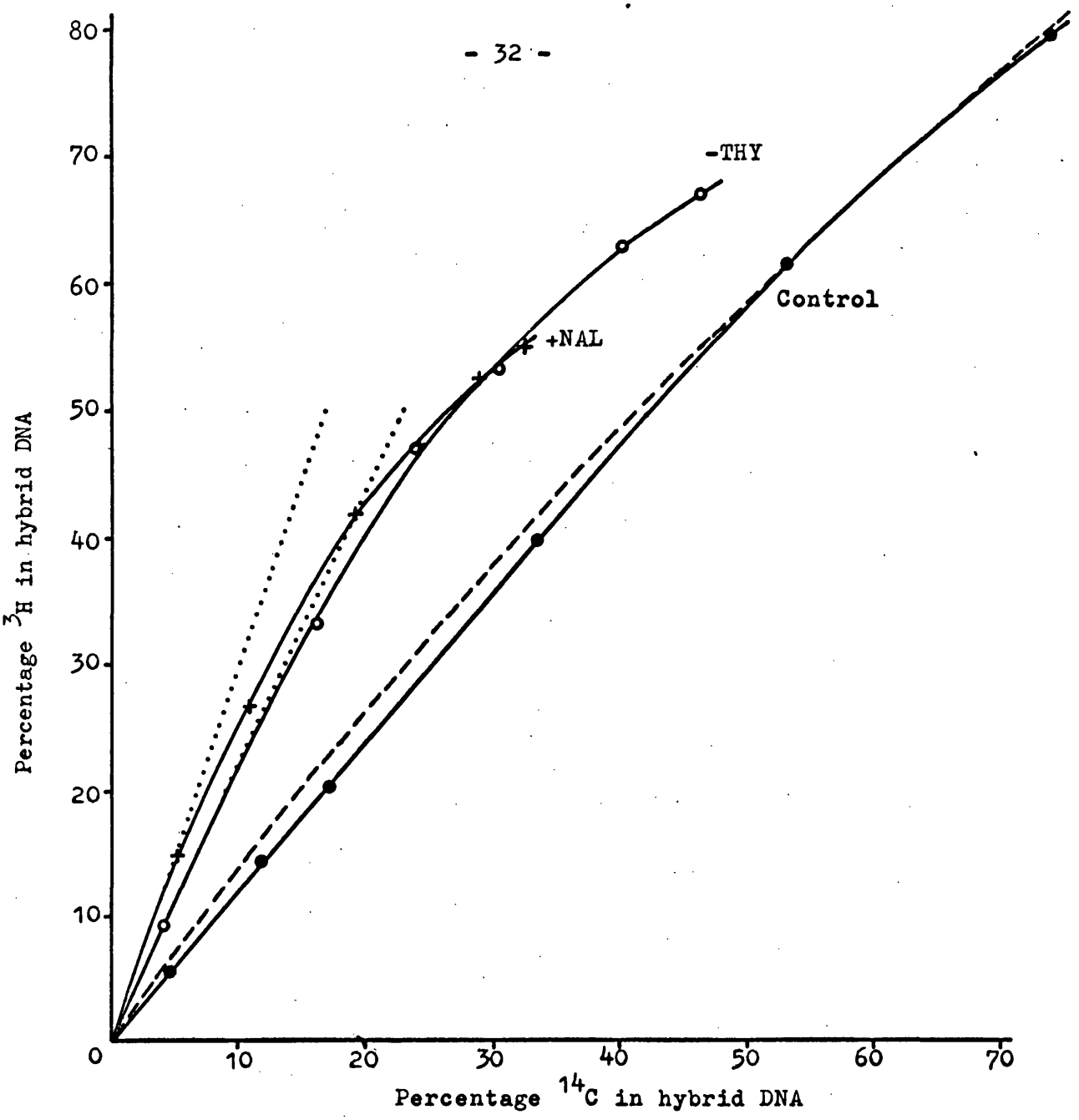


Figure 4 Effect of nalidixate treatment and thymine starvation on the replication of DNA ^{3}H-pulse-labelled after amino acid starvation.

Control = culture (1)

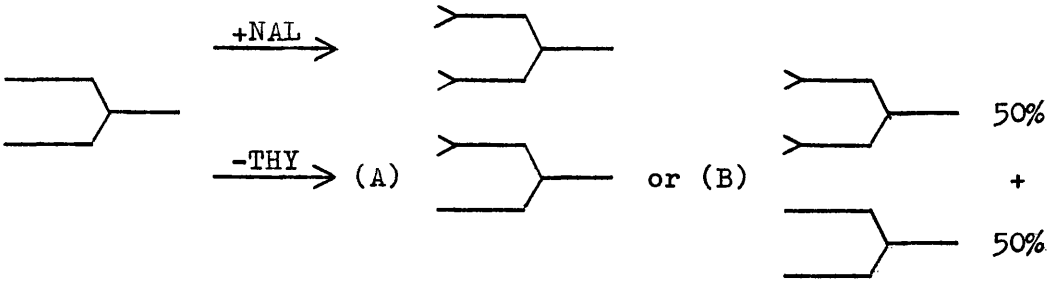
-THY = " (2) ie. after 40 min thymine starvation

+NAL = " (3) ie. after 40 min nalidixic acid treatment

---- = predicted curve for random replication of the pulse (see Appendix 2)

It will be noticed that nalidixate treatment produces a steeper initial gradient than thymine starvation (Figure 4). This difference results from large differences in the isotope ratios, well beyond differences ascribable to sampling or other experimental errors. The whole experiment was carried out twice, and essentially the same results were obtained each time. The difference therefore seems to be real, which is consistent with the results presented in Figure 2 and similar experiments described below. We can consider two alternatives to explain this difference:

(1) Nalidixate treatment causes reinitiation of replication from the origins of both of the partical replicas of a replicating chromosome, while as Pritchard & Lark (1964) and Lark & Bird (1965a) have proposed, thymine starvation causes reinitiation at only one. Neither treatment inactivates the pre-existing replication fork. This proposal is shown diagrammatically in Figure 5



Proposed effects of nalidixate treatment and thymine starvation on chromosome replication. The alternatives A and B shown for thymine starvation are considered below.

(2) Nalidixate treatment causes more DNA damage than thymine starvation, this being repaired during subsequent replication thus increasing the rate of DNA synthesis. Nalidixate does indeed cause DNA degradation as shown by Cook, Deitz & Goss (1966) and by experiments described in the next chapter (Figures 18-20), but this is not very extensive, amounting to 3.5% of the total DNA in $15T^-$ (555-7) after 40 minutestreatment with 50 µg nalidixate/ml (from Figure 19). The CsCl density-gradient analysis (examples shown in Figure 3) showed very little material between the hybrid and light bands, and not noticeably more in the samples from the nalidixate-treated culture than in the others. Such material would be expected if a significant amount of repair synthesis were taking place (Pettijohn & Hanawalt, 1964). Repair synthesis cannot, therefore, account for the observed difference between nalidixate treatment and thymine starvation in causing stimulation of DNA synthesis (Figures 2 and 14). Even if repair synthesis were taking place significantly in the density-label experiments just described, there seems to be no reason why this should cause preferential replication of the ^{3}H-labelled region.

The proposal for the effect of nalidixate treatment shown in Figure 5 is also favoured by a model to be discussed later, which says, briefly, that after the specific inhibition of DNA synthesis for a suitable time, a cell temporarily replicates dichotomously, ie., in the same way as fast-growing cells.

Evidence suggests that such replication is symmetrical (Yoshikawa, O'Sullivan & Sueoka, 1964; Cooper & Helmstetter, 1968) as seems *a priori* probable, rather than unsymmetrical as shown for thymine starvation (Figure 5,A). Thus the effect of thymine starvation should also be symmetrical according to this model, alternative B in Figure 5 being a possibility. Pritchard & Lark (1964) admitted that none of their data distinguish between alternatives A and B.

Are the results of the density-label experiments (Figure 4) compatible with the proposed difference between nalidixate treatment and thymine starvation in causing reinitiation (Figure 5)? The initial slopes of the two treated cultures in Figure 4 can be compared for this purpose. The *initial* slopes are used as the lines rapidly deviate, probably as a consequence of heterogeneity in the length of the ^{3}H pulse incorporated after amino acid starvation (Lark *et al*, 1963). The model for nalidixate treatment predicts that all the ^{3}H-labelled section will have been replicated when DNA equivalent to three times the length of this section has been replicated. Thus from the initial slope of curve (3) in Figure 4, the mean length of the ^{3}H section can be calculated as $\frac{33.7}{3} = 11.2\%$. (This estimate is similar to that obtained by Pritchard & Lark (1964) viz, 10% for a 30 min pulse of ^{3}H-thymine). Now the model for thymine starvation predicts that 50% of the ^{3}H section will have been replicated when DNA equivalent to twice the length of this section has been replicated (Figure 5, A and B are indistinguishable in this respect). Thus from the

initial slope of curve (2) in Figure 4, the length of the ^{3}H section would be $\frac{23.0}{2}$ = 11.5%. The close agreement between the two estimates shows that the difference between the two initial slopes is compatible with the model described. Possible reasons for this difference will be discussed later. It should be noted that the difference is not maintained long, the two lines coincide before a third of the total DNA has replicated.

(3) Agents that cause reinitiation

The results of the density-label experiments above, show that nalidixate treatment, like thymine starvation, causes reinitiation of replication from the origin. Evidence was produced that exposure of a culture of $15T^{-}$(555-7) to X-rays (Billen, Hewitt & Jorgensen, 1965) or ultraviolet light (Hewitt & Billen, 1965) also has this effect. These authors used the type of experiment with which Lark *et al* (1963) demonstrated the sequential nature of chromosome replication (described in section (2) of the Introduction). Irradiation of the culture immediately after the ^{3}H pulse randomizes the subsequent replication of the labelled section, suggesting that new replication cycles have been initiated from sites placed randomly with respect to the ^{3}H-labelled sections. They also demonstrated that the DNA region immediately following the origin (ie. that region replicated after a period of amino acid starvation, or by a stationary-phase culture on dilution into fresh medium (Ginsberg & Jagger, 1963) has a higher probability of replication

after irradiation than a region displaced from the origin. The simplest explanation for these data is that irradiation causes reinitiation from the origin, although the number of replication forks induced cannot be decided from their data. The phenomenon is not restricted to 15T⁻(555-7): similar results were obtained with E.coli B/r and C (Hewitt, Billen & Jorgensen, 1967).

It was mentioned in the Introduction that deoxyriboside starvation of L.acidophillus R26 probably also causes reinitiation (Soska & Lark, 1966). Thus we now have evidence that four other treatments that specifically inhibit DNA synthesis, apart from thymine starvation, induce reinitiation. It therefore seems unlikely that thymine has a special role in the control of initiation. As pointed out by Maaløe & Kjeldgaard (1966) there is another reason for thinking this: the time taken for thymine starvation to cause its maximum effect (40-50 minutes, Pritchard & Lark, 1964), is very much longer than the time taken for the concentration of thymidine nucleotides in the cell, to fall to their minimum (about 2 minutes, Neuhard & Munch-Petersen, 1966).

The generality of this reinitiation effect suggests that it is due to a mechanism in the cell for maintaining the DNA to mass ratio, an increase of the mass or mass/DNA ratio to a critical value causing a new cycle of replication to be initiated. A prediction of this hypothesis is that it should take one generation period of treatment (assuming normal mass increase during this time) for all the cells in a random exponentially-

growing culture, to reinitiate. This is because each cell must initiate the replication of each of its chromosomes (if it has more than one) once in each cell division cycle, in order to maintain its chromosomal complement.

This prediction was tested with a study on the relationship between the length of nalidixate treatment, and the resulting rate of DNA synthesis. The latter was taken as a measure of the proportion of cells that had been reinitiated in view of the data and arguments in the previous section.

(4) The effect of the duration of nalidixate treatment on the subsequent rate of DNA synthesis

This was examined in the following way: exponentially-growing cultures of $15T^-$(555-7) were treated with 50 µg nalidixate/ml for various periods. They were then washed free of the drug and resuspended in growth medium containing ^{14}C-thymine (8 µM, 0.0319 µC/ml or 20 µM, 0.08 µC/ml; these specific activities are the same). Samples were taken at intervals to measure the incorporation of radioactivity in DNA as described in Methods. The kinetics of incorporation in two such experiments are recorded in Figures 6 and 7.

For the first generation period, the rate of DNA synthesis in all the cultures was exponential, ie. the radioactivity incorporated was proportional to $2^{t/g}-1$ (Appendix 1). After one generation period, the rates in the pretreated cultures declined from this, although the control maintained an exponential rate.

DNA synthesis was reduced to a rate 4.5% of the control during nalidixate treatment.

Increasing the period of nalidixate treatment increased the subsequent rate of DNA synthesis, a maximum of about three times the control rate being reached after about 40 minutes of treatment. The mean generation times (given in Figures 6 and 7) of the cultures were also about 40 minutes. These results are therefore consistent with the prediction, and thus with the postulate outlined above.

The threefold stimulation is compatible with the assumption that all the chromosomes in the population have a single replication fork during balanced growth at this particular rate. Treatment with nalidixate for a generation time increases this to three, by the addition of a replication fork at the origin of each of the two partial replicas present. On this assumption also, a sudden decrease in the rate of DNA synthesis would be predicted to occur when these newly-induced replication forks had traversed the entire chromosome, ie. after C minutes (using the terminology of Cooper & Helmstetter, 1968). Since the replication forks start synchronously (when nalidixate is removed), and the rate of travel of each fork is presumably the same (evidence for this is discussed in the Introduction), they should terminate synchronously. That such a decrease was always found and always approximately at the same time, is good evidence in favour of this interpretation.

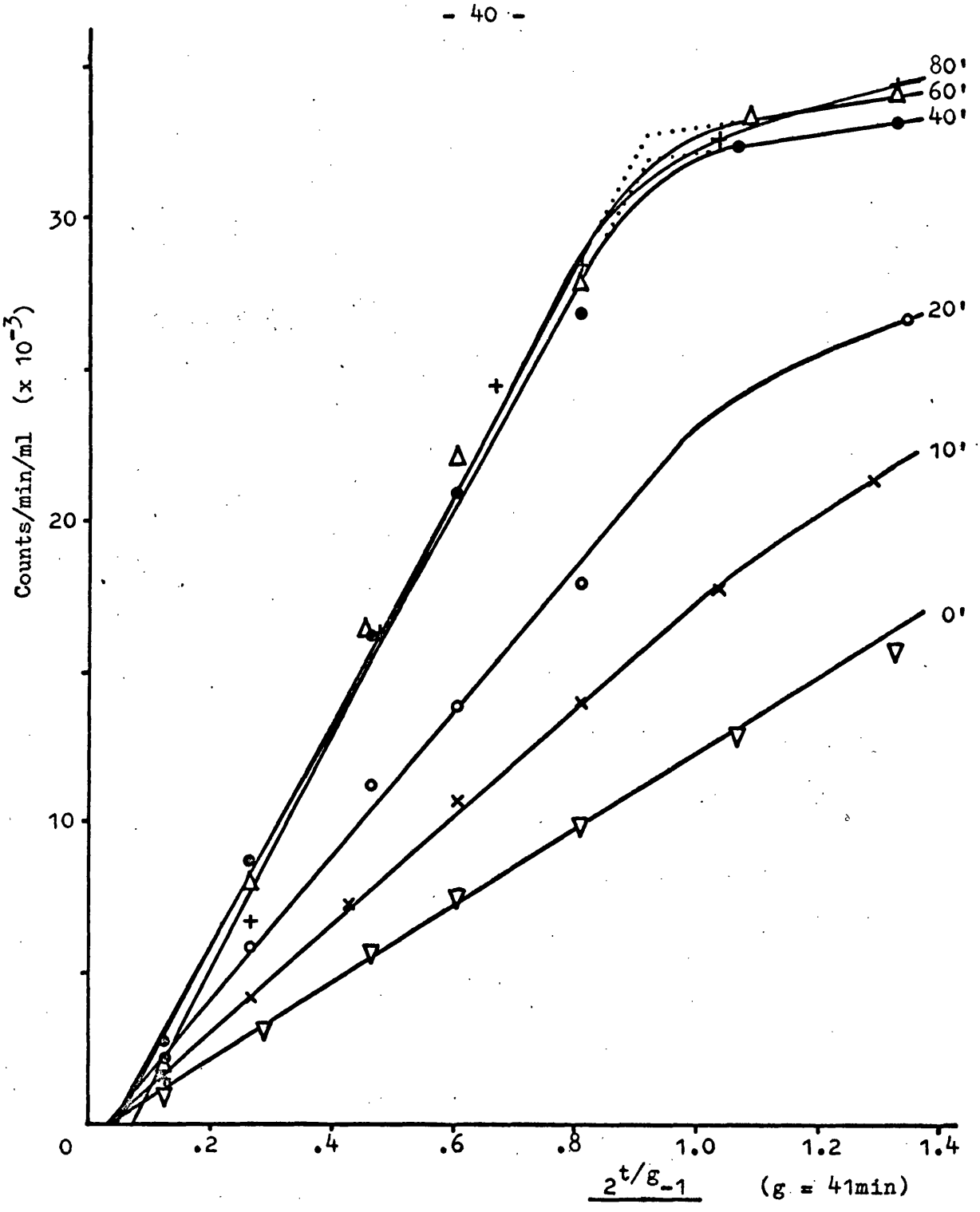


Figure 6 The effect of pretreatment with nalidixate on the subsequent rate of DNA synthesis. The numbers on the figures give the time (in minutes) of nalidixate treatment of $15T^-$(555-7) before transfer to drug-free, ^{14}C-thymine-containing medium at time(t) = 0.

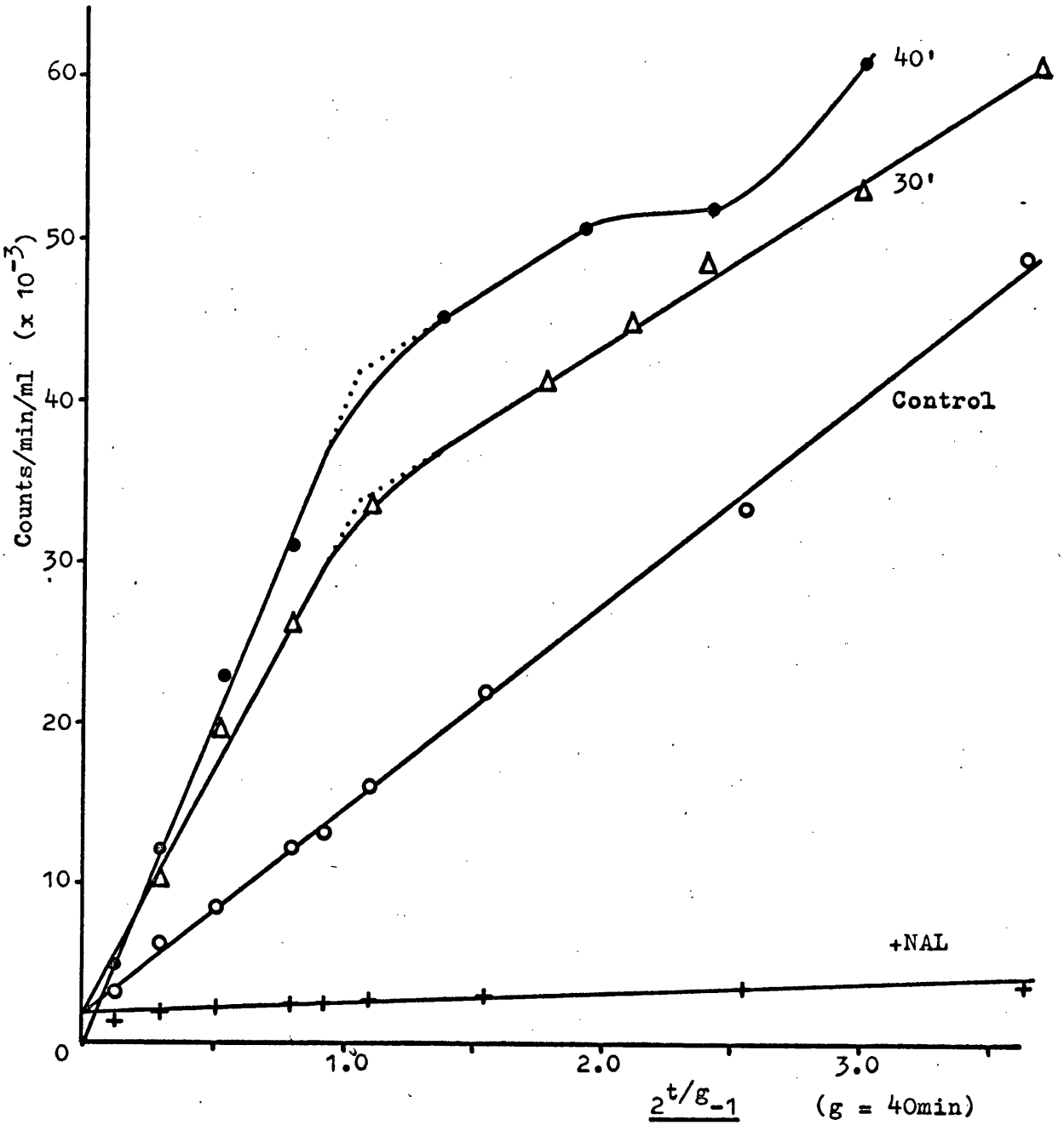


Figure 7 The effect of pretreatment with nalidixate on the subsequent rate of DNA synthesis. Method as under Figure 6. The rate of DNA synthesis during nalidixate treatment is also included (+NAL).

NB. The abscissa has a smaller scale than that of Figure 6.

In Table 1 below, the times of this decrease are collected together from the various kinetic experiments of the type shown in Figures 6 and 7. All the data refer to strain $15T^-$(555-7).

Table 1: Times of rate decrease in the stimulated DNA synthesis experiments

Figure	Generation time (min)	Times of first rate decrease (min)
2	70	41.0, 43.4, & ca. 42
6	41	38.5, 38.5, & ca. 38.5, 42 & 43
7	40	41.6, 41.2
12	40	40.0
13	40	41.2
14	40	42.1, 43.8

The average of these times is 41.2 minutes suggesting that if the interpretation above is correct, $15T^-$(555-7) has the same value of C as B/r (ie. 41 minutes, Cooper & Helmstetter, 1968).

After the change in rate, it can be seen from Figure 7 that DNA synthesis continues, but at a slightly lower rate than the control. The 30 minute treated culture has an exponential rate 80% of the control. To make a meaningful comparison of this rate with the rate that would have been found, had there been no treatment, we must effectively move the curve to the right by 30 minutes. This can be done by dividing the rate by

$2^{30/40} = 1.682$.* This gives a value a little less than half the control rate. A slightly lower value is given by a similar calculation on the 40 minute-treated culture.

Before considering in more detail the values of the gradients in Figures 6 and 7, and especially why the initial rate of DNA synthesis is exponential after pretreatment with nalidixate, the kinetics of growth during nalidixate treatment will be considered.

(5) Growth during nalidixate treatment

The cell number and mass (as $A_{600\ m\mu}$) of an exponentially-growing culture of $15T^-$(555-7) were measured before and after the addition of nalidixate. The results are shown in Figure 8.

The increase in cell number ceases about 20 minutes after addition of the drug, the total increment reaching 38% at the plateau. In contrast, cell mass continues increasing at the previous exponential rate for 50-60 minutes before reaching its plateau at about 70 minutes. The absence of any further effect on the rate of DNA synthesis (Figure 6) after 40 minutes of drug treatment, is not therefore due to mass increase ceasing at this time.

*The equation in Appendix 1 shows that increasing the function $2^{t/g}$ to $2^{(t+ti)/g} = 2^{t/g} \times 2^{ti/g}$, decreases the gradient by this factor. The line remains straight.

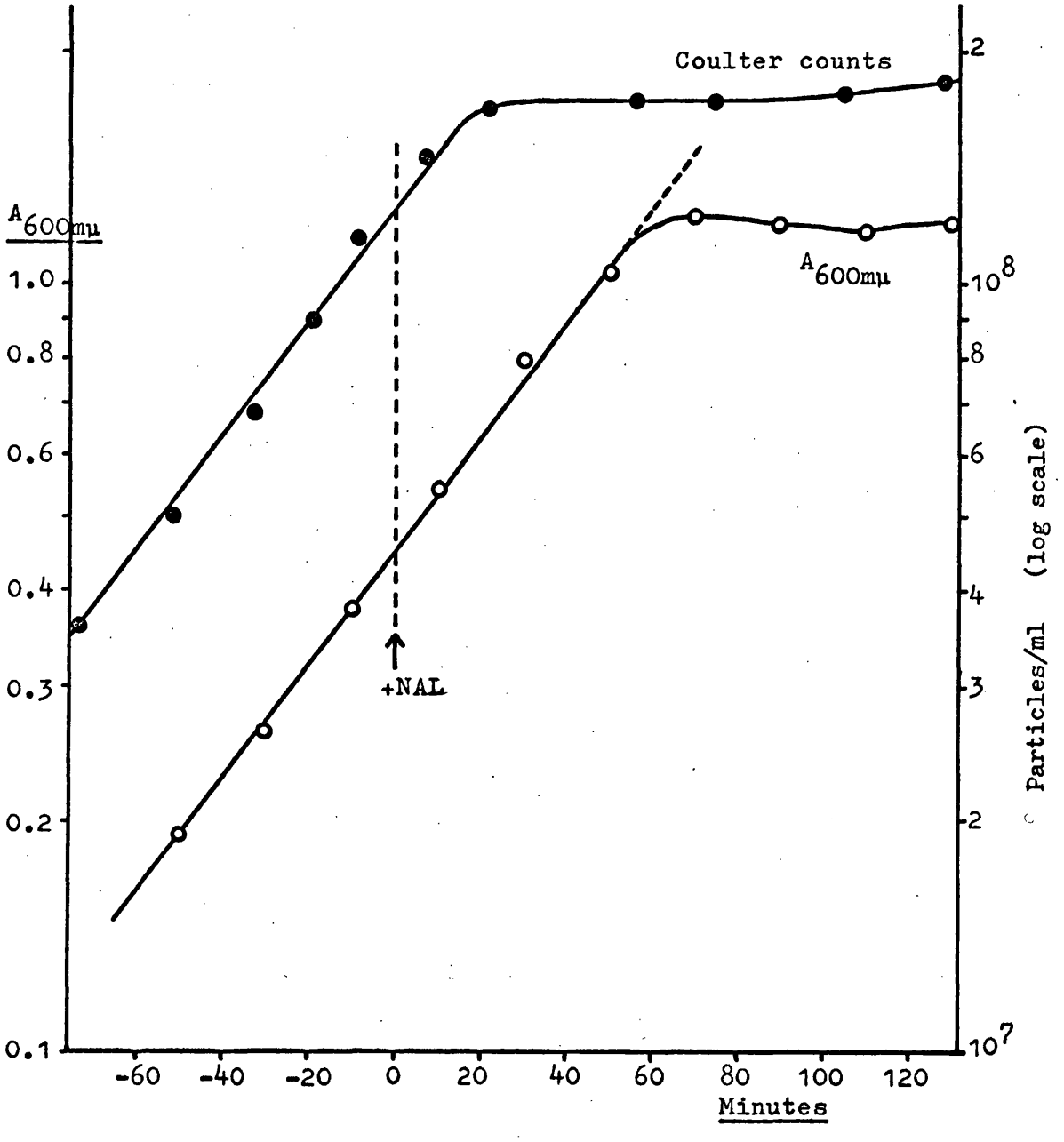


Figure 8 The cell titre (Coulter counts) and mass ($A_{600m\mu}$) of cultures of $15T^-$(555-7) growing exponentially with a generation time of 41 min. 50 µg nalidixate/ml was added at the time shown by arrow.

(6) The relationship between the duration of nalidixate treatment and the stimulation of DNA synthesis induced

This relationship is predicted in Appendix 3, on the model that, increasing the mass of a cell to a critical value, causes reinitiation in some way. It is proposed that the absence of DNA synthesis does not affect this process. The absence of any significant effect of nalidixate on the exponential increase in mass for a considerable period (Figure 8), therefore means that the rate of initiation during this period will be unchanged. Equation (5) in Appendix 3 predicts, from this model, that the rate of DNA synthesis will be exponential for a period = C after pretreatment with nalidixate. This has been consistently found (Figures 2,6,7,12,13 & 14).

The model predicts that after this period, the rate should decrease sharply (as the induced replication forks terminate) and return to control rate, if the mass of the culture has been increasing exponentially throughout. However, this rate has always been found to be less than that of the control, this being more marked the longer the period of treatment. This is presumed to be due to nalidixate ultimately affecting the rate of mass increase. It has been found (not shown) that after long periods of nalidixate treatment (40-60 minutes), removal of the drug does not reverse this effect. The initial exponential rate is not thought to be decreased very markedly by this effect, however, because of the delay before its significant onset. Repair synthesis is also considered not to

have much effect on the observed rate of DNA synthesis: little DNA degradation is observed for the doses and times of nalidixate treatment used here (Figure 19).

Equation (6) in Appendix 3 predicts that the ratio of the initial rate of DNA synthesis in the pretreated culture to that in the control, is proportional to $2^{ti/g}$ (where ti = time of inhibition of DNA synthesis). These ratios were therefore calculated from the results in Figures 2,6,7,12 and 14, and are plotted in Figure 9 against $2^{ti/g}$ -1 (to conform with previous practice).

Consider first the nalidixate-treated cultures where g = 40-41 minutes. For the first generation period of treatment, a straight line is produced, which is thus consistent with the model. At this point (where ti = g) the value of the ratio is 3.09. From equation (6), this gives an estimate of the value of C/g = 0.94, which means that g is predicted to be $2\frac{1}{2}$ minutes greater than C under these growth conditions. This is reasonably close to the value of C estimated from different criteria in Table 1.

The isolated point in Figure 9, where g was 70 minutes, gives a value of C = 45 minutes (from Equation (6)). This is consistent with the value of C increasing somewhat with g, when g is greater than about 60 minutes (Cooper & Helmstetter, 1968). The result for thymine starvation is well below prediction, and will be considered later.

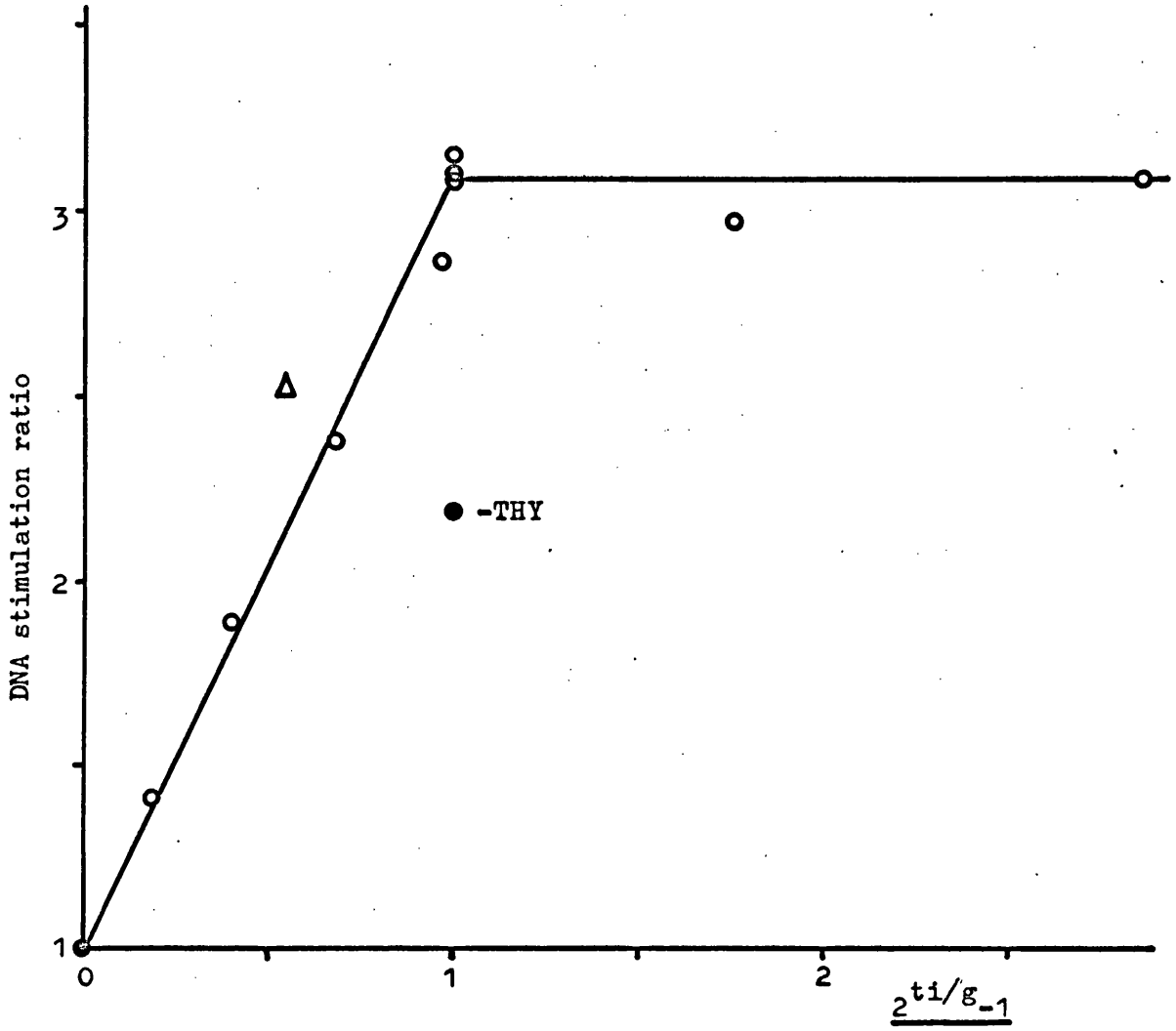


Figure 9 The relationship between the duration of DNA inhibition and the resultant stimulation of DNA synthesis.

Ordinate = ratio of rate of DNA synthesis in the pretreated culture to that in the control. ti = time of DNA inhibition; g = mean generation time of the culture.

○ = nalidixate treatment, g = 40-41 min.

Δ = " " g = 70 min. (from Figure 2).

● = thymine starvation, g = 40 min. (from Figure 14).

A surprising result is the plateau found in Figure 9 when ti is greater than g. It was pointed out that mass increase in the presence of nalidixate does not cease at ti = g. A similar result was obtained by Pritchard & Lark (1964) for thymine starvation. This could be interpreted as meaning that there is:

(a) a physical restriction of some kind preventing a third round of initiation on a chromosome already engaged in two cycles of replication. Such an initiation would increase the number of origins from 4 to 8; would these all be able to remain attached to the terminus?

or (b) a limitation in the supply of DNA precursors, preventing DNA synthesis at a rate faster than that carried out by (fortuitously) about three replication forks. This may apply specifically to the uptake of thymine and conversion to its nucleotides (see Chapter III). Further work is necessary to distinguish between these alternatives.

(7) The use of 5-bromouracil as a density-label

Criticism has been levelled against the use of BU as a density-label (Maaløe & Kjeldgaard, 1966) because of its toxity to E.coli (Hanawalt et al, 1961). The loss of viability of $15T^-$(555-7) on transference to BU-containing medium is shown in Figure 10, where it is compared with the thymineless death of the same culture.

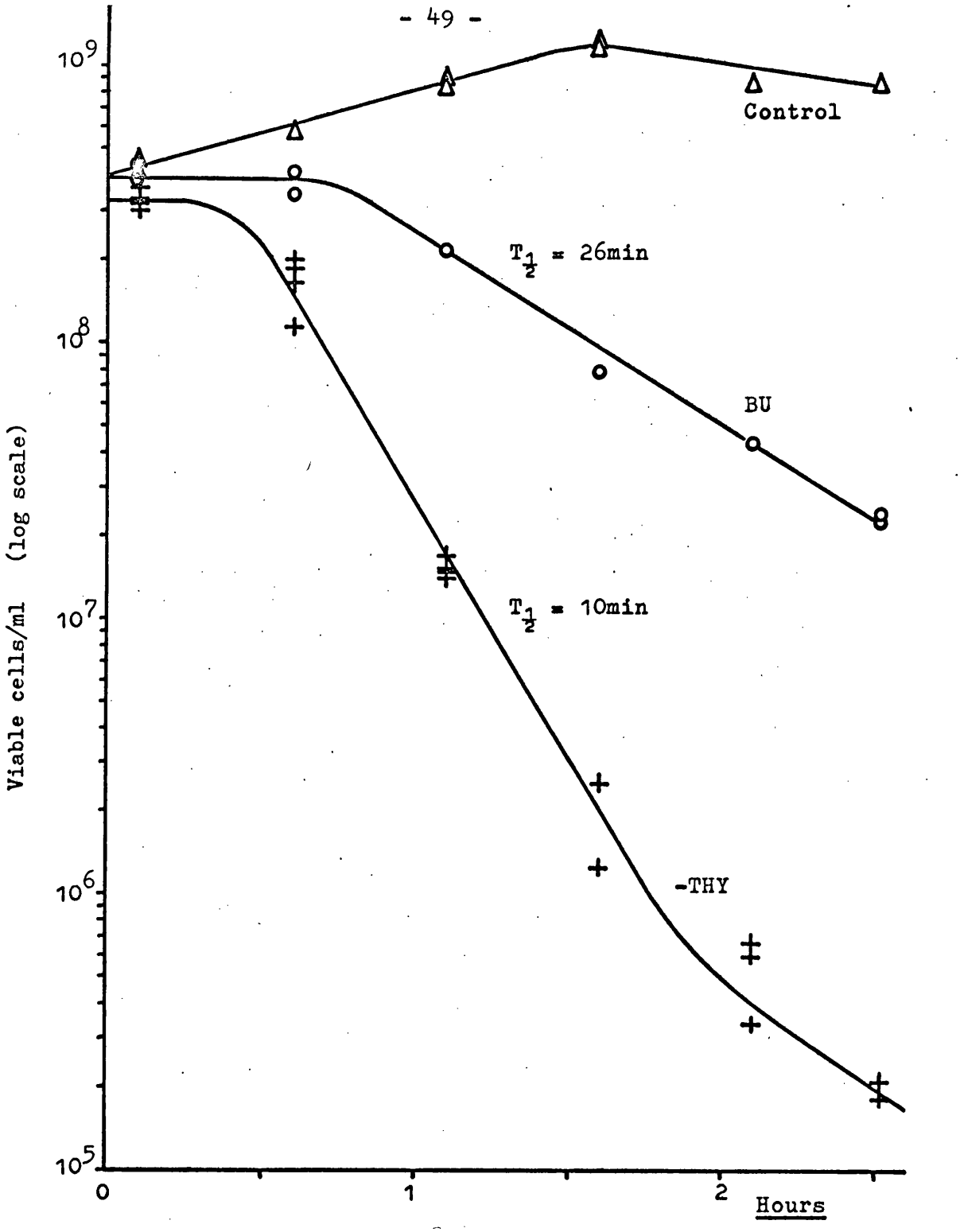


Figure 10 An exponential-phase culture of $15T^-$(555-7) was divided and transferred to medium lacking thymine (-THY) or with 20μM BU replacing thymine. Measurement of viability is described in Methods. $T_{\frac{1}{2}}$ = half life.

The rate of BU death is markedly less than that of thymineless death and the initial lag before its onset is longer. No death occurs until about 45 minutes, by which time the density-label experiments (Figures 3 and 4) were completed. Nevertheless, it is necessary to consider whether the use of BU significantly affected the conclusions drawn from these experiments.

The data shown in Figure 4 have been replotted in Figure 11 as the percentage of the ^{14}C label that had become hybrid in density, against $2^{t/g} - 1$. This gives a measure of the rate of DNA synthesis in the three cultures after transfer into BU-containing medium.

The control culture replicated DNA exponentially throughout the sampling period, but the rates in the pretreated cultures declined progressively from this exponential rate. This is in contrast to the results described above: a stimulated rate of DNA synthesis is found in the pretreated cultures when replication takes place in the presence of thymine. Thus BU affects the kinetics of replication but the important point to establish is whether it alters the pattern of replication.

Experiments conducted by Hewitt, Billen & Jorgensen (1967) were designed to answer this point. As mentioned in section (3) of this chapter, they noted that the pattern of replication in BU-medium of a culture was altered following irradiation of the culture. They found substantially the same pattern using ^{13}C and ^{15}N as density-labels in place of BU, and thus concluded that it

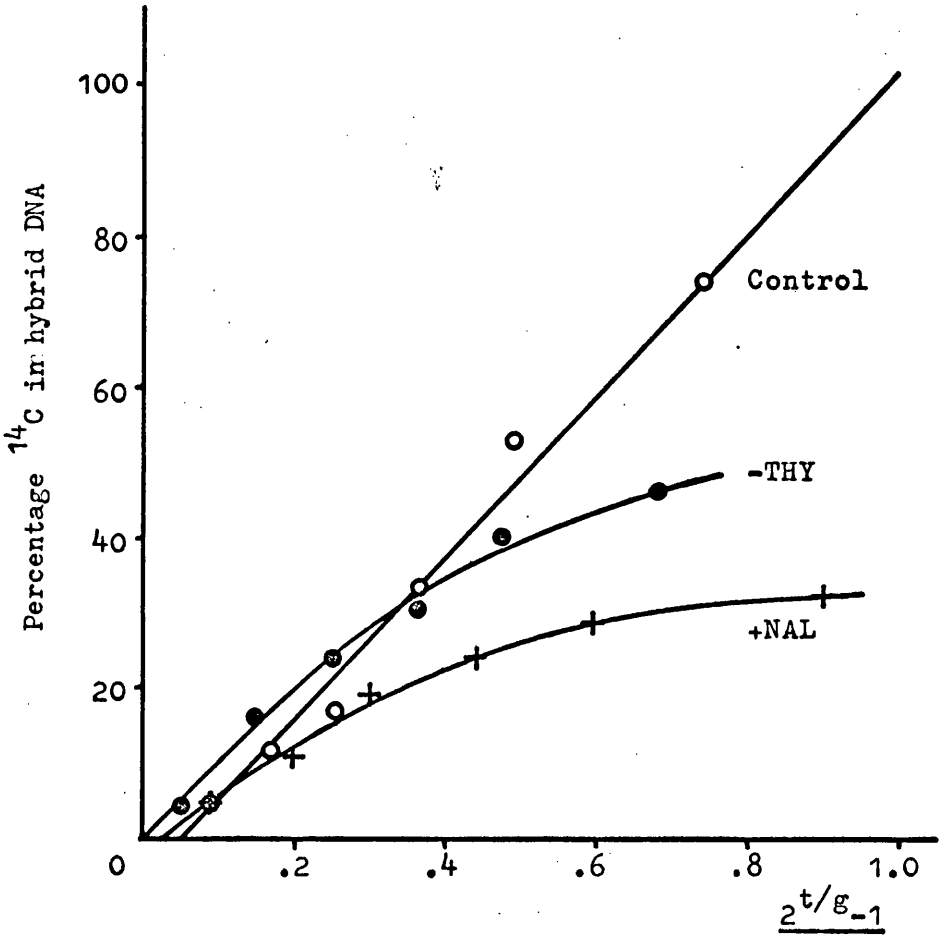


Figure 11 DNA replication of $15T^-$(555-7) in BU-medium: data taken from Figure 4. The cultures used were: a control and two cultures pretreated for 40 minutes with nalidixate (+NAL) or by thymine starvation (-THY). g = 40 min.

was not an artifact produced by BU, despite the fact that the rate and extent of DNA replication is reduced in medium with BU substituted for thymine. More recently, Eberle (1968) repeated two of the experiments on sequential replication of Lark, Repko & Hoffman (1963) also using ^{13}C and ^{15}N instead of BU. The patterns of replication she observed were precisely the same as those of Lark et al, (1963).

BU has been shown to cause reinitiation in a culture after a period of incubation (Abe & Tomizawa, 1967). This effect would also apply to the control culture in the experiment shown in Figure 4. Thus despite this effect, BU's toxity and its reduction of the rate of DNA synthesis, it does not alter the pattern of replication significantly in the type of experiment considered above.

(8) The effect of 5-bromouracil on the kinetics of DNA synthesis after nalidixate treatment

The inhibitory effect of BU on DNA synthesis was further studied as the mechanism of this effect is not understood. The kinetics obtained in Figure 11 are only approximate because the experiment was not designed for this purpose. The method described in section (4) was used for further experiments, resuspending the cultures after nalidixate treatment in medium containing ^{14}C-BU (20 μM, 0.08 μC/ml) or, as a control, ^{14}C-thymine (also 20 μM, 0.08 μC/ml). Samples were taken to measure their incorporation

into DNA. The results of such an experiment are shown in Figure 12.

40 minutes pretreatment with 50 µg nalidixate/ml (40' Nal, into thy) caused an initial stimulation of DNA synthesis (3.1 times the control rate) as found in previous experiments. The same pretreated culture in BU medium (40' Nal, into BU), synthesized DNA at only a third of this rate, however. The "BU control" rate was also less than the corresponding "thymine control" rate (about 75% of it), and it did not remain exponential throughout. A decline was seen after about 70 minutes (ie. less than two generation periods).

Another effect that BU has, is to shift to a later time the first decrease in rate in the pretreated cultures (discussed in section (4) above). As was seen in Table 1, this point extrapolates to 40 minutes for the culture growing in thymine, but in BU it is delayed to 56 minutes after nalidixate removal. A delay is predicted on the model discussed in section (4): the time of the decrease is argued to be equal to C, thus if BU reduces the rate of replication, C will be greater, and so the decrease will come later. As the BU control rate also declines, however, 56 minutes may be an underestimate of C (during replication in BU).

The results show that substituting BU for thymine causes a proportionally greater inhibition of the rate of DNA synthesis when the number of replication forks has been increased by nalidixate pretreatment. As it has been shown that BU does not

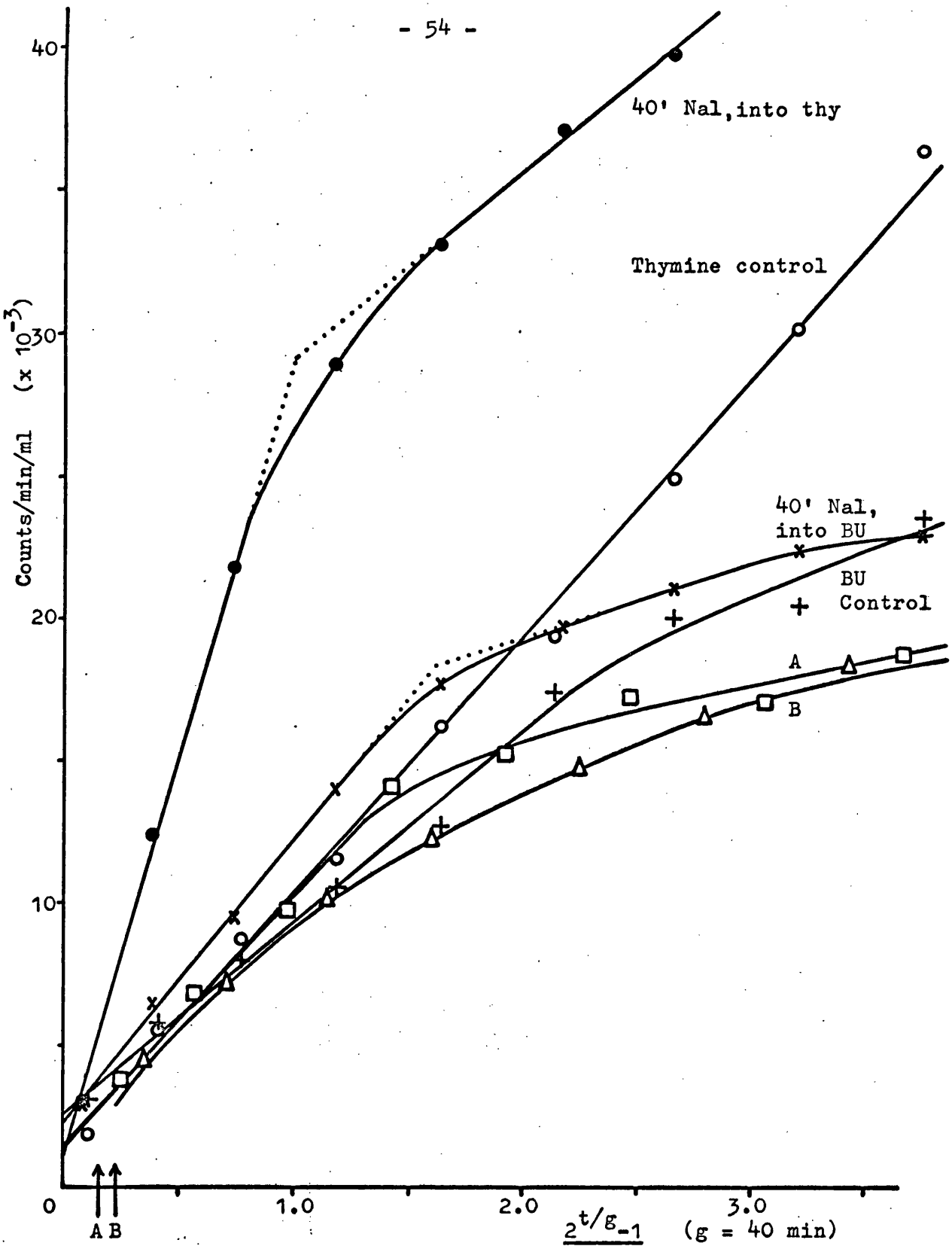


Figure 12 The effect of BU on the rate of DNA synthesis after nalidixate treatment. Description is in text.

alter this pattern of replication, ie. does not preferentially prevent or impair replication at these newly induced forks, it seems probable that this effect is due to substrate limitation. That is, the rate of conversion of BU to its nucleotides becomes limiting when replication is taking place with more than one replication fork per chromosome. An alternative, that BU reduces the rate of replication at the polymerase level, would not give proportionally greater inhibition during multifork replication. The experiments of Richardson, Schildkraut & Kornberg (1963) also weigh against this alternative as they found very little reduction, in vitro, of the rate of incorporation of dBUTP (compared to dTTP) by the DNA polymerase of E.coli.

The two lines, labelled A and B in Figure 12, have not been mentioned yet. Although previous experiments strongly suggested that BU does not prevent replication of the newly-initiated forks, this point was directly tested, by allowing a period of replication in thymine, after the nalidixate treatment, before transfer into the ^{14}C-BU medium. This will shift the forks from the origin and thus allow replication in BU at the stimulated rate, if BU is restricting only newly-initiated replication. A and B are portions of the nalidixate-treated culture (40' Nal) that were incubated for 8 and 12 minutes respectively, in medium containing unlabelled thymine, before being transferred into the ^{14}C-BU medium. This does not stimulate the rate of DNA synthesis above that of the culture without this period of growth in thymine (ie. the "40' Nal, into BU" curve).

This type of experiment also eliminates another possibility which is, that after nalidixate treatment, repair of the bacterial DNA is necessary to obtain the full replication rate observed, the substitution of thymine by BU restricting this process and thus reducing the replication rate. The experiment was repeated, allowing longer periods of growth in ^{14}C-thymine (10 and 20 minutes) before transfer to ^{14}C-BU medium, in case the 12 minutes used before was not sufficient to allow significant repair. The results, presented in Figure 13, show that the periods of growth in thymine do not permit replication in BU medium at the stimulated rate. The change to BU medium did not instantaneously decrease the rate: this may be due to the utilization of a ^{14}C-thymine nucleotide pool in the cell.

The effect of BU will be considered further in the next section.

(9) Comparison between the effects of nalidixate treatment and thymine starvation on the subsequent DNA replication

The density-label experiments (Figures 3 and 4) demonstrated that thymine starvation is less effective than nalidixate treatment in causing reinitiation of replication. Pritchard & Lark (1964) found an approximate doubling of the rate of DNA synthesis after 40 minutes thymine starvation, whereas, the results in Figure 9 show slightly more than a threefold stimulation after this period of nalidixate treatment. The effects of the two treatments were

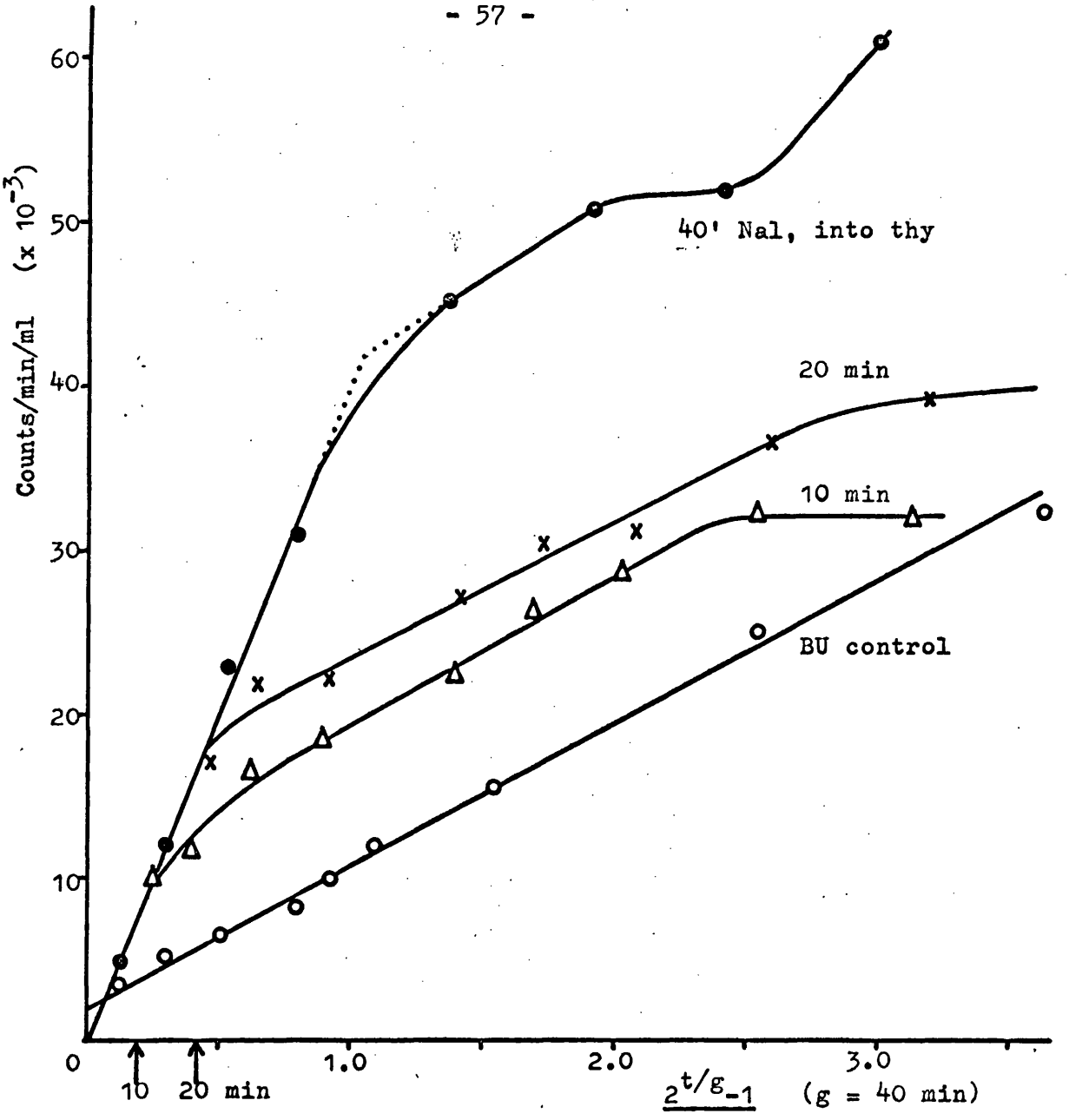


Figure 13 The effect of growth in thymine medium on the rate of DNA synthesis, before transfer of a nalidixate-pretreated culture into BU-medium. BU control = untreated culture of $15T^-$(555-7) in medium containing ^{14}C-BU (20 µM, 0.08 µC/ml); 40' NaI, into thy = culture pretreated for 40 min with 50 µg nalidixate/ml in ^{14}C-thymine (20 µM, 0.08 µC/ml); 10 min and 20 min = portions of previous culture transferred to ^{14}C-BU-medium after 10 and 20 min respectively.

therefore re-examined to make certain that this difference is real.

(9a) The rate of DNA replication

Kinetic experiments of the type described in sections (4) and (8) above were carried out, using the two treatments. The effect of BU on the kinetics of DNA replication after thymine starvation was also examined to see if the BU effect is specifically associated with nalidixate treatment or not. The results of these experiments are shown in Figure 14, where the abbreviations used denote the following:

"Thymine control" and "BU control" - the incorporation into acid-insoluble material of ^{14}C-thymine and ^{14}C-BU respectively (20 μM, 0.08 μC/ml), by an untreated exponential-phase culture of 15T$^-$(555-7) (at 1.4 x 10^8 cells/ml);

"Nal 40', into T" - the incorporation of ^{14}C-thymine by this culture after 40 minutes nalidixate pretreatment (50 μg/ml);

"-T 40', into T" and "-T 40', into BU" - the incorporation of ^{14}C-thymine and ^{14}C-BU respectively after a 40 minute period of thymine starvation;

"-T 40', +T 10', into BU" - the incorporation of ^{14}C-BU by the previous culture, but after a further 10 minutes incubation in unlabelled thymine-medium.

40 minutes thymine starvation or nalidixate treatment gave, respectively, a 2.19 and 3.08-fold stimulation of the control rate of DNA synthesis (with thymine as substrate). This

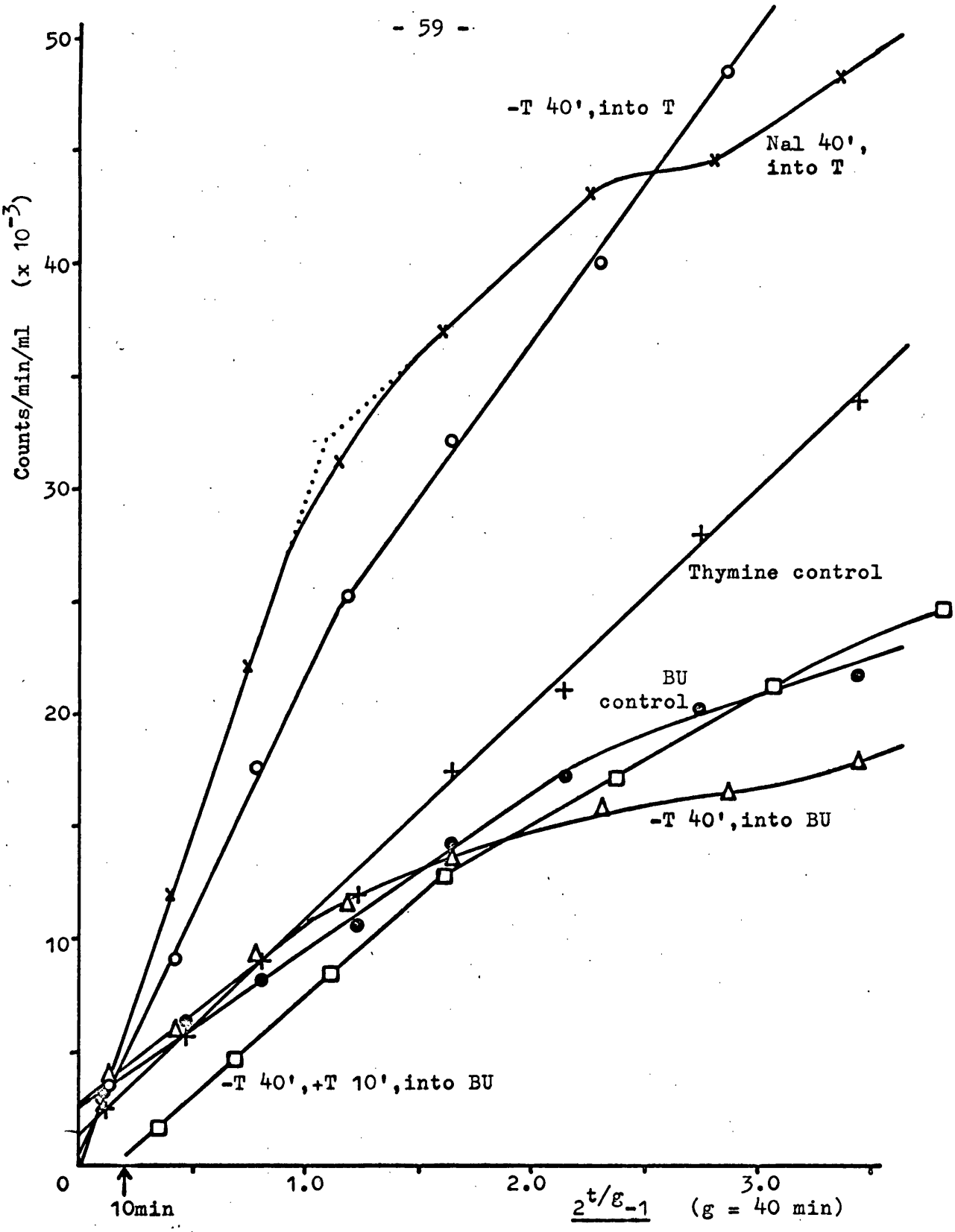


Figure 14 Effect of thymine starvation on the subsequent rate of DNA synthesis and the effect of BU on this; description in text.

confirms the previous findings that these treatments have different quantitative effects.

The results in Figure 14 also show that the effect of BU on DNA replication after thymine starvation is similar to that found following nalidixate treatment (Figures 12 and 13). The rate of DNA synthesis of the control culture in BU medium was 72.5% of the rate in thymine (similar to Figure 12). After thymine starvation, the rate in BU was hardly stimulated (1.12 times the BU control), and was thus only 37% of the rate in thymine. A repeat curve (not shown) for the replication in BU of the nalidixate-pretreated culture, gave a similar rate to that shown in Figure 12. A 10 minute period of growth with thymine, after thymine starvation and before transfer into BU-medium, allowed only a marginally greater rate of DNA synthesis (1.26 times the BU control). This inhibition by BU seems, therefore, to be unaffected by such treatments, and not dependent on whether stimulation was induced by thymine starvation or nalidixate treatment. This is consistent with the previous suggestion that this inhibition is inherent in the use of BU, because it has a lower rate (and lower maximum rate) of conversion into its deoxynucleotide triphosphate. In support of this theory is the observation of Hackett & Hanawalt (1966) on the selectivity that 15TAU bar exhibits for thymine over BU incorporation, and also that of Razzel and Khorana (1958) that BU has only a third of the reaction rate of thymine, when used as a substrate for the enzyme, thymidine phosphorylase, extracted from E.coli (see Chapter III).

(9b) The amount of DNA synthesized in the absence of required amino acids

Section (4) of the Introduction discussed the evidence for assuming that in cultures deprived of required amino acids, existing cycles of DNA replication are completed, but new ones cannot be initiated. A measurement of the amount of DNA synthesized during this deprivation can thus be used to estimate the number and distribution of replication forks in existence at the onset of starvation.

A culture of 15T⁻(555-7) was uniformly labelled by growth with ^{14}C-thymine. Its further uptake of ^{14}C-thymine was measured during amino acid starvation without pretreatment, and after 40 minutes thymine starvation or nalidixate treatment. The results are shown in Figure 15. The percentage increments of DNA synthesized during starvation were:

control	-	82.5%
prestarved of thymine	-	247%
pretreated with nalidixate	-	293%

These increments are considerably higher than expected. A culture with g = 40 minutes is predicted to produce an increment of 40% (Maaløe & Hanawalt, 1961). Reinitiation at half, and at all, of the available origins in such a culture is predicted to increase the increment to 110% and 180% respectively. (These and other increments have been calculated using the equation derived in Appendix 4). The high increments suggest, perhaps that the culture

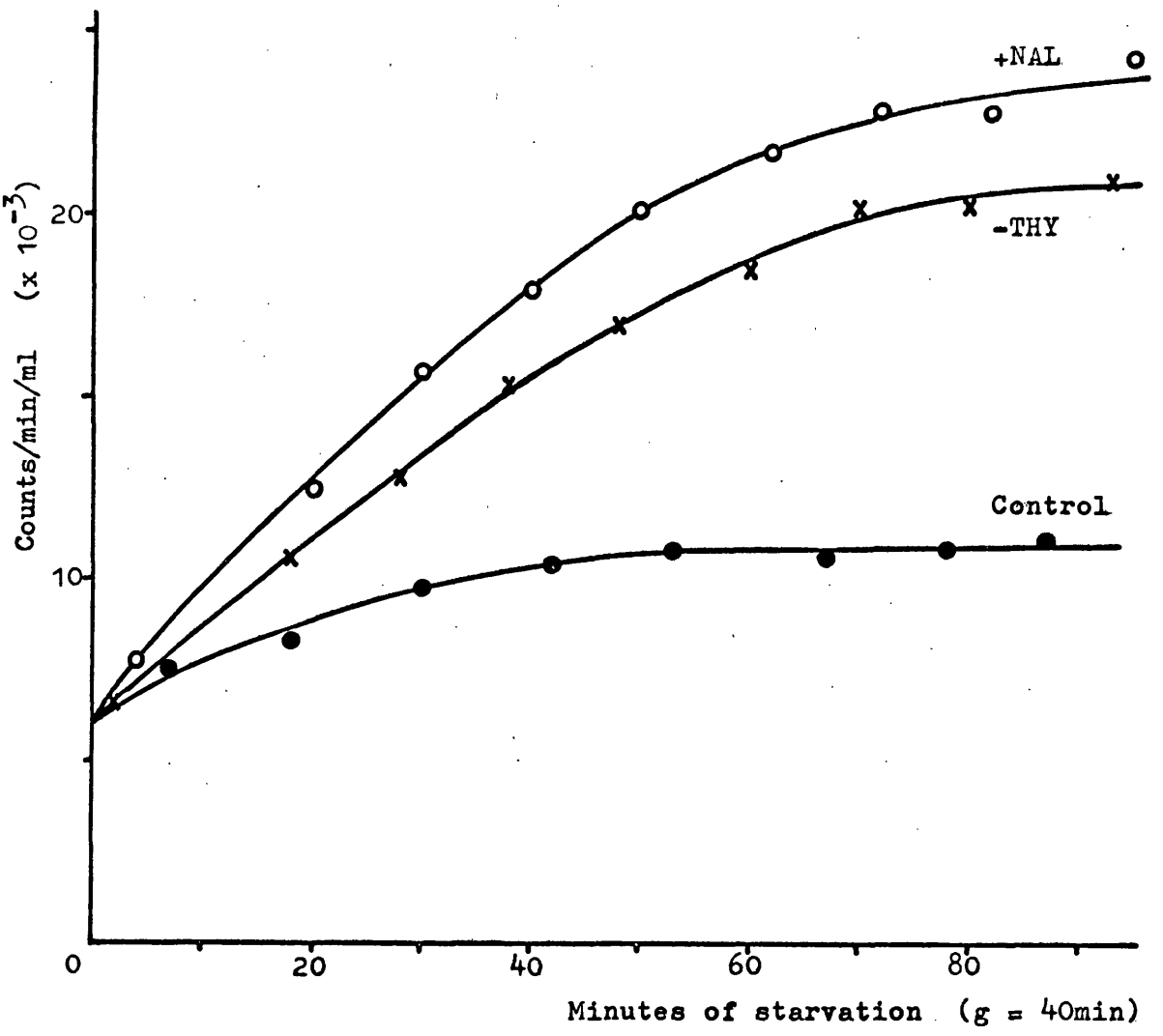


Figure 15 DNA synthesis during amino acid starvation in previously thymine starved (-THY) or nalidixate treated (+NAL) cultures of $15T^{-}$(555-7). This strain was prelabelled by five generations of growth in medium containing ^{14}C-thymine (20 µM, 0.08 µC/ml). At a titre of 3 x 10^{8} cells/ml, a portion was transferred to the same medium but lacking amino acids. Two other portions were respectively thymine starved or treated with 50 µg nalidixate/ml for 40 minutes, and then also transferred to this medium. The incorporation of label into DNA was measured as in Methods.

was growing with a multifork replication pattern. For example, putting C = 80 minutes with g = 40 minutes gives expected increments of 85%, 177% and 270% for the control, half reinitiation and full reinitiation respectively. These values are much closer to my results than the first set of values.

However, we must consider how meaningful these observed increments are. There is a great deal of discrepancy in the literature on the value of the increment. Thus Lark & Lark (1964, 1965) report increments of about 40% during the starvation of strain 15T⁻(555-7), while Billen & Hewitt (1966) report 74% for the same strain. The growth rates reported were g = 40 minutes and 45 minutes respectively. Thus the increment obtained by Billen & Hewitt is expected to be less than that of Lark & Lark, if anything. The increments reported by Maaløe & Hanawalt (1961) for 15TAU⁻ vary between 40 and 55%. The precise conditions of starvation are probably very important to the value obtained. It has been shown, contrary to the expectations of Maaløe & Hanawalt's model, that the increment depends on which amino acid, or combination of amino acids, is withdrawn (Billen & Hewitt, 1966).

Since there is good alternative evidence that the amino acid starvation model is correct (Wolf et al, 1968, Cerda-Olmedo et al, 1968), the simplest way to account of the discrepancies is probably to consider that the minimum increment obtained is meaningful, and that higher increments are due to growth not being entirely prevented, some initiation of new cycles thus occurring.

Only a few percent of the cells in the population behaving this way would make a large difference to the increment. The minimum increment of 40% for $15T^-$(555-7) (Lark & Lark, 1964, 1965) predicts that C is about 40 minutes (Appendix 4) which is in agreement with estimates of C in sections (4) and (6). A multifork replication pattern (as favoured by Billen & Hewitt, 1966) however, would give C a larger value than this, and thus not be compatible.

In the experiment shown in Figure 15, the cell titre at the start of the experiment was greater than 3×10^8 cells/ml, which was rather high for rapid and efficient filtration and washing. All this leads me to consider it reasonable then, that between 30% and 40% of the cells in each culture reinitiated new cycles of replication during the period of starvation: 30% in the control culture giving a theoretical increment of 80%, and 40% reinitiation in the nalidixate-pretreated culture, (taking it that this has already undergone complete reinitiation during the treatment) giving an increment of 290%. A similar level of reinitiation during amino acid starvation in the thymine-starved culture, would mean that thymine starvation had effected about 70% reinitiation.

Whether one accepts these complexities of interpretation or not, two conclusions arise from these results:

1. Both thymine starvation and nalidixate treatment increase the number of replication forks on the chromosomes. This supports the conclusion based on the density-label experiments and on the observed kinetics of DNA synthesis, that these two treatments

cause reinitiation of chromosome replication.

2. Nalidixate treatment is more effective in this than thymine starvation, thus supporting the conclusion of the preceding section that this difference is real and reproducible in $15T^-$(555-7).

(9c) Mass increase during thymine starvation

Because of the difference between nalidixate treatment and thymine starvation, it was interesting to observe whether there is a similar difference in the mass increase of a culture during the two treatments. In Figure 16 is shown the increase in $A_{600\ m\mu}$ of a culture of $15T^-$(555-7) during an hour's thymine starvation. (Thymine was restored to the medium after this for another purpose - section (3a), Chapter II). The mass increase during nalidixate treatment was shown in Figure 8. Comparison of the two graphs reveals some difference between them, but nothing substantial. The thymine-starved culture's mass falls below the extrapolated exponential rate sooner than the other, but the difference is small up to 40 minutes, which was the time most commonly used. At this point the thymine starved culture has increased its mass by 89% (100% for the extrapolate) compared to 100% for the nalidixate-treated culture.

Let us compare this difference with the difference in effectiveness of the two treatments in causing reinitiation. From Figure 4 on reinitiation, Figure 14 on the stimulation of DNA synthesis, and consistent with the data of Pritchard & Lark (1964), it seems that thymine starvation is only about 50-60%

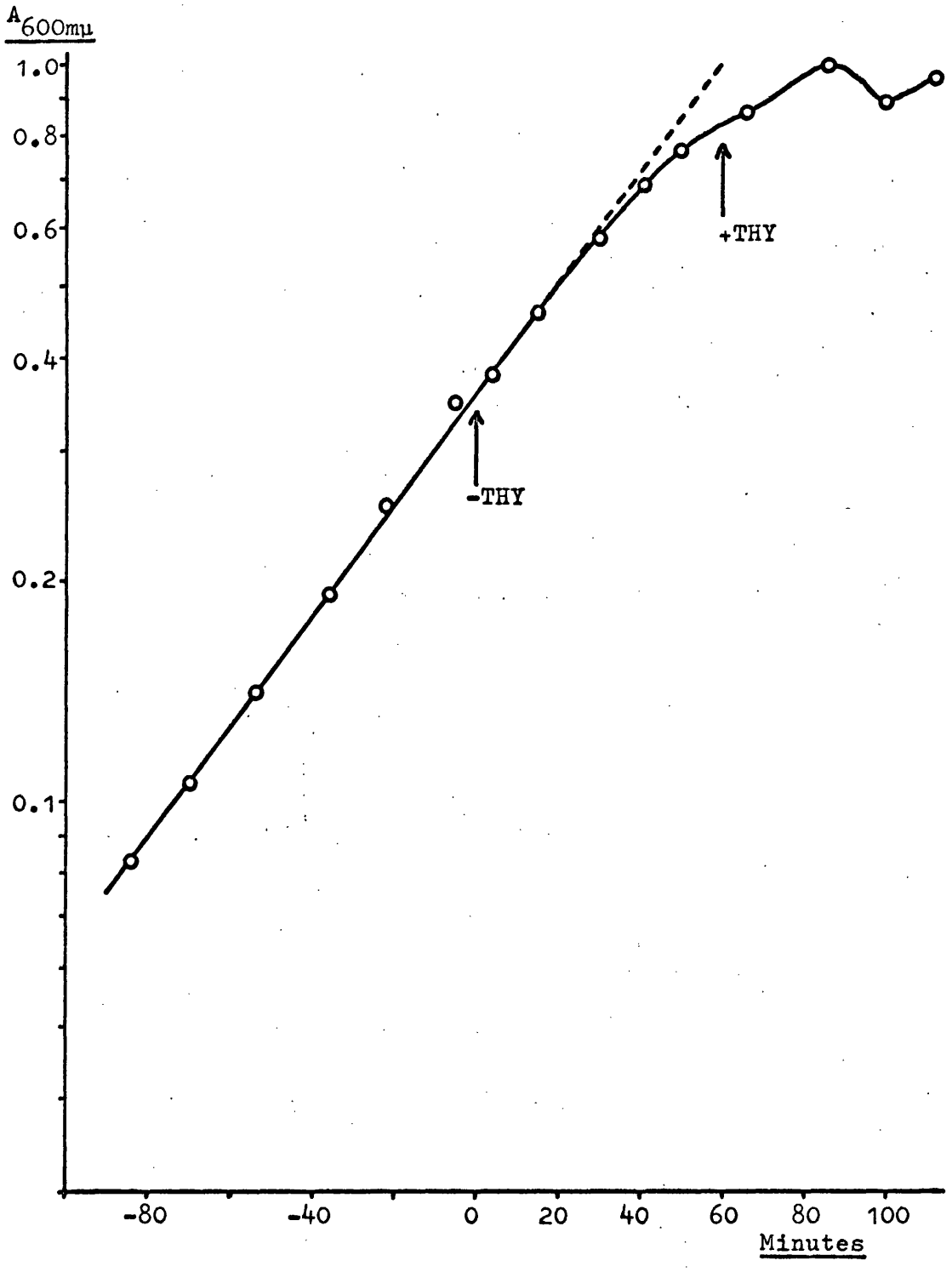


Figure 16 The effect of thymine starvation on the mass ($A_{600\ m\mu}$) increase of an exponential-phase culture of $15T^-$(555-7). Thymine was removed (-THY) and restored (+THY) at times shown.

as efficient in causing reinitiation of replication as a similar period of nalidixate treatment. Thus on the model discussed in section (6) and Appendix 3, the difference in the kinetics of mass (or rather $A_{600\ m\mu}$) increase cannot provide a sufficient explanation for the difference in effectiveness in inducing reinitiation.

It has been suggested (Pritchard, Barth & Collins, 1968) that the lower effectiveness of thymine starvation may be caused by a DNA precursor limitation effect (analogous to that described for the BU effect). The period of starvation may upset the deoxynucleotide control system (which is known to involve control by a thymine deoxynucleotide - see Chapter III) and thus reduce the rate at which thymine can be incorporated or perhaps some other deoxynucleotide be synthesized. This mechanism could explain the lower stimulation of DNA synthesis caused by thymine starvation (Figure 14), but unless the precursor limitation preferentially affected the newly initiated replication forks, it would not explain the difference between the two treatments observed in Figure 4 on the extent of reinitiation, or the data of Pritchard and Lark (1964).

Three other points argue against this scheme. In Figure 14 it can be seen that the decrease of rate in the thymine starved and nalidixate treated cultures come at 43.8 and 42.1 minutes respectively. From the arguments in section (4) this suggests that the replication rate of the newly induced forks is about the

same after both treatments. The precursor limitation model would predict the decrease to come at about 60 minutes in the thymine starved culture. Secondly, it is difficult to see why an upset deoxynucleotide control system would not return quickly to normal when thymine is replaced. Finally, it seems more likely that thymine starvation would increase the availability of DNA precursors, through derepression of ribonucleotide reductase (Biswas, Hardy & Beck, 1965), rather than decrease it.

For the above reasons, it seems to me that thymine starvation is more likely to have the effect shown diagramatically in Figure 5,B, ie. the cell population behaves heterogeneously. Consistent with this are the observations of McFall & Magasanik (1964) on the capacity of individual cells in a culture of $15T^{-}PA^{-}$ starved of thymine for 60 minutes, to synthesize protein. They found that about 40% of the cells lost this capacity while the remainder continued to synthesize protein. The cause of this heterogeneous response is unknown, but it could lead to a similar response to reinitiation of DNA replication.

C H A P T E R II

Studies on the Properties of Nalidixate

Nalidixate has played a central role in the preceding experiments on the control of initiation. Some of its properties, such as its specificity of action in inhibiting DNA synthesis, and the speed and reversibility of this effect, have already been described. None of the work published on the effects of nalidixate (Goss et al, 1964, 1965; Deitz et al, 1966; Cook, Deitz & Goss, 1966; Cook, Goss & Deitz, 1966; Boyle, Goss & Cook, 1967; and Taketo & Watanabe, 1967) has, however, given any information on the mechanism of its action. It is clear from the references above, that both bacterial and phage (Taketo & Watanabe, 1967) DNA synthesis are inhibited, and that this effect is not dependent on protein or RNA synthesis (Deitz et al, 1965).

(1) In vitro DNA-nalidixate interaction: a spectroscopic test

A possible mechanism by which nalidixate could cause inhibition of DNA synthesis, is by combining with DNA in some way. If such a combination involves a hypochromicity effect, this would provide a simple means for its detection.

Nalidixate (Figure 17) shows a strong absorption in the ultraviolet region with a maximum at about 258 mμ. The molar absorptivity at 260 mμ is 2.5×10^4 (95% of the maximum), 260 mμ is therefore a suitable wavelength to use.

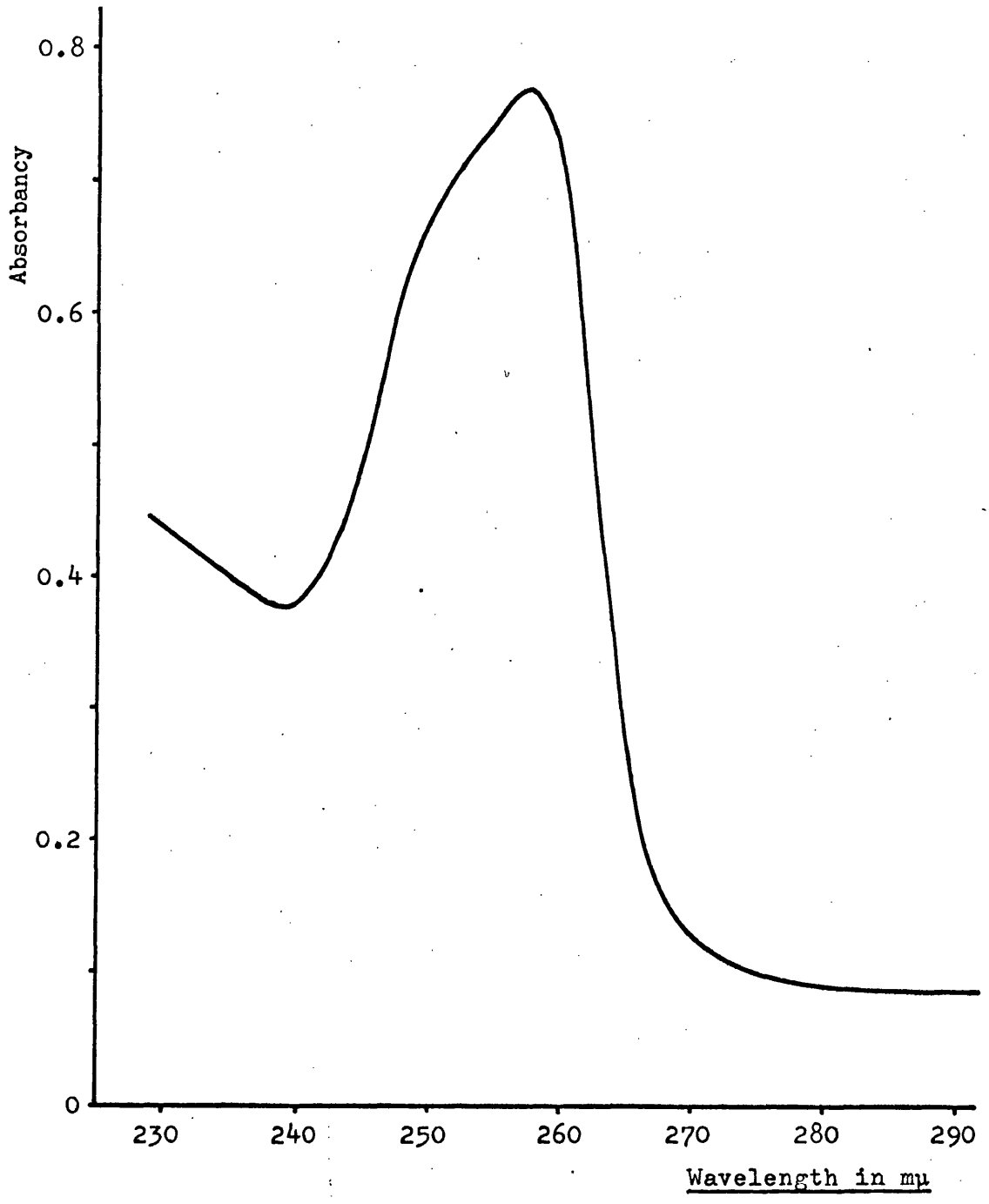


Figure 17 The absorption spectrum of a 28.8 μM solution of sodium nalidixate at pH 6.2-6.3, measured with a Unicam SP500 spectrophotometer. The light path was 1 cm.

The separate absorptions, at this wavelength, of a solution of nalidixate (about 10 μM) and of DNA (about 10 μg/ml, isolated from *E.coli* 15TAU$^-$ as described in Methods) were measured. Equal volumes of the two solutionswere mixed together and their absorption measured again. No hypochromicity effect was detectable. This does not rule out DNA-nalidixate interaction as too few nalidixate molecules may combine to be detectable, or combination may take place without hypochromicity, or nalidixate may not combine *in vitro*.

(2) DNA degradation

A property of nalidixate, mentioned previously, is its induction of DNA degradation. In this it mimics the selective DNA inhibitors - mitomycin C and ultraviolet radiation, but unlike mitomycin C-induced breakdown, which is enhanced by the inhibition of protein synthesis with chloramphenicol (Constantopoulos & Tchen, 1964), nalidixate-induced breakdown is restricted by the latter treatment (Cook, Deitz & Goss, 1966). Indeed, these authors showed that in *E.coli* 15TAU$^-$, amino acid starvation almost completely abolishes both nalidixate-induced DNA breakdown and loss of viability.

It was considered interesting and necessary to repeat these studies on *E.coli* 15T$^-$(555-7), because at the time my experiments on the stimulatory effect of nalidixate on DNA synthesis had been carried out (with this strain) my observations

had not been confirmed by the Sterling-Winthrop group (Goss et al, 1965; Deitz et al, 1966*) working with 15TAU$^-$, although a recent paper has confirmed them (Boyle et al, 1967).

Differences in the extent of DNA degradation in these two strains could obviously alter the subsequent stimulatory effect. The degradation of prelabelled DNA was measured in the absence (Figure 18), and presence (Figure 19) of amino acids. The degradation induced by nalidixate was compared with that induced by ultraviolet radiation (see Figure 22 for the ultraviolet death kinetics of 15T$^-$(555-7)). The DNA degradation occurring in an irradiated culture during incubation with nalidixate was also measured. The DNA was prelabelled by growing the culture through at least four generations with ^{14}C-thymine (10 µM, 0.1 µC/ml). At a cell titre of 2-3 x 10^8 cells/ml, the culture was filtered and resuspended in M9 salts medium without glucose or supplements. A sample was taken, to measure the total radioactivity incorporated by the culture. The culture was divided, and half was irradiated with an ultraviolet dose of 1000 ergs/mm^2. Each half was further divided, one portion of each being supplemented with 50 µg nalidixate/ml. In the experiment shown in Figure 18, the only further supplement was thymine (to give 20 µM), whereas in the experiment shown

*Actually Figure 5 in Deitz et al, 1966, shows some stimulatory effect although it is not mentioned in their text.

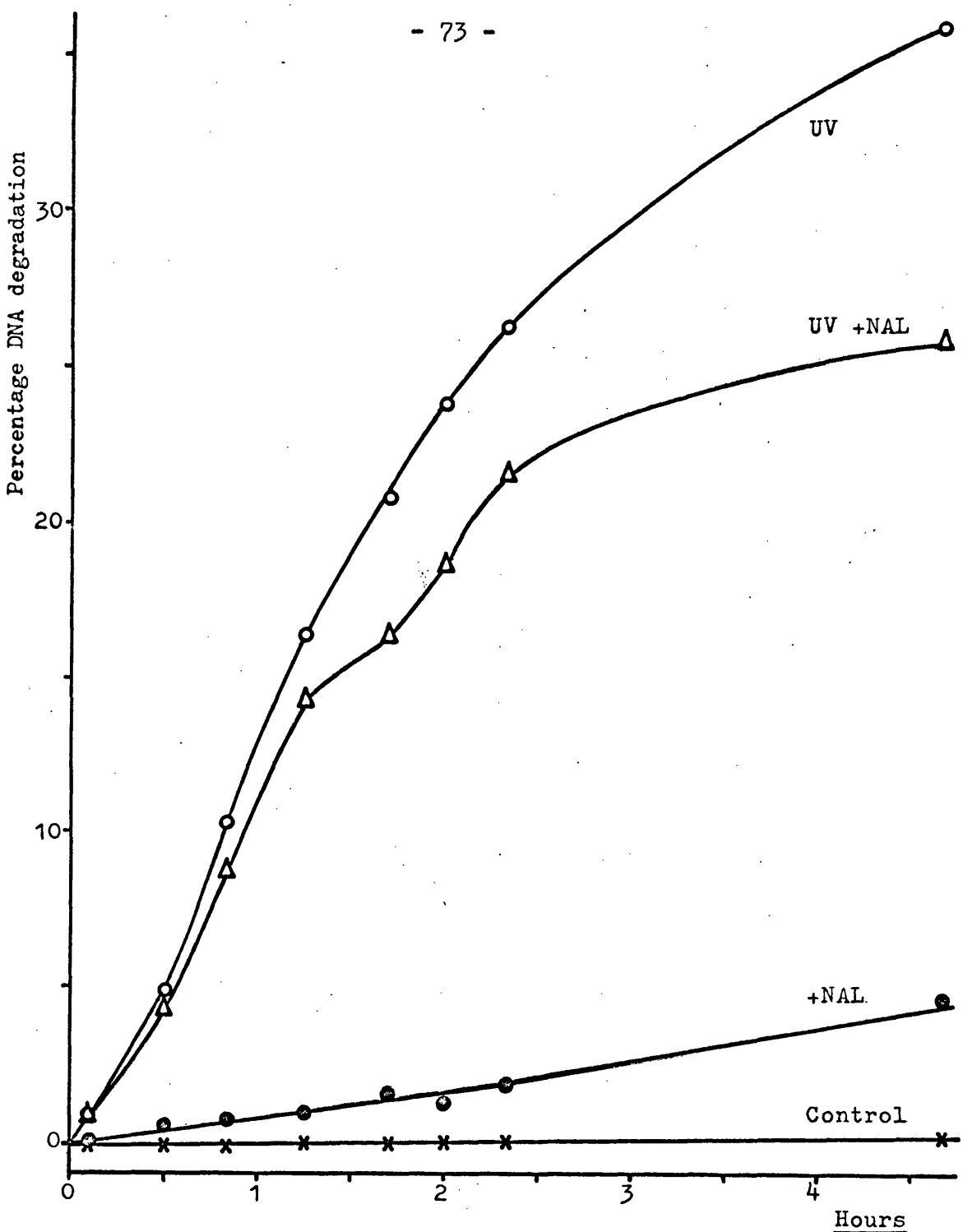


Figure 18 The release of acid-soluble radioactivity from prelabelled DNA, during the incubation of a culture of 15T⁻(555-7) in the absence of glucose and amino acids, and in the presence of 50 µg nalidixate/ml (+NAL), after a dose of 1000 ergs/mm^2 of ultraviolet irradiation (UV), and after this dose in the presence of nalidixate (UV +NAL). Total radioactivity incorporated was 44,200 counts/min/ml.

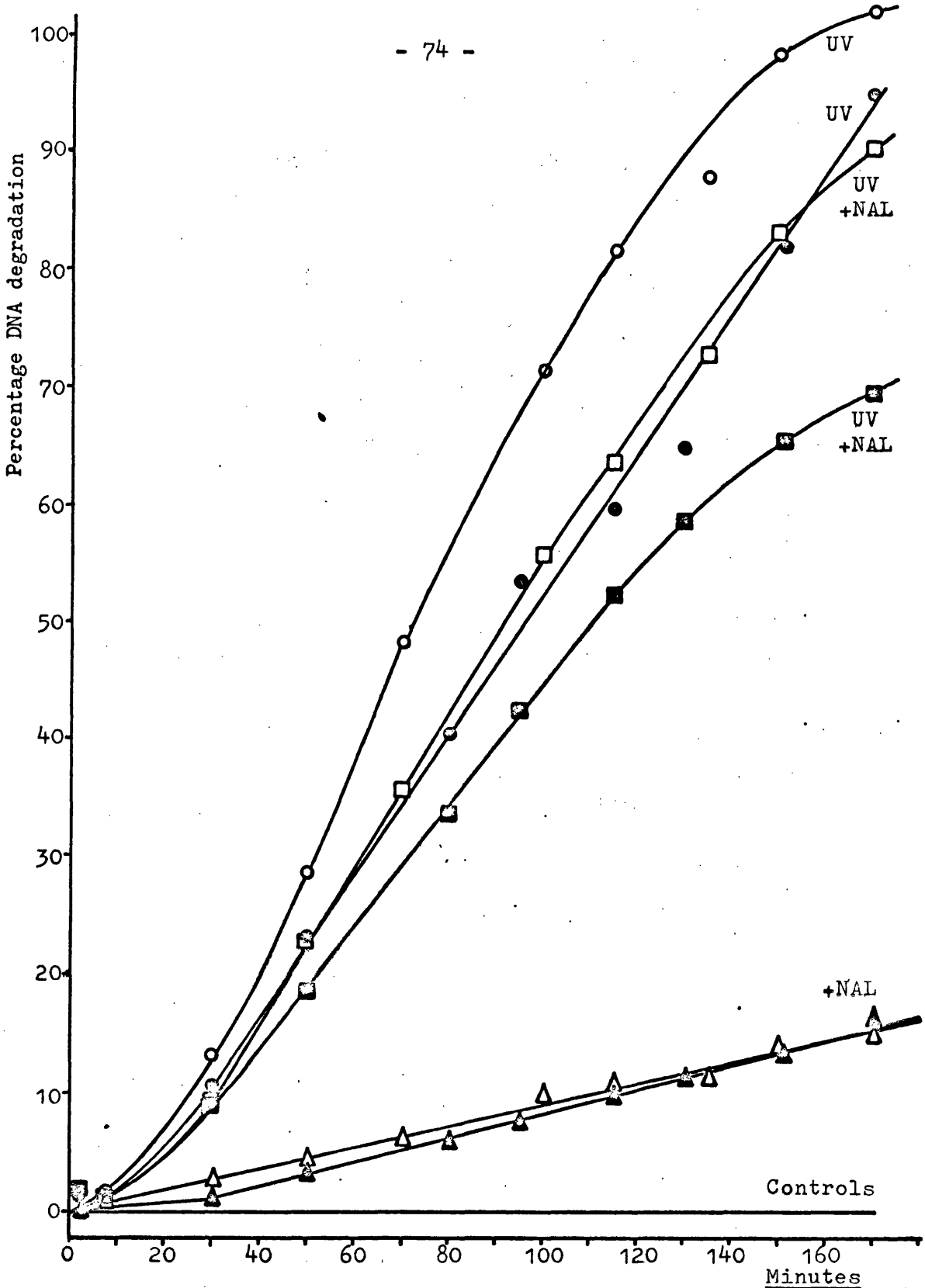


Figure 19 DNA degradation in 15T⁻(555-7) (open symbols) and 15TAU⁻ (closed symbols) during incubation in full growth medium, with the same treatments as in Fig.18. Total radioactivity incorporated was (555-7) - 29,300 cts/min/ml and TAU⁻ - 50,000 cts/min/ml.

in Figure 19, supplements were added to give the usual growth medium. All the cultures were then further incubated, samples being taken at intervals to determine the release of acid-soluble radioactivity (as described in Methods). This is expressed in the Figures as a percentage of the initial acid-insoluble radioactivity.

Several conclusions emerge from the results which are shown in Figures 18 and 19. Broadly, the findings of Cook, Deitz & Goss (1966) are confirmed: treatment of these strains with nalidixate results in degradation of their DNA, this being approximately 6 times as fast in full growth medium as in glucose and amino acid deficient medium. They obtained a rate of degradation about twice as fast as that shown in Figure 19, however. A second point is that the ultraviolet-induced DNA degradation is also reduced in the deficient medium, although by a smaller factor - about 3.5. This phenomenon is not, therefore, unique to nalidixate-induced degradation. Thirdly, the two strains $15T^-$(555-7) and $15TAU^-$ have the same rate of nalidixate-induced DNA degradation; there is no strain difference in this respect.

Finally, the surprising result was found that the effects of irradiation and nalidixate are not additive, but that nalidixate consistently restricts the DNA breakdown induced by irradiation. In the three cases shown in Figures 18 and 19, after $2\frac{1}{2}$ hours incubation, DNA breakdown in the doubly treated culture is restricted by about 25-30% of the sum of the two treatments separately. This

interesting result suggests that nalidixate may combine with DNA and thus interfere with the nucleases involved in DNA degradation.

DNA degradation during thymine starvation

In the following experiment, DNA degradation in $15T^-$(555-7) and $15TAU^-$ was measured during thymine starvation, during simultaneous thymine starvation and nalidixate treatment, and following half the previously used dose of ultraviolet irradiation (ie. 500 erg/mm^2). Incubation was in otherwise fully-supplemented medium and the method used was essentially that described for the last two figures.

The results shown in Figure 20, demonstrate that, in contrast to nalidixate treatment, thymine starvation induces a negligible amount of net DNA degradation. After 3 hours of starvation of the two cultures, only $\frac{1}{2}$-1% of the labelled DNA had appeared as acid-soluble radioactivity, as opposed to 16% for nalidixate treatment (Figure 19). The failure to detect degradation under these conditions could, however, be due to reutilization of the degradation products, their appearance permitting some DNA synthesis. Such reutilization should, however, be prevented in the thymine starved culture containing nalidixate, so this should show what degradation thymine starvation induces.

The two strains unfortunately gave different results: in $15T^-$(555-7) the breakdown was greater than with nalidixate alone (at 3 hours 19% compared to 16%), while in $15TAU^-$, it was less

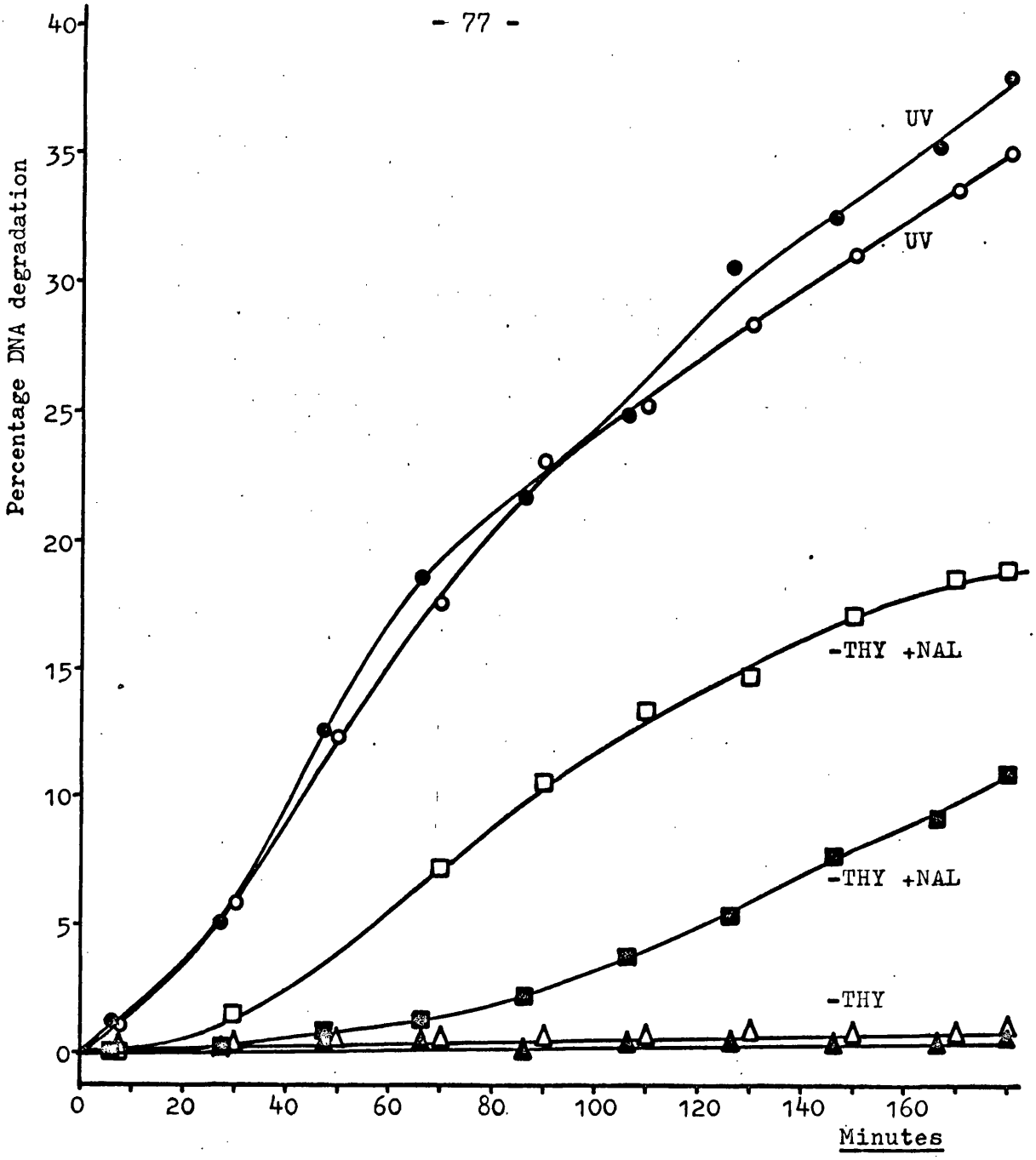


Figure 20 The percentage DNA degradation in $15T^-$(555-7) (open symbols) and $15TAU^-$(closed symbols) during thymine starvation (-THY) also in the presence of 50 ug nalidixate/ml (-THY +NAL), and after 500 erg/mm^2 ultraviolet irradiation (UV). Total radioactivity incorporated was: (555-7), 28,400 cts/min/ml and TAU^-, 28,300 cts/min/ml. The control (untreated) cultures gave no detectable DNA degradation.

(11% compared to 16%). This makes it difficult to draw a solid conclusion, but taking the average of the two curves (which is almost identical to the nalidixate-only curves in Figure 19), would mean that thymine starvation induces no degradation in the presence of nalidixate. At most, in these conditions, it induces 20% of the DNA breakdown induced by nalidixate alone.

The effect of a 500 erg/mm^2 dose of ultraviolet irradiation was included in the experiment shown in Figure 20, to provide a comparison with this effect on another strain (Figure 23), for reasons explained in the next section. It may be of interest to note that the DNA degradation induced by 500 ergs is less than half that induced by 1000 ergs.

(3) The induction of colicin 15 and death

Derivatives of E.coli strain 15 were shown by Ryan, Fried & Mukai (1955), and Mukai (1960) to be induced by ultraviolet irradiation to lyse and produce a bacteriocidal factor, to which they are themselves sensitive, although other E.coli strains tested are not sensitive. As no bacteriophages could be detected in the lysates, this factor was called a colicin (col15). Later work has shown that col15 is probably a defective prophage (Endo et al, 1965; Sandoval Reilly & Tandler, 1965; Mennigmann, 1965; Frampton & Brinkley, 1965).

Specific inhibitors of DNA synthesis which are known to induce both colicinogenic and lysogenic bacteria (Fredericq, 1963)

such as X-ray and ultraviolet irradiation (Mukai, 1960; Frampton & Brinkley, 1965), mitomycin-C (Endo et al, 1965) and thymine starvation of a thymine auxotroph (Mennigmann, 1964) induce col15 and cause cell death. In the case of thymine starvation, it has been established that col15 induction in strain $15T^-$ is a major cause of the "thymineless death" first observed by Cohen & Barner (1954). Thus Mennigmann (1964) showed that there is a correlation between the decrease in viable number, and the concentration of col15 and level of cell lysis, produced by periods of thymine starvation followed by replacement. When this strain does not produce colicin in the absence of thymine (Sicard and Devoret, 1962), death is considerably reduced, as it is in a mutant of $15T^-$ and hybrids between $15T^-$ and K12 that can no longer produce col15 (Ishibashi & Hirota, 1965). These latter authors concluded that colicinogeny is a major factor responsible for thymineless death. It has been suggested that the remaining lethal effect (always observed) which is not due to col15 induction, is reversible and due to an increased sensitivity to plating (Donachie & Hobbs, 1967). No explanation for this increased sensitivity has been proposed.

It was considered likely that nalidixate also induces col15 because phage induction seems to be a common property of DNA inhibitors. Two questions were therefore asked: does nalidixate induce col15, and if so, what effect does this have on the properties of nalidixate already described?

(3a) The induction of col15 by thymine starvation

The first question proved difficult to answer with certainty. Firstly, as a control, the induction of col15 by thymine starvation was examined in the two strains used for the nalidixate studies ($15T^-$(555-7) and $15TAU^-$) using the method of Mennigmann (1964). Both strains have been shown to produce defective phage on irradiation (Frampton & Brinkley, 1965), but $15T^-$(555-7) does not subsequently lyse. It was selected for this property by Weatherwax and Landman (1960).

Two experiments were carried out which differed only in the growth medium used. The first was the usual supplemented M9 medium (described in Methods) while the second also contained 1.5% casamino acids. The generation times of the strains growing in the two media were 40 and 30 minutes respectively. At a cell titre of 2-3 x 10^8 cells/ml they were transferred to the same medium lacking thymine and incubated for an hour. Thymine was restored and the incubation continued for a further 4-5 hours. Throughout this time the absorption at 600 mμ of the cultures was monitored. Part of this graph for $15T^-$(555-7) in the first medium is shown in Figure 16 above, the $A_{600\ m\mu}$ continued to rise slowly after the readdition of thymine, while for $15TAU^-$, a small decrease was observed. The cultures were finally chloroformed and centrifuged to bring down cell debris. Col15 was detected by placing undiluted spots of these lysates onto indicator lawns (both $15TAU^-$ and (555-7) were used: about 10^8 cells in a soft

agar layer on tryptone agar plates). After overnight incubation, clearings were observed at some of the spots.

The results obtained indicate that col15 was induced in both strains (growing in the medium without casamino acids) by the above treatment, but whereas 15TAU$^-$ is also sensitive to col15 as expected, 15T$^-$(555-7) is resistant. (This resistance may have some bearing on its resistance to lysis following induction, although a mutation affecting a phage lytic mechanism, as suggested by Frampton and Brinkley (1965) would seem more likely.)

(3b) The induction of col15 by nalidixate treatment

The difficulty in determining this question arises from the fact that a 50 µg/ml solution of nalidixate itself causes a clearing on the indicator lawns, and the sensitivity of col15 detection was too low to permit dilution of the lysate to eliminate the nalidixate effect, without also eliminating the col15 effect. Col15 was therefore detected in the presence of nalidixate by comparing the clearing produced on a 15TAU$^-$ lawn with that on a 15T$^-$(555-7) lawn (ie. col15 resistant). This method was not very satisfactory, but attempts to isolate a nalidixate-resistant mutant of 15TAU$^-$ were not successful.

Experiments were carried out as just described for thymine starvation, but only in the medium without casamino acids, and using treatment with 50 µg nalidixate/ml instead. The clearings on the 15TAU$^-$ lawn were clearer than on the 15T$^-$(555-7) lawn

for lysates made from both strains during the nalidixate treatment, suggesting that col15 induction was indeed occurring. Lysates made from samples taken after the removal of nalidixate gave very little clearing on 15TAU$^-$ and none on 15T$^-$(555-7) lawns. Treatment with 20 µg nalidixate/ml which it was hoped would avoid the problem of clearing caused by nalidixate *per se*) gave no discernable col15 induction, either during or after treatment. The balance of these results suggests, however, that nalidixate does induce col15. The finding of Taketo and Watanabe (1967) that nalidixate induces phage in a lysogenic strain of *E.coli* K12 supports this conclusion since an inducer of one phage is generally found to be an inducer of other inducible phages.

(4) Comparison with a Col15$^-$ Strain

(a) Nalidixate-induced death

Since nalidixate probably induces col15, and this has been shown to be a major cause of the death induced by thymine starvation (Ishibashi & Hirota, 1965), it was of interest to see whether nalidixate-induced death is also affected by the presence of the colicinogenic factor. The col15$^-$ mutant used for comparison was JG151, kindly supplied by Drs Ishibashi and Hirota, and isolated by them from the survivors of 15T$^-$ which had been repeatedly subjected to thymine starvation (1965). The kinetics of death of JG151 and 15T$^-$(555-7) in the presence of 50 µg nalidixate/ml, and of JG151 in the absence of thymine are shown in Figure 21. Cultures

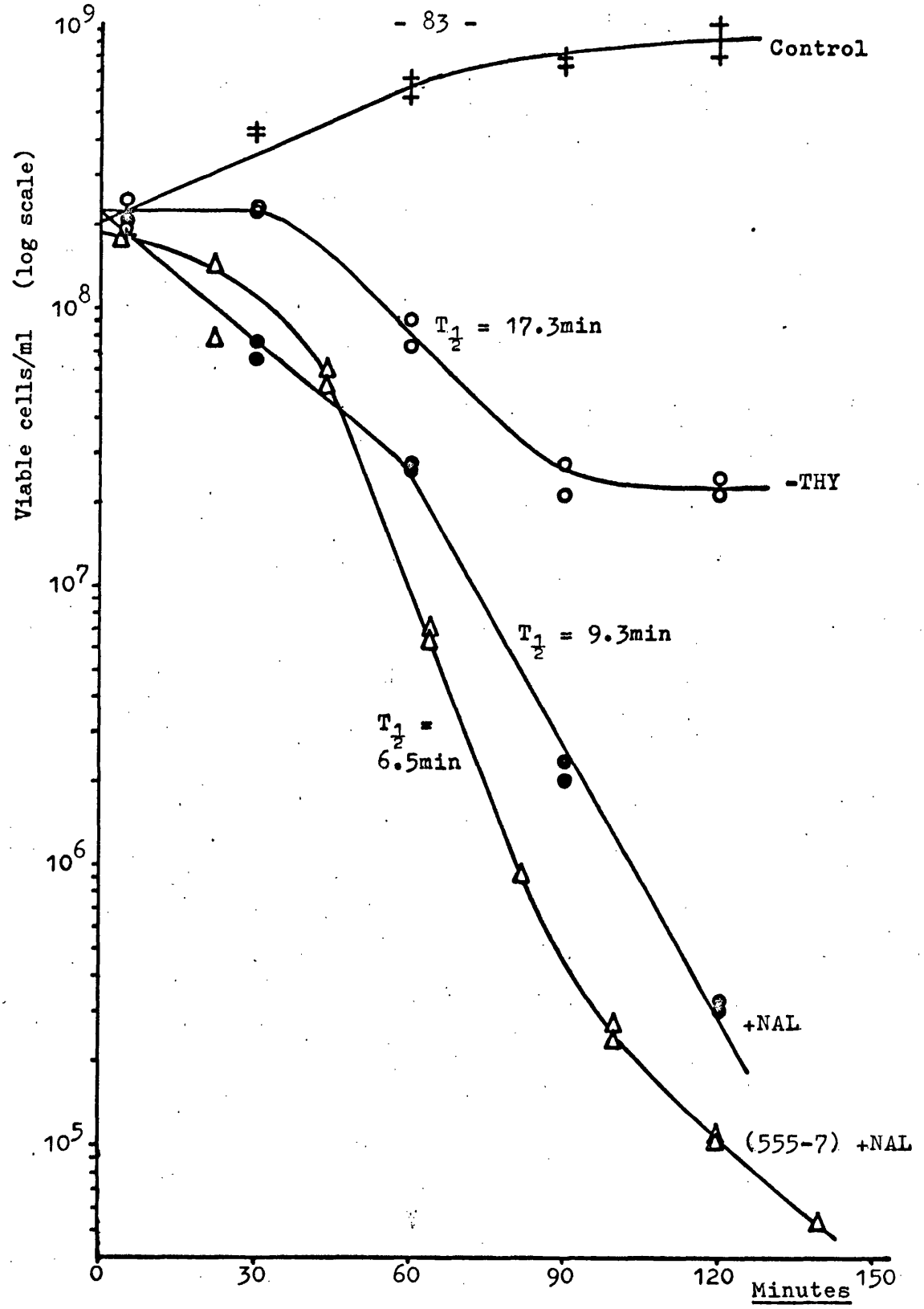


Figure 21 The kinetics of change of viability of JG151 during normal growth (Control), or thymine starvation (-THY), or treatment with 50µg nalidixate/ml (+NAL), and of 15T⁻(555-7) during nalidixate treatment. $T_{\frac{1}{2}}$ = half life.

of both strains were growing exponentially with generation times of about 40 minutes before the treatments were imposed.

As noted by Ishibashi and Hirota (1965), the rate of thymineless death of JG151 is considerably less than that of col15$^+$ strains (Figure 10 shows the death of 15T$^-$(555-7) during thymine starvation). The rate of death induced by nalidixate is also reduced by the loss of colicinogeny, but far less markedly. Col15 induction therefore plays a part in the nalidixate-induced death of a col15$^+$ strain, but the difference between the thymine- and nalidixate-induced death rates in JG151, shows that the remaining mechanisms are different for the two treatments. A convincing indication of this was the lysis, several hours later, of the nalidixate treated culture of JG151 while the thymine starved culture did not lyse.

(4b) Ultraviolet sensitivity

The nature of the mutation in JG151 was investigated. Its loss of viability with ultraviolet irradiation is shown in Figure 22. From this, the half-life doses of the three strains used are:

JG185	:	112 erg/mm^2
15T$^-$(555-7)	:	34 "
JG151	:	4.0 "

The surprising result is that JG151 is very much more ultraviolet sensitive than its col15$^+$ counterpart, whereas one would suppose that simply curing a strain of col15 would decrease

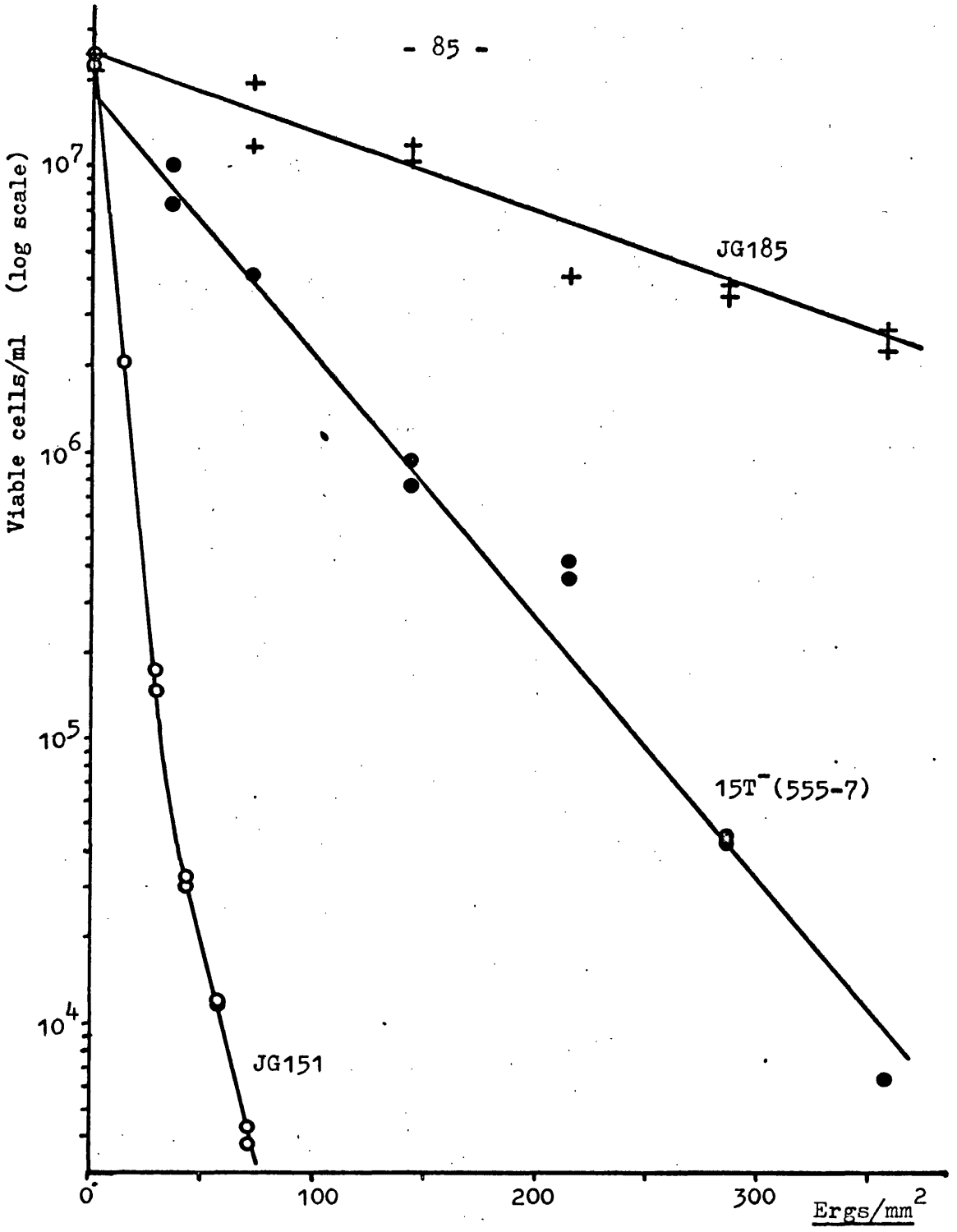


Figure 22 The loss of viability with ultraviolet irradiation of 15T⁻(555-7) = col15^{+S}: JG151 = 15T⁻col15^{-S} and JG185 = K12 thy$^-_1$ col15^{-R} (g = 70 min). Genetic symbols: col15, + and -, ability and inability to produce col15; R and S, resistance and sensitivity to col15. thy$^-_1$, gene with the thymine-requirement mutation of strain 15T⁻.

its sensitivity to ultraviolet irradiation, since its induction leads to death. JG185, in contrast to JG151, behaves as expected in this respect. We know that in one class of ultraviolet-sensitive mutants of E.coli, induction of prophages does not occur, either spontaneously or after ultraviolet irradiation (Fuerst & Siminovitch, 1965; Hertman & Luria, 1967). Such mutants are also defective in genetic recombination competence (rec$^-$) (Clark & Margulies, 1965; Howard-Flanders & Theriot, 1966). It therefore seems possible that JG151 is not cured, but has become rec$^-$. If JG151 is a "reckless" rec$^-$ mutant (Howard-Flanders & Boyce, 1966), then the extent of DNA degradation it undergoes after ultraviolet irradiation, will be greater than its rec$^+$ parent. This property was therefore examined.

(4c) DNA degradation in JG151

The degradation of prelabelled DNA of JG151 was measured as described for Figures 18 and 19, during incubation in full growth medium (g = 41 min). It was measured during treatment with nalidixate and after a dose of 500 erg/mm^2 ultraviolet irradiation.

The results, presented in Figure 23 will be compared with equivalent results for 15T$^-$(555-7) and 15TAU$^-$ (Figure 19 for nalidixate treatment and Figure 20 for the rest). As suspected, the extent of DNA degradation following irradiation, or during nalidixate treatment is greater in JG151 than the other two strains, being approximately double, and 3.5 fold respectively. There was no measurable degradation in the control (contrary to expectation,

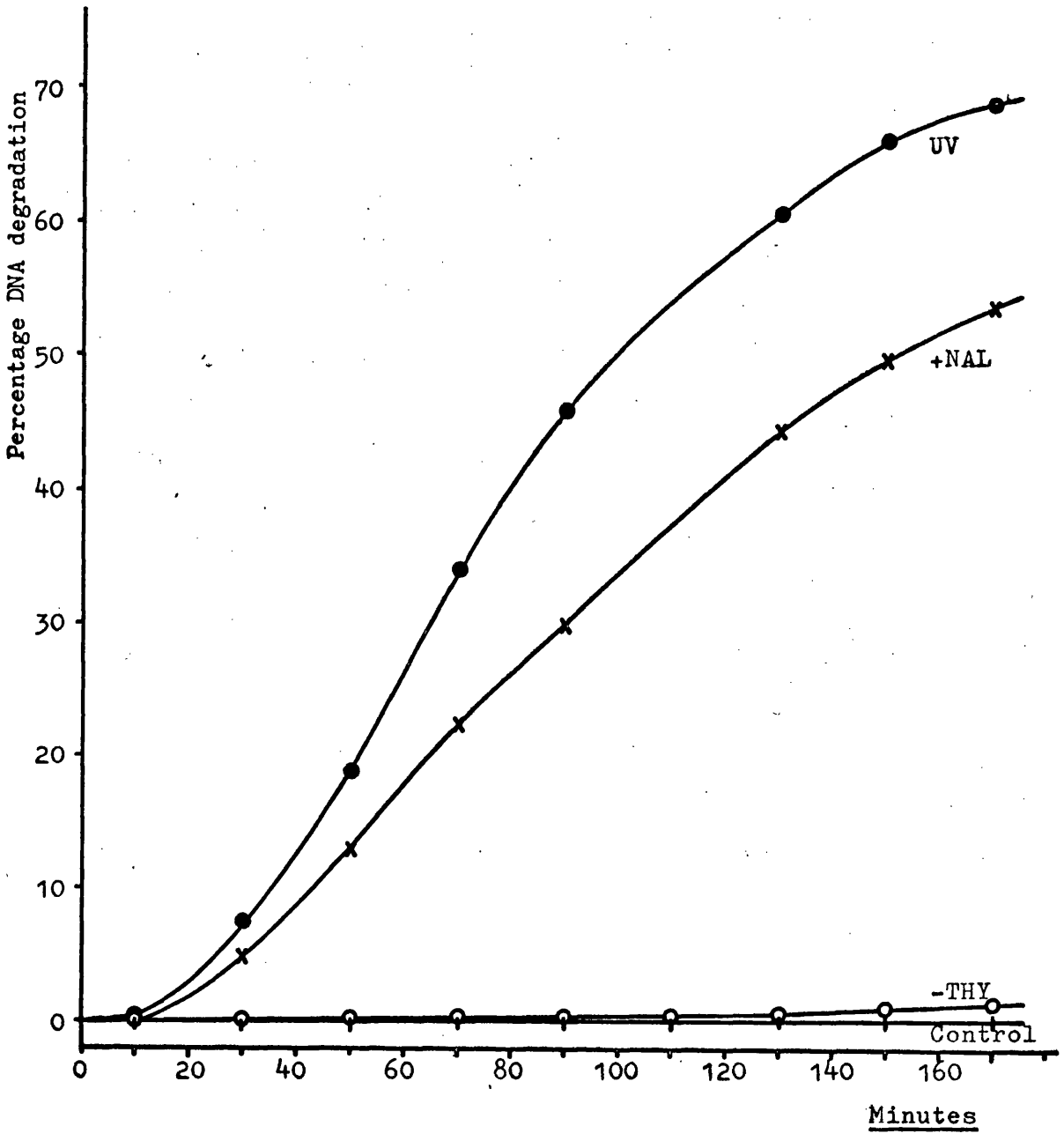


Figure 23 The percentage DNA degradation in JG151 during normal growth (Control), thymine starvation (-THY) or treatment with 50 µg nalidixate/ml (+NAL), and also after 500 ergs/mm^2 of ultraviolet irradiation (UV). Total radioactivity incorporated was 29,800 counts/min/ml.

Clark et al, 1966) and only about 1% after 3 hours of thymine starvation. Thus it seems that JG151 is rec$^-$, probably still carrying col15.

The work on colicinogeny leads to certain conclusions about the properties of nalidixate. Cook, Deitz & Goss (1966) showed that there was a correlation between bacterial survival and DNA degradation in 15TAU$^-$ after nalidixate treatment, and suggested that the latter caused the former. Considering JG151, however, we have seen that its DNA degradation is very much greater than that of 15TAU$^-$ and 15T$^-$(555-7) during nalidixate treatment, and yet its death rate is somewhat less. The simplest explanation for this, is to say that nalidixate causes death by at least two, separate processes: 1. by inducing col15 (which, from the results during thymine starvation, causes little or no DNA degradation itself), and 2. by inducing DNA degradation in some unknown manner. The results in Figures 18 and 19 suggested that nalidixate combines with DNA, this perhaps being recognised as a lesion by the repair enzymes, and so being stripped out (Howard-Flanders, Boyce & Theriot, 1966). Consistent with this is the observation that nalidixate treatment (Figure 21) causes a faster death rate than thymine starvation (Figure 10) in col15$^+$ or col15-inducible strains.

C H A P T E R III

The relationship between thymine and deoxynucleoside metabolism

The Introduction described briefly the reason for thinking (at the time this work was started) that thymine plays a special role in the control of the initiation of DNA replication. Evidence had appeared that suggested that a thymine-containing compound exerted control over deoxynucleotide synthesis, and this could certainly be an important factor in control of initiation. Thus Munch-Petersen & Neuhard (1964) showed that E.coli 15TAU$^-$ accumulated nucleotides (mainly dATP and dADP) during thymine starvation, while Biswas et al (1964) reported an increase in the ribonucleoside diphosphate reductase system of 15T$^-$ under such conditions. (This was published fully subsequently: Biswas, Hardy & Beck, 1965). Also it had been reported that cultures of 15T$^-$ and related strains excreted bases, nucleosides and nucleotides, synthesised de novo (Cohen & Barner, 1954; Breitman & Bradford, 1964), and free deoxyribose (Smith, 1964; Breitman & Bradford, 1964) into the medium, during thymine starvation.

Breitman & Bradford (1964) noted that there was essentially no deoxyribose excretion by the originally isolated thymineless strain (70-462), during starvation, but only by the secondary mutant viz, 70V3-462, which differs from the parent strain in being able to grow with a low concentration of thymine (about 20 μM).

$15T^-$ and its derivatives are also low requiring double mutants of this sort. (The history of the isolation of the above mutants from E.coli 15 has been described by Roepke (1967)). Thus it seemed that the second mutation, the nature of which was not known, might be altering a deoxynucleotide control system dependent on a thymine compound. Its study, described in this chapter, therefore seemed interesting and relevant.

It is clear that thymineless strains of E.coli fall into two classes, in both of which the gene specifying thymidylate synthetase is defective (Barner & Cohen, 1959; Mantsavinos & Zamenhof, 1961; Breitman & Bradford, 1967a):-

1. Some, like 70-462, will grow normally only in the presence of a relatively high concentration (about 200 μM) of thymine (Breitman & Bradford, 1964, 1967a), and
2. Others, like $15T^-$, derived from the first as a result of the above mentioned second mutation (Breitman & Bradford, 1964, 1967a; Harrison, 1965; Alikhanian et al, 1966), have gained the ability to grow normally with a lower concentration of thymine (about 20 μM).

Mutations of the gene(s) concerned in this ability have been mapped by Alikhanian et al (1966) and Okada (1966) close to, and left of, the threonine (thr) locus in E.coli, ie. quite separate from the thymine (thy) locus. The former authors designated this new locus tlr (thymine low requirement).

What is the nature of the change involved in tlr mutations? Matsavinos & Zamenhof (1961) showed that the inability of strain 15,

unlike tlr$^-$ strains, to incorporate significant amounts of exogenous thymine into DNA (first observed by Crawford, 1958) is not due to a deficiency of the enzymes necessary to convert thymine into dTMP. They postulated that the difference between the two types of strain lay, rather, in the control mechanisms regulating the levels of enzymes and key intermediates. They suggested that the first step in the pathway of thymine incorporation into DNA is the conversion of thymine into thymidine. This could be catalysed either by thymidine phosphorylase (Rachmeler, Gerhart & Rosner, 1961; Razzell & Casshyap, 1964);

$$\text{thy} + \text{dR-1-P} \longleftrightarrow \text{thy-dR} + P_i \qquad \text{- - - - (a)}$$

or by a trans-N-deoxyribosidase (Hoffmann, 1952; Lampen, 1952; Koch, 1956):

$$\text{thy} + \text{pyr-dR} \longleftrightarrow \text{thy-dR} + \text{pyr} \qquad \text{- - - - (b)}$$

(Abbreviations: thy = thymine; thy-dR = thymidine; pyr and pyr-dR = pyrimidine and its deoxynucleoside; dR-1-P = deoxyribose-1-phosphate; P_i = orthophosphate).

It seems very probable that either or both of these reactions are involved in thymine incorporation for the following reasons. Thymine uptake is enhanced in high thymine requiring (tlr$^+$) strains by the addition of deoxyadenosine (Boyce & Setlow, 1962) or by any purine or pyrimidine deoxynucleoside, normal growth being then permitted in the presence of a low thymine concentration (Pritchard & Stacey, unpublished results). Similar effects have been reported with purine deoxynucleosides in E.coli B (Kammen,

1967). Since E.coli is reported to have two types of trans-N-deoxyribosidases, one specific for purines and one for pyrimidines (Hoffmann, 1952; Lampen, 1952), reaction (b) could not account for the effects of purine deoxynucleosides. Rather, these must involve reaction (a), dR-1-P being formed by an analogous reaction catalysed by purine nucleoside phosphorylase (Lampen, 1952; Koch, 1956; Rachmeler et al, 1961). This has been confirmed by Budman & Pardee (1967) who observed that thymine incorporation in the presence of deoxyadenosine was abolished in a thymidine phosphorylase negative mutant (isolated by Fangman & Novick, 1966).

These data, and the fact that both high and low thymine requirers utilize thymidine with equal efficiency (Rachmeler et al, 1961), and also the data on deoxynucleotide accumulation in tlr^- strains during thymine starvation (described above) lead to the idea that in contrast to tlr^+ strains, tlr^- mutants have a high pool size of deoxynucleosides and dR-1-P, which, by promoting the conversion of thymine to thymidine by reactions (a) and/or (b), is responsible for the efficient thymine uptake of these mutants (Breitman & Bradford, 1964; Pritchard, 1966). The control of this pool by a thymine compound in tlr^- strains was postulated by Breitman & Bradford (1964), its enhancement during thymine starvation being consistent with the deoxyribose excretion and induction of thymidine phosphorylase they observed, dR-1-P having been suggested to be the inducer of this enzyme (Razzell & Casshyap, 1964).

Results

(1) Deoxyribose excretion during thymine starvation

The theory above predicts that deoxyribose excretion during thymine starvation should take place with all *tlr*$^-$ strains, and not with any *tlr*$^+$ strains. A number of unrelated *tlr*$^+$ and *tlr*$^-$ strains were therefore tested for this capacity.

It was found that by plotting the concentration of deoxyribose in the medium against the time function $2^{t/g} - 1$, a straight line was produced (as predicted from the considerations in Appendix 1) during 3-4 generations of growth. A few examples of such graphs are shown in Figure 24. Using the gradients of these lines, the excretion rates in mμ moles deoxyribose per 10^8 cells per mean generation time, have been calculated, and are given below.

Table 2 Excretion of deoxyribose by thymineless *E.coli* strains during growth with and without thymine

Strain	*tlr*	g(min)	Thymine concentration 800 μM	Nil
P56	+	60	0.075	0.20
P90	+	60	0.066	0.21
CR34	-	62	0.095	1.10
TK201	-	49	0.13	1.09
15T$^-$	-	46	0.055	1.20
15TAU$^-$	-	45	0.57	1.59

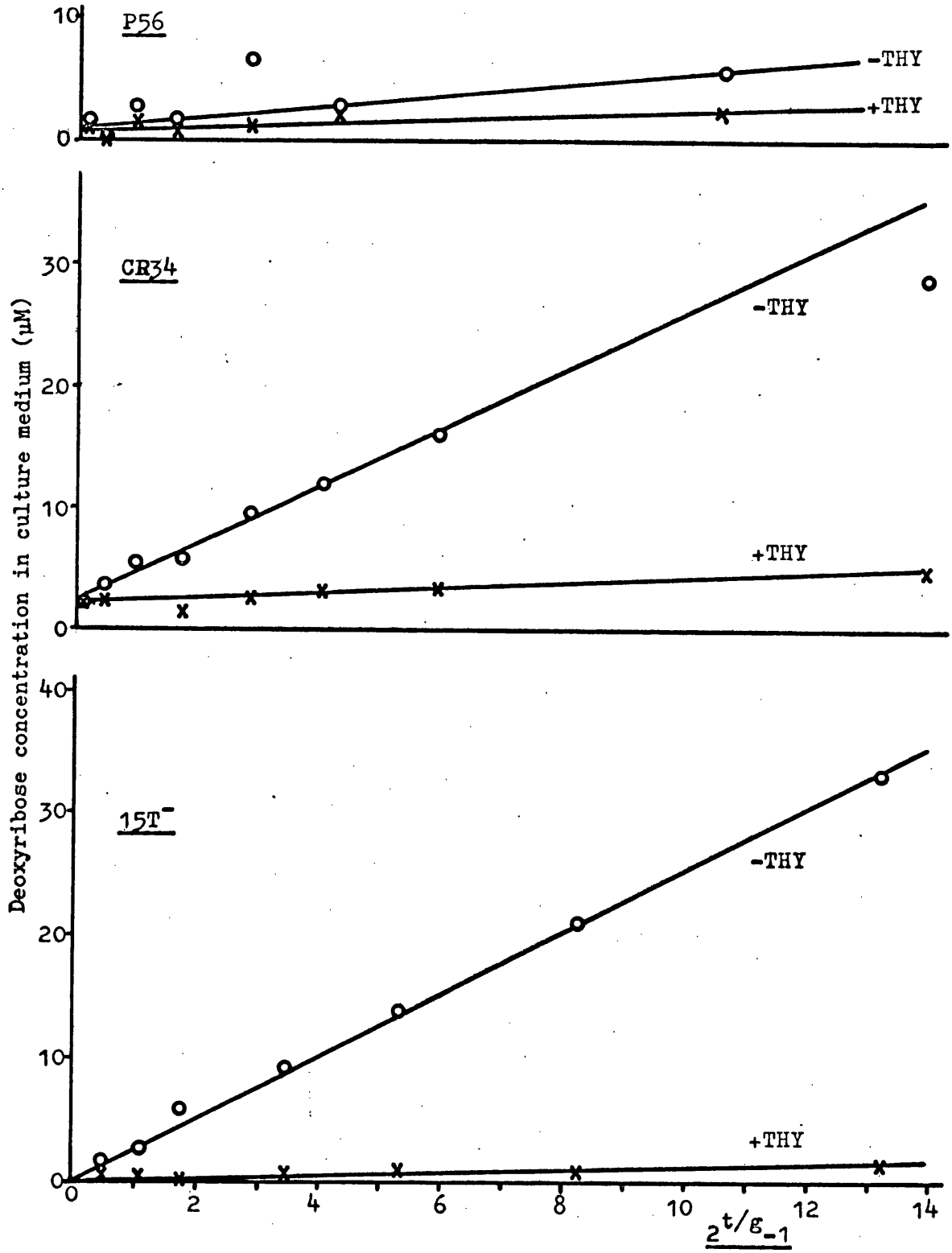


Figure 24 Excretion of deoxyribose by three thymineless strains of E.coli during growth with and without thymine. The method is described under Table 2.

Table 2: Rates are expressed in mμ moles deoxyribose/10^8 bacteria/ mean generation time. Exponentially growing cultures, at about $2x10^8$ cells/ml, were transferred into medium without thymine, half this culture being immediately resupplemented with thymine. Samples were withdrawn from both for deoxyribose measurement as described in Methods. The first four strains are K12 derivatives.

Differences due to the different growth rates of the various strains used are eliminated by this expression of rate, although differences due to differences in cell mass are not. Table 2 shows that the strains tested behaved according to expectation: only and all the tlr$^-$ strains excreted significant amounts of deoxyribose during thymine starvation.

(2) The effect of thymine concentration and nalidixate treatment on deoxyribose excretion

A question that arises from the results above is: what is the importance of thymine in this phenomenon? Is deoxyribose excretion in tlr$^-$ strains dependent only on the depletion of thymine nucleotides in the cell (as a result of a change in a control system dependent on thymine, as postulated by Breitman & Bradford (1964)), or does inhibiting DNA synthesis itself cause deoxyribose excretion in these strains?

A suitable set of mutants for this study was kindly made available by Dr A. Eisenstark. These are thymineless mutants of Salmonella typhimurium, which fall into the same two classes as those of E.coli (Beacham, Eisenstark, Barth & Pritchard, 1968).

The rate of deoxyribose excretion during growth on high and low concentrations of thymine, as well as during the inhibition of DNA synthesis with nalidixate or by thymine starvation, were compared. The results are shown below.

Table 3 Excretion of deoxyribose by S.typhimurium during growth under various conditions

Strain	Genotype thy	Genotype tlr	g(min)	Thymine Concentration 300-800 µM	20 µM	Nil	Nalidixate treated
2793	+	+	50			0.06	0.06
2006	-	+	45	0.05		0.04	
2462	-	+	45	0.05		0.05	0
2456	+	-	45			0.07	0.96
2477	+	-	51			0.14	0.83
2454	-	-	45	0.05	0.40	1.12	0.86
2001	-	-	51		0.52	0.84	0.87

Rates are expressed in mµ moles deoxyribose/10^8 bacteria /mean generation time (linear kinetics of excretion against $2^{t/g}$ -1 were obtained over about 3 hours of growth as before). Some of the results are the average of 2 or 3 measurements. The method was as previously described, nalidixate being added to the growing culture to give a concentration of 50 µg/ml. The relationships between the strains are described in Methods.

The data show that these S.typhimurium strains behave in the same way as the E.coli strains, ie. significant amounts of deoxyribose are excreted only by the tlr$^-$ mutants. Two important

conclusions can be drawn from these data. Firstly, thymine starvation is not unique in causing deoxyribose excretion, inhibiting DNA synthesis with nalidixate causes about the same rate of excretion in *tlr*$^-$ strains (whether *thy*$^+$ or *thy*$^-$), as thymine starvation in *tlr*$^-$ *thy*$^-$ strains.

Secondly, it can be seen that the concentration of thymine in the growth medium of *thy*$^-$ *tlr*$^-$ strains affects their rate of deoxyribose excretion. When a high concentration is present, no appreciable excretion occurs, but with 20 μM, which is sufficient for normal growth, excretion occurs (at about half the rate found when DNA synthesis is inhibited). It should also be noted that *thy*$^+$ *tlr*$^-$ strains do not excrete deoxyribose during normal growth. It seems reasonable to consider that the rate of deoxyribose excretion is a measure of the intracellular accumulation of dR-1-P, which is presumably the precursor of deoxyribose. Such accumulation was argued to be responsible for the efficient uptake of thymine via reaction (a) in *tlr*$^-$ strains, and further evidence in favour of this view will be given later. These results therefore indicate that the *tlr*$^-$ mutation does not of itself lead to dR-1-P accumulation. *tlr*$^-$ strains apparently still retain the regulation of deoxynucleotide synthesis dependent on a thymine derivative, ie. the inhibition of ribonucleoside diphosphate reductase by dTTP - synergistically with dATP, or indirectly - (Larsson & Reichard, 1966a,b,c), and the repression of this enzyme probably by the same compound (Biswas *et al*, 1965). (Cannon &

Breitman (1967) have shown that such regulation occurs *in vivo*.) However, the *tlr*$^-$ mutation leads to deoxyribose excretion when this regulation is upset (by a low external concentration of thymine in a *thy*$^-$ strain) whereas the *tlr*$^+$ mutation does not. This argument, with further supporting data, has been more fully expressed elsewhere (Beacham, Barth & Pritchard, 1968).

(3) Deoxyribose excretion during inhibition of DNA synthesis

We have seen that inhibiting DNA synthesis with nalidixate causes deoxyribose excretion in *tlr*$^-$ strains. The effect of ultraviolet irradiation on excretion was also studied to verify that excretion is brought about by DNA inhibition *per se*, rather than an unknown effect of nalidixate on deoxynucleotide metabolism: it is known that ultraviolet light inhibits DNA synthesis directly by damaging the DNA.

Two *thy*$^-$ strains were used for this study: an ultraviolet-sensitive (*uvrB*) derivative of CR34 viz, KMBL90 (Van De Putte *et al*, 1965) which is unable to excise photoproducts from its DNA (Boyce & Howard-Flanders, 1964; Mattern, Van Winden & Rörsch, 1965) and, in contrast, the apparently "reckless" strain - JG151 (Chapter II, section 4).

Firstly the effect of ultraviolet irradiation on the subsequent rate of DNA synthesis was measured in KMBL90. This is described in Figure 25. The rate was expressed as a percentage of the rate in the unirradiated culture (control). A sufficient

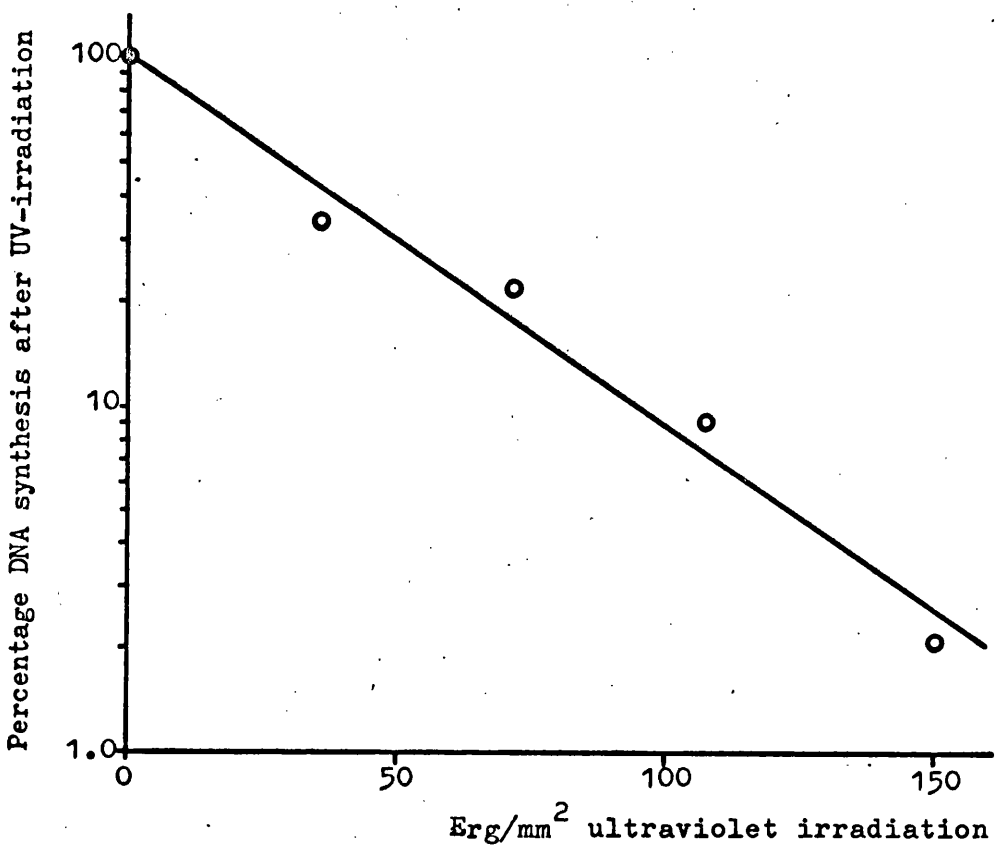


Figure 25 The effect of irradiation on the subsequent rate of DNA synthesis in KMBL90. An exponential-phase culture (g = 46 min), at a titre of 2.5 x 10^8 cells/ml, was filtered, washed and resuspended at 9 x 10^8 cells/ml in basic M9 medium (without nutrients). Portions were diluted into full growth medium containing ^{14}C-thymine (40 µM, 0.16 µC/ml) at once, and after various periods of ultraviolet irradiation. Incorporation of label into DNA was measured by the usual methods, over about onegeneration period. This rate was expressed as a percentage of the rate in the unirradiated culture.

inhibition of DNA synthesis is produced by a dose of 150 erg/mm^2 (reducing the rate to 3% of the control rate). The deoxyribose excretion of a culture of KMBL90, after being irradiated with this dose, was then measured. For comparison, the excretion of this culture during thymine starvation, nalidixate treatment (50 μg/ml) or normal growth, were also measured. Similar measurements were made on cultures of JG151 during these treatments, and also after an ultraviolet dose of 500 erg/mm^2 (as the DNA degradation induced by this dose had been measured; see Figure 23). The results are given below.

Table 4 Deoxyribose excretion

Strain	g(min)	Thymine concentration		Nalidixate treated	Irradiated
		20 μM	Nil		
KMBL90	46	0.66	2.91	2.49	3.15
JG151	41	0.52	2.35	2.54	2.12

Rates are given in mμ moles deoxyribose/10^8 bacteria/mean generation time. The strains and methods are described above.

In both strains, the initial rate of deoxyribose excretion was about the same for all three methods of inhibiting DNA synthesis. This suggests that stopping DNA synthesis leads to an accumulation of deoxynucleotides, which are then degraded: the deoxyribose moiety at least, being excreted in tlr$^-$ strains. This probably means that the deoxynucleotide feedback control system is rather

inefficient, a conclusion that has also been reached by Smith & Davis (1967) from their studies on the effect of novobiocin (a DNA inhibitor) on the pool size of deoxynucleotides. The excretion from the irradiated culture of KMBL90 shows that the deoxyribose probably does not originate from preformed DNA, since this strain cannot degrade its DNA, and certainly its rate of excretion is no less than that of JG151 (which degrades its DNA excessively).

In order to give the reader an idea of the magnitude of these excretion rates (in Tables 2,3 & 4) let us compare these rates with the expected rates of deoxyribose synthesis (as deoxynucleotide diphosphate) necessary for maintaining DNA synthesis. Taking an estimate of the number of nucleotides in a non-replicating chromosome of E.coli as 8×10^6 (Cairns, 1963a; Cooper & Helmstetter, 1968), then the net synthesis of one chromosome per cell per generation time requires 1.35 mμ moles deoxyribose/10^8 cells/g. This rate of synthesis is predicted, from Cooper & Helmstetter's model, to occur in a culture growing with g = 63 minutes, whereas for g = 42 minutes, $1\frac{1}{2}$ chromosomes/cell/g are predicted, ie. 2.0 mμ moles deoxyribose/10^8 cells/g. (It was noted in the Introduction section (7), that strain $15T^-$ has a higher DNA content per cell than predicted on this model. Its rate of DNA synthesis would also be greater: estimated at 2 chromosomes/cell/ g when g = 42 minutes, ie. 2.7 mμ moles deoxyribose/10^8 cells/g). Thus the observed rates of excretion during the inhibition of DNA synthesis, were about 50% of the predicted normal rate of deoxyribose output (with the exception of

the two strains in Table 4 where it was about 100%).

(4) Deoxynucleoside catabolism

From the data and arguments above, it has become clear that tlr⁻ mutants have an altered catabolism of deoxynucleosides. Enzymes known, that are capable of catalysing this catabolism, and use of the deoxyribose moiety as a carbon source, are: thymidine phosphorylase (reaction (a) above), purine nucleoside phosphorylase (Manson & Lampen, 1951b; Lampen, 1952), deoxyribomutase (Manson & Lampen, 1951a; Hoffmann & Lampen, 1952) and deoxyriboaldolase (Racker, 1952). The latter three enzymes catalyse respectively the following reactions:

$$\text{pur-dR} + P_i \longleftrightarrow \text{pur} + \text{dR-1-P} \qquad \text{- - - - (c)}$$

$$\text{dR-1-P} \longleftrightarrow \text{dR-5-P} \qquad \text{- - - - (d)}$$

$$\text{dR-5-P} \longleftrightarrow \text{glyceradehyde-3-P} + \text{acetaldehyde} \qquad \text{- - - - (e)}$$

(Abbreviations: pur and pur-dR = purine and its deoxynucleoside; dR-5-P = deoxyribose-5-phosphate; dR = deoxyribose). All the deoxynucleosides are capable of being catabolised by these four enzymes, deoxycytidine requiring prior deamination to deoxuridine (Koch & Lamont, 1956) which is also a substrate of thymidine phosphorylase (Razzell & Casshyap, 1964).

The accumulation of the dR-1-P in tlr⁻ strains could come about by an enhanced breakdown of deoxynucleotides, or by a block in its degradation. To differentiate between these possibilities, the ability of the S.typhimurium strains shown in Table 3 to

utilize deoxyribose as a sole carbon source, was tested. It had been shown by Domagk & Horecker (1958) and Ginsberg (1959) that Lactobacillus plantarum ferments deoxyribose using a deoxyribokinase catalysing the reaction:

$$dR + ATP \longleftrightarrow dR\text{-}5\text{-}P + ADP \qquad ----(f)$$

the dR-5-P then being cleaved by deoxyriboaldolase ("aldolase") as in reaction (e). Thus it was argued that if the species S.typhimurium can also ferment deoxyribose, the loss of this ability in any tlr^- mutants will demonstrate a block in the catabolism of dR-5-P, (unless this fermentation took place via an entirely different pathway).

It was found that the three tlr^+ strains grew, but the four tlr^- strains did not (on plates containing 5mM deoxyribose as sole carbon source). It has since been confirmed that S.typhimurium utilizes the same pathway as L.plantarum for deoxyribose fermentation (Hoffee, 1968). Thus it seems that these tlr^- mutants lack aldolase, ie. they are "dra^-". This finding is in agreement with the report of Breitman & Bradford (1967b) of the absence of aldolase in a tlr^- mutant of E.coli. (None of the E.coli strains tested was able to grow on deoxyribose, although a strain capable of doing so has been reported by Jonsen, Laland & Strand, 1959).

The addition of thymidine to a culture of E.coli rapidly induces thymidine phosphorylase which cleaves the thymidine according to reaction (a) (Rachmeler et al, 1961; Razzell & Casshyap, 1964). If the further degradation of the dR-1-P formed,

is blocked in *tlr*$^-$ mutants, we expected that this would lead to the excretion of deoxyribose. Figure 26 shows the appearance of diphenylamine-reacting material (Burton, 1956) in the medium, after the addition of thymidine to cultures of the *S.typhimurium* strains used above. One of the cultures (2001 = *thy*$^-$ *tlr*$^-$) also had nalidixate (50 µg/ml) added at the same time, to prevent the incorporation of thymidine or dR-1-P into DNA.

The *tlr*$^+$ strains excreted negligible amounts of deoxyribose into the medium (in accordance with the findings of Hoffmann & Lampen (1952) using a strain of *E.coli*). The *tlr*$^-$ strains, in contrast, rapidly excreted a maximum concentration 55-75% of the initial thymidine concentration after 80-90 minutes. At this point, 2001 and 2477 then reutilized the deoxyribose. The fact that nalidixate prevented this, suggests that, rather than being catabolized, the deoxyribose was being incorporated into DNA (via reactions f,d,c and a etc.). The rates of reutilization are considerably greater than predicted for this alone, however (see next section). These patterns of thymidine metabolism, and the inability of *tlr*$^-$ mutants to utilize deoxyribose as sole carbon source, are consistent with the *tlr*$^-$ mutants 2001 and 2477 being aldolase defective (obliging them to excrete deoxyribose from the dR-1-P accumulating) but retaining mutase (to permit the observed deoxyribose uptake), ie. being *dra*$^-$ *drm*$^+$. The lack of reutilization by 2454 suggests it may be *dra*$^-$ *drm*$^-$.

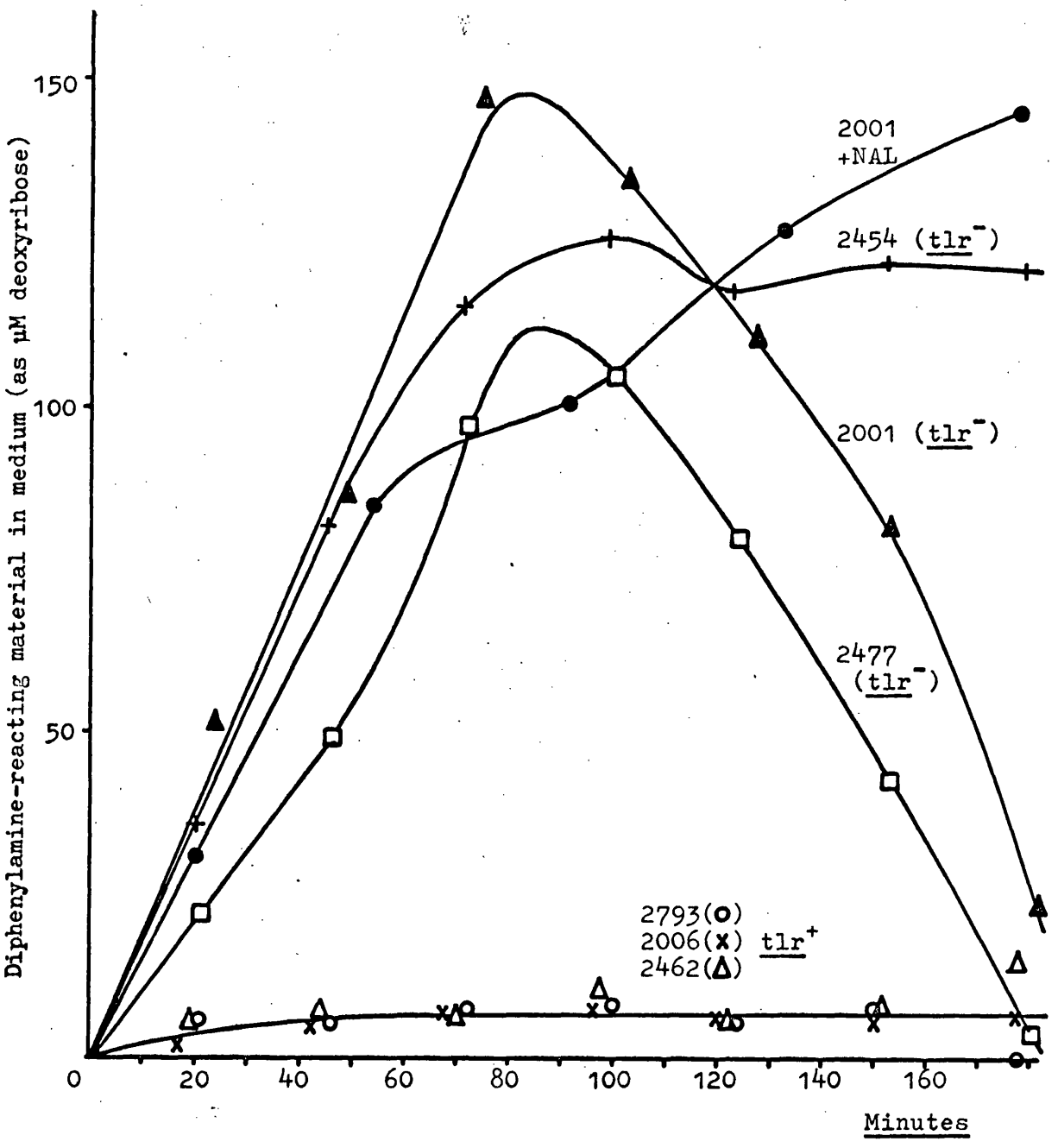


Figure 26 The release of deoxyribose into the medium on addition of thymidine. Cultures of the strains listed in Table 3, growing exponentially in medium containing 200 µM thymine were transferred, at a titre of about 2 x 10^8 cells/ml, to medium containing 200 µM thymidine (time = 0), and for one culture of 2001, 50 µg nalidixate /ml also. Deoxyribose was measured as before.

(5) Deoxyribose uptake

The defective deoxyribose phosphate metabolism observed in the experiment above was examined more directly by observing the uptake of deoxyribose added to the medium of the S.typhimurium strains. The results, shown in Figure 27, are consistent with the conclusions above. The tlr$^-$ mutants are defective in their mode of deoxyribose utilization: 2001 and 2477 take it up at about a fifth, and 2456 at a tenth, of the rate of uptake in tlr$^+$ strains, while 2454 apparently cannot utilize deoxyribose at all.

These results are consistent with the existence of the two pathways for deoxyribose metabolism described: catabolic, via the kinase and aldolase, and anabolic, via the kinase, mutase and phosphorylases. 2001 and 2477 have lost the catabolic (faster) route, while 2454 and 2456 are also defective for the anabolic route.

The rates of uptake by 2001 and 2477 are about 6-7 fold greater than the calculated rate of deoxyribose incorporation into DNA (in S.typhimurium at this growth rate - see section (3)). Most of the deoxyribose is probably, therefore, reacting (as dR-1-P) with thymine from the medium to produce thymidine (via reaction (a) - thymidine phosphorylase). This was not measured. The observation that nalidixate prevented this uptake in the previous experiment (Figure 26), therefore, leads to a paradox. However, nalidixate treatment causes deoxyribose excretion (Table 3) which would therefore mask some of the expected conversion of

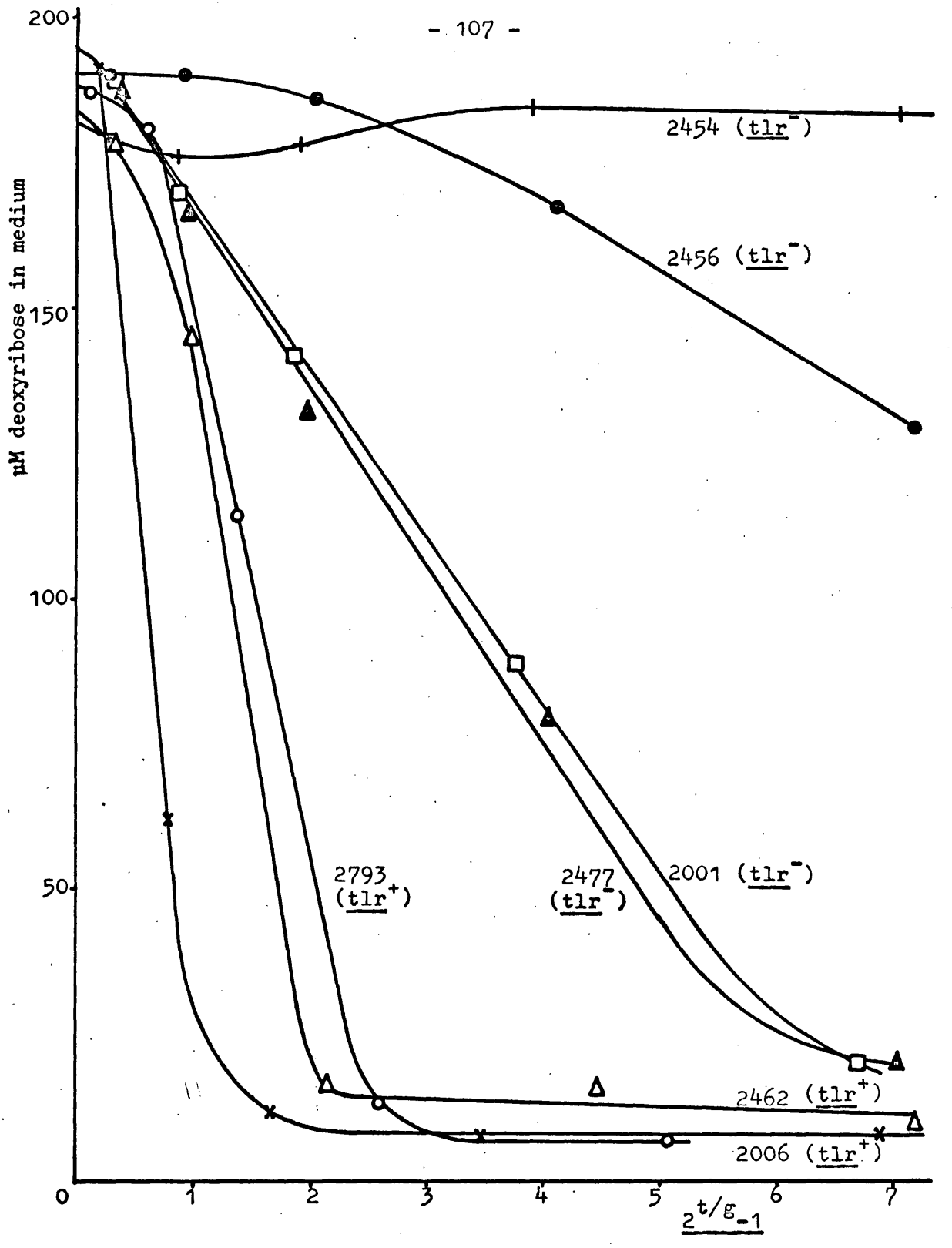


Figure 27 The disappearance of added deoxyribose. The experiment was performed as described in Figure 26, but deoxyribose (giving about 200 µM) was added to the cultures at zero time.

deoxyribose into thymidine. Also the inhibitory effect of nalidixate on growth (Figure 8) may inhibit this conversion.

This scheme means that 2454 and 2456 may be defective for mutase or thymidine phosphorylase (as well as aldolase). Figure 26 shows that 2454 catabolizes thymidine at the same rate as 2001 and 2477; 2454 must therefore be mutase defective. As 2456 is a $\underline{thy}^+$ revertant of 2454, the nature of the $\underline{tlr}$ mutation should be the same in the two strains. The difference between the two strains seen in Figure 27 is therefore puzzling. As 2456 had also reverted spontaneously to $\underline{thr}^+$ (2454 = $\underline{thr}^-$), and the $\underline{tlr}$ locus is close to $\underline{thr}$, it is possible that this caused a simultaneous partial reversion of a $\underline{drm}^-$ mutation in 2454.

(6) Properties of spontaneous tlr mutants

The model of thymine low requirement proposed, predicts that $\underline{dra}^-$ or $\underline{drm}^-$ mutants ought to be low requirers. In a search for these and any other possible class, spontaneous $\underline{tlr}^-$ mutants of 2006 ($\underline{thy}^-$ $\underline{tlr}^+$) were selected (by spreading overnight cultures onto plates containing 20 μM thymine). The mutants were purified and then screened for their ability to grow on various deoxynucleosides or deoxyribose as sole carbon source. $\underline{dra}^-$ mutants will lose the ability to grow on both of these, but $\underline{drm}^-$ mutants will be distinguishable by retaining the ability to grow on deoxyribose. If a class of $\underline{tlr}^-$ mutants exists that is not defective in dR-phosphate catabolism, but has, for example, enhanced deoxynucleotide breakdown, such mutants would retain the capacity to grow on

deoxynucleosides and deoxyribose.

The spontaneous tlr$^-$ mutants were also tested for their sensitivity to these compounds. Alikhanian et al (1966) noted that some of their E.coli tlr$^-$ mutants had gained the property of being inhibited by thymidine, the mutation leading to sensitivity also being located close to the thr locus. Thymine low requirement and thymidine sensitivity (also found in S.typhimurium) have been shown to be pleiotropic effects of a single mutational event in the tlr locus (Beacham et al, 1968). The properties of these tlr$^-$ mutants are summarized below:

Table 5 Properties of spontaneous tlr$^-$ mutants of 2006

		Class		
		1	2	3
Growth on:	dR	+	-	-
	thy-dR	-	-	-
	gua-dR	-	-	-
Sensitivity to:	dR	R	S	S
	thy-dR	R	S	R
	gua-dR	R	R	R
Numbers obtained		23	13	1

Overnight cultures were streaked onto solid medium containing 20 µM thymine and 5 mM dR, thy-dR or deoxyguanosine (gua-dR) as sole carbon source. Sensitivity was tested on plates containing 10 mM glucose, 20 µM thymine and about 1 mM dR, thy-dR or gua-dR. Growth was recorded after about 12 hours.

As predicted, the mutants fall into two classes on the basis of their ability to grow on deoxyribose. None of the mutants retained the ability to grow on either purine or pyrimidine deoxynucleosides. This observation has also been made of tlr$^-$ mutants by Stacey (in Bockrath, Osborn & Person, 1968). Thus it seems that only mutants with a block in the catabolism of deoxyribose phosphates were found as low thymine requirers. From these properties it was predicted that the mutants in class 1 are drm$^-$ while those in classes 2 and 3 are dra$^-$. Measurement of the activities of the enzymes aldolase and mutase in a sample of these mutants (Beacham et al, 1968) has confirmed this interpretation of the mutations involved (see also next section).

"Thymidine" sensitivity has been shown to be a phenomenon produced also by other deoxynucleosides with varying degrees of effectiveness, deoxyguanosine having only a small effect (Beacham et al, 1968). This explains the lack of any observed sensitivity to gua-dR on plates in Table 5. Some tlr$^-$ mutants (of S.typhimurium) also show sensitivity to deoxyribose itself, although the effect is not very marked, recovery taking place quickly. Observation of this sensitivity is improved by incubation of the plates at a lower temperature, for a shorter time, or especially by using ribose as carbon source in place of glucose.

The interesting observation on sensitivity that emerges from the data in Table 5, is that only the mutants unable to grow on deoxyribose are sensitive to thymidine and deoxyribose, and

apart from the single exception (class 3, which will be considered later), they are all sensitive. The same holds true for sensitivity to deoxyuridine and deoxycytidine. Thus it appears that sensitivity is due to the absence of aldolase.

(7) Assay of deoxyriboaldolase and deoxyribomutase

This conclusion suggested a means for classifying tlr$^-$ mutants of E.coli which cannot be classified by their growth on deoxyribose as carbon source. E.coli C600 and its two tlr$^-$ derivatives: P152 (which is resistant to thymidine) and CR34 (which is sensitive) were used to test this classification. P152 was predicted to be drm$^-$ and CR34, dra$^-$.

Enzymes were assayed using toluenized or osmotic shock (Neu & Heppel, 1965) extracts from cultures induced with thymidine. Aldolase was estimated from the rate of disappearance of diphenylamine-reacting material using dR-5-P as substrate, and was found in both extracts. Mutase proved to be more difficult to assay. Several methods were tried. Firstly the catabolism of thymidine was utilized. In the presence of phosphate both thymine (measured by its absorbancy at 300 mμ at pH 13 - Hotchkiss, 1948) and diphenylanine-reacting material are produced (ie. thymidine phosphorolysis). Disappearance of the latter would indicate the activity of both mutase and aldolase. Although this method has been successfully used (Bockrath et al, 1968) no reaction was observed in my studies because, it is now realized, the mutase

reaction is extemely slow in comparison to that of thymidine phosphorylase. This method could only indicate mutase activity in the presence of aldolase. A modification attempting to overcome this was made by measuring the acid-labile phosphate in the reaction mixture (hydrolysis with 0.5N $HC1O_4$ at 37° for 5 min, phosphate being measured according to Lowry & Lopez, 1946). This distinguishes dR-1-P from dR-5-P, as the former is very acid-labile (Friedkin & Kalckar, 1950). The disappearance of phosphate is thus a measure of mutase. Although a positive reaction was observed, using a toluenized extract of CR34, indicating the presence of mutase, the (lesser) reaction observed in a control without thymidine, made the method unsuitable for reasonable measurements of activity. (The estimated activity of 7.4 mμ moles/min/ml extract is similar, however, to the value obtained by the method used for the results in Table 6).

A second approach was made using dR-5-P as a substrate, and measuring the appearance of acid-labile phosphate. Aldolase activity would of course deplete the substrate, and because this activity is considerably greater than that of mutase after induction by thymidine (Table 7), this is unfavourable for measuring mutase activity. Nevertheless, this method showed that, as predicted, strain 2006 and one of its class 2 $\underline{tlr}^-$ derivatives (Table 5) have mutase activity. (The latter's activity was estimated as 20 mμ moles/min/mg protein). These assays were performed on osmotic shock extracts, and therefore indicate that mutase is also a

"surface" enzyme. An attempt to "capture" the dR-1-P produced from dR-5-P by mutase was made by adding thymine and looking for its conversion to thymidine (by reversal of thymidine phosphorolysis). This proved unsuccessful for no apparent reason.

Finally, dR-1-P was used as a substrate in an assay mixture containing NaF to inhibit phosphatases. Four things were assayed during the reaction: (a) the disappearance of acid-labile phosphate (ie. dR-1-P), (b) the disappearance of acid-hydrolysable diphenylamine-reacting material after precipitation of unhydrolysed sugar phosphates with $ZnSO_4$ and $Ba(OH)_2$ (Manson & Lampen, 1951a) ie. dR-5-P is precipitated leaving dR-1-P for assay, (c) the appearance of dR-5-P in this precipitate (being released by acidification and measured with the diphenylamine reaction), and (d) the disappearance of total dR (ie. free dR + dR-phosphates). The measurements (a) and (b) directly assay mutase, while (c) is only a measure of mutase in the absence of aldolase, which otherwise rapidly removes dR-5-P. A loss of total dR in (d) demonstrates the presence of both mutase and aldolase. The results are shown in Table 6.

These data show that, as predicted, P152 has lost mutase activity and CR34 has lost aldolase activity. These results are in agreement with a recent study of several *tlr*$^-$ mutants of *E.coli* by Munch-Petersen (1968). Using techniques equivalent to those in Table 6 marked aldolase and (d), she concluded that her strains were deficient in mutase and/or aldolase.

Table 6 Aldolase and mutase activities in extracts of thymidine-induced cultures of C600, P152 and CR34

Strain	Aldolase	Reaction of dR-1-P			
		(a)	(b)	(c)	(d)
C600	122	5.0	5.0	0	5.0
P152	11.4	0	0	0	0
CR34	0	15.6	11.2	12.5	0

Activities are expressed in mμ moles/min/mg protein (0 means activity was not detectable). Exponential-phase cultures were induced for about $1\frac{1}{2}$ generations with 1-2 mM thymidine. A toluenized extract was used for aldolase, and an ultrasonicate for mutase. Protein was measured according to Lowry et al (1951). Rate (a) is loss of acid-labile phosphate, (b) loss of dR-1-P, (c) appearance of dR-5-P and (d) loss of total dR. Full details are given in Methods.

(8) The inducer of the deoxynucleoside phosphorylases and deoxyriboaldolase

The characterization of P152 and CR34 enabled us to attempt to find out what the inducer of thymidine phosphorylase is. Razzell and Casshyap (1964) proposed that dR-1-P might be the inducer, because they observed induction with pyrimidine or purine deoxynucleosides, although the latter are not substrates of thymidine phosphorylase. If this is so, both *drm*$^-$ and *dra*$^-$ strains ought to be inducible by these compounds, whereas if dR-5-P, for example, were to be the inducer, induction would not occur in the

drm⁻ strains.

The activity of thymidine phosphorylase before and after induction by thymidine was therefore compared in the three strains: C600, P152 and CR34. The activities of purine phosphorylase and aldolase were also compared (Table 7).

Table 7 Inducibility by thymidine of deoxynucleoside catabolic enzymes

Strain	Induced	Thymidine phosphorylase	Purine phosphorylase	Aldolase
C600	-	14.4	82	7.4
	+	136	255	122
P152	-	22.9	73	11.4
	+	35.9	78	11.4
CR34	-	110	343	0
	+	1400	520	0

Activities are expressed in mμ moles/min/mg protein. Induction was performed as described under Table 6. Assays were performed as described in Methods, on toluenized extracts. The three enzymes were assayed using different extracts, induction ratios cannot therefore be strictly compared. Deoxyguanosine was used as substrate for purine phosphorylase. Negligible phosphorylase activity was observed in the absence of arsenate or substrate.

It can be seen that P152 differs from the parental strain in that the three enzymes assayed are no longer induced by thymidine. CR34, in contrast, is constitutive for the phosphorylases, these being hyper-induced by thymidine. The simplest interpretation of these data is that dR-5-P is the inducer of all three enzymes. On this hypothesis drm$^-$ strains are uninducible because they cannot synthesize dR-5-P from thymidine, and dra$^-$ strains are constitutive because as a result of a block in its further catabolism, they possess an increased pool of dR-5-P, when growing with a low concentration of thymine. (These strains were grown with 40 μM thymine). We saw in section (2) above, that this increased pool is thought to occur only during growth with a low thymine concentration, because of the controlling effects of a thymidine nucleotide on deoxynucleotide synthesis. We have argued (Beacham, Barth & Pritchard, 1968) that this effect explains, at least partly, why Breitman & Bradford (1967b) found only a repressed level of thymidine phosphorylase in their dra$^-$ mutant: they used a high concentration of thymine for growth (1 mM). This interpretation is supported by the finding that the activity of thymidine phosphorylase is decreased in dra$^-$ strains by increasing the thymine concentration in the growth medium (Beacham, Barth & Pritchard, 1968).

Confirmation that dR-5-P is the inducer is provided by theinducibility of thymidine phosphorylase in the tlr$^-$ mutants of S.typhimurium. As predicted, deoxyribose is also found to be an

inducer in these strains, both in the dra⁻ and drm⁻ mutants. Induction of a dra⁻ mutant with 1 mM thymidine or deoxyribose for two cell doublings, produced thymidine phosphorylase activities of 780 and 1840 mμ moles/min/mg protein respectively (the uninduced activity being 470). Further data on this (including a tlr⁺ and drm⁻ strain) has been published (Beacham et al, 1968).

(9) Sensitivity to deoxyribosides

The finding that only aldolase negative strains are sensitive to deoxyribosides suggested that this sensitivity must be due to an accumulation of dR-5-P (as a consequence of the phosphorolysis of the added compounds). However, in view of our finding that dR-5-P is the inducer of the deoxyriboside phosphorylases, one cannot conclude that dR-5-P is also the toxic compound. Thus in drm⁻ strains the activities of both phosphorylases are very much lower than in dra⁻ strains, especially when induced (Table 7). As a consequence, the pool size of dR-1-P (which will be determined by the rates of phosphorolysis and excretion as free deoxyribose (Figure 26) is probably also much lower in drm⁻ strains than in dra⁻ strains. Either or both dR-1-P and dR-5-P may therefore be the toxic compound.

The behaviour of 2454, however, is consistent with both compounds being toxic. As would be expected on the hypothesis that sensitivity is due to dR-phosphate accumulation, we saw (Table 5) that thymidine sensitive strains of S.typhimurium are

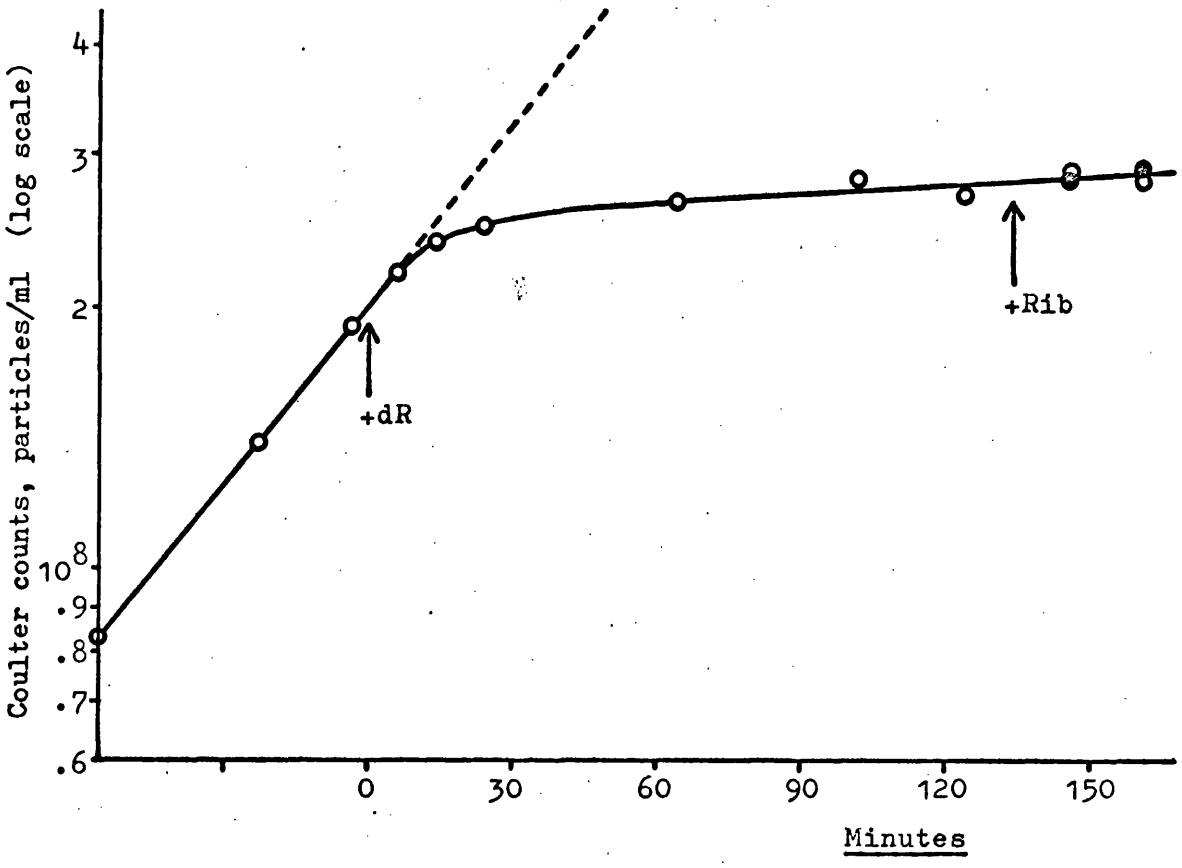


Figure 28 The effect of deoxyribose (giving 200 µM) added to an exponential-phase culture of KSU2454. The strain was growing in M9 medium containing 10 mM glucose, 200 µM threonine, 300 µM leucine and 200 µM thymine. Ribose (400 µM) was added to half this culture at the time indicated. No significant growth took place in either half during subsequent incubation overnight.

also sensitive to deoxyribose itself, although this is less marked (presumably because the kinase reaction is slower than thymidine phosphorolysis under these conditions). 2454, however, was found to be exceptionally sensitive to deoxyribose when this was added to the culture medium (Figure 28). Considering the evidence above (Figures 26 & 27) that 2454 is both dra$^-$ and drm$^-$, this exceptional sensitivity is understandable (the dR-5-P formed cannot be catabolized or removed anabolically), and demonstrates that dR-5-P is a toxic compound.

Surprisingly, 2454 is also sensitive to thymidine. This is not expected for drm$^-$ strains on the hypothesis above. However, the catabolism of thymidine by 2454 (Figure 26) suggests that it has thymidine phosphorylase activities similar to those of the dra$^-$ drm$^+$ strains (2001 and 2477) which is also not expected of a drm$^-$ strain. The simplest way to account for these properties seems to be to postulate that 2454 has a mutation making it constitutive for thymidine phosphorylase. Such constitutive tlr$^-$ mutants of S.typhimurium have been reported by Hoffee (1968); 2454 may be of this type. On this interpretation, therefore, the sensitivity of 2454 to thymidine means that dR-1-P accumulation is also toxic. (On the other hand, there may be a way to account for the apparent lack of mutase activity in 2454 without it being drm$^-$).

It has been suggested (Beacham et al, 1968) that sensitivity to dR-phosphates may be related to numerous other cases in E.coli of sensitivity to sugars, in mutants unable to catabolize the

phosphorylated form of the sugar, viz, ribulose-5-P (Englesberg et al, 1962), glycerol-1-P (Cozzarelli, Koch & Lin, 1965), fructose -1,6-diphosphate (Böck & Neidhardt, 1966), rhamnulose-1-P (Barkulis, 1949; Englesberg & Baron, 1959), and galactose-1-P (Kurahashi & Wahba, 1958). The latter authors suggested that the sensitivity to galactose is due to phosphate deficiency, caused by its being trapped in a non-utilizable form. This scheme has the merit of providing an explanation for the generality of this phenomenon; sensitivity caused by either dR-1-P or dR-5-P accumulation would of course be consistent with it. Studies on the nature of sensitivity and its reversibility (Beacham et al, 1968) have shown that it is unlikely to be due to the competitive inhibition by dR-5-P of enzymes concerned with the metabolism of ribose-5-phosphate. The absence of reversal by ribose (Figure 28), even when this is used as carbon source in place of glucose, also supports this conclusion, and therefore makes a general mechanism of sensitivity (eg. phosphate trapping) seem more likely.

(10) An operon for the catabolism of nucleosides?

Several lines of evidence support the idea that the deoxynucleoside phosphorylases, mutase and aldolase are part of a group concerned with the catabolism of deoxynucleosides, rather than their synthesis as has been suggested (Racker, 1952). Thus Hoffmann & Lampen (1952) showed that these enzymes enable E.coli to utilize deoxynucleosides as a carbon source. The demonstration

of a deoxyribonucleotide synthetic pathway via ribonucleotide reductase (Reichard, 1962; Larsson & Reichard, 1966b,c), and the difficulty with which wild-type E.coli strains convert thymine to thymidine (Crawford, 1958) argue against the necessity for, and ability of, this group to act anabolically. Two properties of these enzymes also support this contention: the osmotic shock procedure of Neu & Heppel (1965), which releases otherwise exclusively degradative enzymes, has been shown to release at least thymidine phosphorylase and aldolase (Kammen, 1967; Munch-Petersen & Vilstrup, 1967) and probably mutase (section (7) above) into the medium. Also, inducibility, a property characteristic of catabolic enzymes, is shown by the phosphorylases and aldolase at least (Table 7). These enzymes, therefore, seem to constitute a catabolic group.

We have suggested (Barth et al, 1968) that the genes specifying the enzymes in this group may constitute an operon. Consistent with this are the data in Table 7 which are taken to mean that the phosphorylases and aldolase share a common inducer (dR-5-P). In addition, the genes specifying aldolase and mutase map close together at about 89 min on the linkage map of E.coli (Alikhanian et al, 1966; Beacham et al, 1968), as does the gene for purine nucleoside phosphorylase (Ahmad, Barth & Pritchard, 1968), and probably that of thymidine phosphorylase (Ahmad, unpublished data).

As deoxynucleosides are probably not encountered in the natural environment of Enterobacteria, except as breakdown products

of DNA, we considered the possibility that this group may be part of a larger one concerned with the catabolism of DNA and its use as a carbon source (a possibility proposed by Racker, 1952). Other enzymes which were considered likely to be in such a group, viz, 5'-nucleotidase (Neu & Heppell, 1965) and cytidine deaminase, have been shown, however, not to be induced by thymidine (Beacham, unpublished data).

Although one operon with at least part of this function seems likely, sufficient information is not yet available to determine its constitution. The situation is complicated by the fact that purine nucleoside phosphorylase seems to accept both ribo- and deoxyribonucleosides as substrates (Koch, 1956; Ahmad et al, 1968), and deoxyribomutase has recently been suggested to accept both ribose and deoxyribose-phosphates (Kammen, personal communication). In agreement with the latter, we have found that strains classified as drm$^-$ (some enzymically, but most by their lack of thymidine sensitivity) are unable to utilize ribonucleosides as sole carbon source in contrast to the dra$^-$ strains (Beacham, unpublished data). This complication creates the difficulty that, if dR-5-P were the only inducer of such an operon, induction by, and therefore probably growth on, ribonucleosides could not occur. Consequently drm (mutase) and pup (purine phosphorylase) have been suggested to be in a different operon to tpp (thymidine phosphorylase) and dra (aldolase) (Kammen and Munch-Petersen, personal communications).

Several observations suggest, however, that *drm* and *dra* are in the same operon. Thus Munch-Petersen (1968) concluded that one out of about a dozen spontaneous *tlr*$^-$ mutants of *E.coli* tested was both *drm*$^-$ and *dra*$^-$. Our own observations are probably similar to this: the one exceptional mutant (2006-A7) in class 3 of Table 5 (out of 37 mutants) has all the properties expected of a *dra*$^-$ *drm*$^-$ mutant (ie. inability to utilize deoxyribose as carbon source and sensitivity to deoxyribose but not to thymidine). It is not resistant to thymidine through lacking thymidine phosphorylase. These mutants could arise by a deletion covering both *dra* and *drm*, but a polar mutation seems more likely as 2006-A7 is not completely mutase negative. This is concluded from a simplified deoxyribose uptake experiment similar to that shown in Figure 27, using lower cell titres and taking samples only after 3½, 6 and 16 hours incubation. At 6 hours, the parent 2006 had taken up 37% of the added deoxyribose (200 µM), the 13 class 2 (*dra*$^-$) mutants had taken up an average of 25.5%, while 2006-A7 had taken up only 6% (considerably less than any individual class 2 mutant). After 16 hours all the cultures had taken up virtually all the deoxyribose. As 2006-A7 shows no detectable growth on deoxyribose as carbon source, this experiment is taken to mean that it has some mutase activity, although considerably lessthan the class 2 mutants.

From this experiment it was also noted that strain 2454 took up little deoxyribose, even after 16 hours incubation, thus supporting the conclusion in section (5) of its *drm*$^-$ *dra*$^-$ nature.

This strain, when plated on medium with deoxyribose as sole carbon source, spontaneously produces revertants that can grow. About 50 such revertants were selected from plates containing also a high concentration of thymine (200 μM) and were then purified on the same medium. These were tested for their high or low thymine requirement (with glucose as carbon source: deoxyribose enables tlr$^+$ strains of S.typhimurium to utilize a low thymine concentration). All of them were found to have simultaneously reverted to being tlr$^+$ (ie. drm$^+$ dra$^+$). This suggests very strongly that 2454 is a polar mutant and, therefore, that drm and dra are in the same operon.

(11) Discussion

The mechanism of thymine low requirement, and the mutations involved that have been described above, make a relatively straightforward picture. It seems that a block in the catabolism of deoxyribose-phosphates, either at the mutase or aldolase position, by allowing a build-up of the pool of dR-1-P, promotes the conversion of thymine to thymidine, and therefore its incorporation into DNA. Parenthetically, it was observed that the class 1 and 3 mutants of Table 5 (ie. drm$^-$) grow slightly better when only 5 μM thymine is added to the medium than the class 2 (ie. dra$^-$) mutants do. This was predicted, since the former, being unable to convert dR-1-P to dR-5-P, should maintain a higher pool of dR-1-P than the latter.

Most of the work published by other laboratories on this topic (Kammen, 1967; Breitman & Bradford, 1967b; Munch-Petersen, 1968) agrees with the picture outlined above. Hoffee (1968) has, however, proposed the existence of two distinct deoxyriboaldolases in S.typhimurium. This is inconsistent with the high frequency of dra$^-$ mutants found and their approximately equal frequency to drm$^-$ mutants (Table 5). Her first two classes of tlr$^-$ mutants correspond in their properties with classes 1 and 2 of Table 5 above. The mutants in her third class are postulated to havelost only a deoxyribose-inducible aldolase, leaving the catabolic route of deoxynucleosides intact. Why such a mutant should be tlr$^-$ or constitutive for thymidine phosphorylase is not explained. From this constitutivity, the inability of these mutants to grow on deoxyribose as carbon source, and their sensitivity to deoxyribose, we would predict them to be dra$^-$. They have aldolase activity, however. Although it was stated above that our enzymic tests on a selection of predicted dra$^-$ mutants showed the absence of aldolase activity in them, one such mutant (2006-A1) had this activity. Because aldolase is a "surface" enzyme, it seems possible that 2006-A1 and these mutants isolated by Hoffee, are aldolase negative by virtue of a changed location of the enzymes, being inaccessible to its substrate in vivo, but having activity when extracted. Her fourth class is also puzzling. Perhaps this is also an enzyme-translocation mutation, also preventing aldolase activity on

intracellular substrate (therefore giving tlr$^-$ phenotype) but permitting catabolism of deoxyribose from the medium (ie. allowing growth on it). The frequency of mutants in the four classes is not given, but Hoffee's proposal would predict those in class 2 to be least frequent (as double mutants) while we would predict a frequency about the same as that of class 1 mutants.

These points serve to indicate that the system studied is genetically and biochemically complex: more data are needed before it can be fully understood.

D I S C U S S I O N

The data in Chapter I showed that initiation of chromosome replication takes place in a growing culture during the inhibition of DNA synthesis. (Initiation means here, of course, the commitment to start a new cycle of replication when this inhibition is reversed.) When nalidixate is used as the inhibitor, the kinetics of the stimulation of DNA synthesis are compatible with initiation continuing at the normal rate during the inhibition. By "normal" is meant the rate calculated from the model of Cooper & Helmstetter (1968) for the particular growth rate used (g = 40 min). (Fortunately at this growth rate, K.G. Lark (1966) proposes an identical rate of initiation.) This therefore leads to the important conclusion that DNA synthesis (in contrast to mass increase) is not necessary for initiation to proceed normally.

Consider what this means. The process of initiation is not dependent on the previous replication cycle first being completed (as has been proposed by K.G. Lark (1966); this will be discussed further below). The existence of multifork replication patterns at fast growth rates (evidence for which was described in the Introduction) also demands the same conclusion. It is also necessary to conclude that initiation is not brought about by the cell reaching a critical mass/DNA ratio, since as DNA synthesis is inhibited, this will occur much sooner in each cell than during normal growth. This leaves us with the possibility that the mass

of a cell itself controls the time of initiation. This assumption was made in Appendix 3 and will now be justified as far as possible.

The relationship between the mean cell mass, $\bar{m}$ (the absorbancy of a culture at 450mμ) and the growth rate (doublings/hour = 60/g), has been reliably established by Schaechter et al. (1958) for S.typhimurium, and can be expressed as:

$$\log \bar{m} = y\frac{60}{g} + z \quad \text{(where y and z are constants)}$$

Measuring the value of y from the gradient of the line superimposed on their data and substituting gives:

$$\bar{m} = k2^{63/g} \qquad \text{- - - - (1)}$$

(where k is a constant). Using the cell age distribution function derived by Powell (1956), it can be shown that if the mass of an individual cell increases exponentially, then its mass m_o at age 0, is related to $\bar{m}$ by:

$$\bar{m} = m_o \times 2 \ln 2$$

and thus equation (1) becomes:

$$m_o = K2^{63/g} \qquad \text{- - - - (2)}$$

(where K is a constant).

This equation is still valid as long as the mass of a cell increases in the same manner at different growth rates, even if this manner is not exponential. Exponential increase seems most likely, however. It it were linear, for example, then when a cell divided, its rate of mass synthesis would have to double abruptly as it

became two cells. Microscopic observations on single E.coli cells by Schaechter et al (1962) is consistent with their volume increasing exponentially. Recent measurements of cell size distributions by Harvey, Marr & Painter (1967), using a sophisticated electronic particle counter, have demonstrated that cellular volume changes in a more complex manner, but approximates to exponential. One can reasonably assume cell density to remain constant throughout the growth cycle, so that these conclusions will apply to both mass and volume.

It will now be assumed that the replication model and parameters C and D. proposed for E.coli B/r, apply equally well to S.typhimurium. This is supported by the similarity in the two strains of the relationship between DNA content per cell, and growth rate (see Cooper & Helmstetter, 1968). We can therefore use equation (2) to calculate the mass of a cell at the time of initiation, i. This time is predicted by their model as:

$$i = ng - (C+D) \qquad ----(3)$$

where n is an integer such that ng is greater than, or equal to C+D. Assuming exponential mass increase of the cell, ie. $m_t = m_o 2^{t/g}$, then its mass at i will be:

$$m_i = m_o 2^{i/g}$$

Substituting from (2) and (3) gives:

$$m_i = K2^{63/g} \times 2^{(ng-(C+D))/g}$$

$$= K2^{(63-(C+D)+ng)/g}$$

As the value of C+D is estimated by Cooper & Helmstetter to be 63 minutes, this simplifies to:

$$m_i = K2^n \qquad \text{- - - - (4)}$$

This means that the mass of a cell at the time of initiation is constant throughout the range of growth rates for which n holds a particular value.

We can go a little further. Cooper & Helmstetter's model predicts that the number of replication forks, R, initiated at i is given by:

$$R = 2^{n-1}$$

Thus,
$$\frac{m_i}{R} = \frac{K2^n}{2^{n-1}} = 2K \qquad \text{- - - - (5)}$$

ie. the ratio of the mass of a cell at the time of initiation to the number of forks initiated, is constant. The possibility of deriving this conclusion from Cooper & Helmstetter's model was pointed out to us by Dr W. Donachie.

Even though this derivation depends on several approximations and (justified) assumptions, it seems too remarkable not to be of significance. The obvious conclusion to draw is that is is important in the control of initiation, ie. a cell "measures" its own mass in some way and then initiates when this reaches a specific value (or multiple of that value, when the cell contains more than one chromosome origin). This, then, is the justification of the assumption used in Appendix 3. Such a mechanism provides an

an adequate control system to ensure that DNA synthesis and cell division are coordinated, by ensuring that initiation occurs only once in the cell cycle. The sequence of events can be visualised as follows: the cell grows and initiates replication at the critical mass. The entire chromosome requires C minutes to replicate; termination signals cell division which occurs D minutes later. Initiation of the subsequent replication cycle does not occur until precisely one mass doubling after the first initiation.

Consider now what happens if the culture is transferred to a growth medium permitting a faster growth rate (ie. a "shift-up"). Mass increase will immediately change to the new rate, but the new rate of DNA synthesis will be delayed somewhat, because it depends on the frequency of initiation, and this will not reach the new rate until all the cells have reached the critical mass after the shift-up. The new rate of cell division will be further delayed until C+D minutes after this (because of its link to initiation by this period). It is this delay of cell division that causes the cells to increase their mass with an increase of growth rate. Shift-up experiments on cultures of S.typhimurium have been performed by Kjeldgaard, Maaløe & Schaechter (1958) and Kjeldgaard (1961). The results show that the sequence of events is the same as proposed above, which thus strongly supports this scheme.

Pritchard has proposed a mechanism which would enable the cell to titrate its own mass, relative to the number of chromosome

origins it contains, and then initiate replication when this reached a critical value, ie. giving a possible explanation for equation (5) (Pritchard, 1968; Pritchard, Barth & Collins, 1968). Briefly, the model proposes the following:

(a) that control of initiation is negative, ie. DNA precursors, attachment sites and "initiation enzymes" are not limiting during normal growth, but initiation is prevented by an inhibitor (H).

(b) H is produced by a gene located at or just past the origin, and is transcribed only at the time of its replication, producing a fixed number of messenger RNA, and thus H, molecules, regardless of the growth rate. The size of the H pulse produced is therefore proportional to the number of origins.

(c) H interacts with the origin, or an "initiator" in such a way that a two-fold change in its concentration effects the transition between complete and zero inhibition.

The volume of the cell (assumed to be proportional to its mass) thus determines the concentration of H, the one is inversely proportional to the other. When growth has lowered the concentration of H to the threshold, initiation occurs, leading to the production of a new pulse of H, which then prevents initiation until growth has diluted it to threshold again. A short dead-time between successive acts of initiation is necessary to prevent more than one initiation occurring before the concentration of H has had time to rise significantly.

During normal growth, the concentration of H can be shown to oscillate between theshold, and twice this value. For a shift-up, we saw that the initial delay of cell division (ie. with respect to the new growth rate) causes the cells to increase in volume, while the number of origins per cell may increase for the same reason. It can be shown that the larger pulse of H that would be produced at initiation under these circumstances, will be exactly compensated by the increase in volume, so that the concentraction of H still oscillates over the same range.

The model also has very interesting self-regulatory properties. If a cell should initiate early (ie. before (H) has reached threshold), then being smaller than otherwise, the pulse of H will cause a correspondingly greater increase in its concentration. This means that the next cycle will take longer for this concentration to reach threshold. Thus there will be a negative correlation between successive inter-initiation times, while daughter cells will be positively correlated in this (as they "inherit" the same H concentration at birth). Both these properties have been observed by Schaechter et al (1962). This in-built self-regulation gives this model an advantage over positive models (Jacob, Brenner & Cuzin, 1963; K.G. Lark, 1966; Maaløe & Kjeldgaard, 1966; Donachie, discussion at Proc. Genet. Soc. April, 1968), which propose, as a generalization, the synthesis of one "initiator"/chromosome origin/mass doubling period. Without further assumptions, such a model does not prescribe the mass that

cells will have at a particular growth rate, although the sequence of events at a shift-up or shift-down will lead to the same predicted overall change of mass. After a sufficient period to allow drift to occur, surely equation (5) will no longer remain true. In Pritchard's model, however, the size of the H pulse prescribes the mass of the cells at any growth rate. It also draws novel conclusions about the relationship between the chromosome and episomes such as F or λ, with regard to their replication, induction and curing. This is fully discussed by Pritchard et al (1968) and will not be considered here.

The interpretation of the difference between the effect of thymine starvation and nalidixate treatment on reinitiation of replication, examined and discussed in Chapter I, is very important to the model discussed above. The studies in Chapter II lead one to the conclusion that the greater effect of nalidixate is not due (except perhaps to a small extent) to artifacts such as repair synthesis or episomal DNA sythesis. Thus, as col15 is induced by both thymine starvation and nalidixate treatment, no difference between the two treatments is expected on this ground. Secondly, the DNA degradation caused by nalidixate is small, reaching only 3½% of the total DNA after 40 minutes. The density-label experiments (Figures 3 and 4) are consistent with this view: they demonstrate that the DNA synthesis induced by these treatments is located specifically at the chromosomal origin (ie. is not associated with phage DNA synthesis to any marked extent), and that little material

of density between that of hybrid and light DNA is found (which would be expected for repair synthesis).

If the nalidixate effect were greater because of an artifact, then the two treatments applied together would probably be expected to cause the greater effect. However, an effect closer to that of thymine starvation alone was found after both treatments (Figure 2), despite the fact that both treatments simultaneously did not reduce the DNA degradation induced by nalidixate alone (Figures 19 and 20). These results suggest, rather, that thymine starvation restricts the initiation process in some way.

As we have seen, the reinitiation effects of nalidixate are entirely consistent with the model derived above mainly from the work of Cooper & Helmstetter (1968) and also from that of Maaløe and his colleagues (extensively reviewed by Maaløe & Kjeldgaard, 1966). K.G. Lark (1966) has proposed a model of replication that is incompatible with our model and conclusions in several respects, however. Thus he has suggested that the termination of replication (by converting a "proreplicator site" into a "replicator site") is necessary before the next replication cycle can be initiated. He has no evidence for this assumption, which is quite incompatible with the data discussed above.

He proposed that the effect of thymine starvation (causing about half the maximum possible level of reinitiation) is possible on this model, because only one strand of the DNA duplex is attached to the "replicator", the free strand thus being available for

reinitiation without termination having first to occur. This requires that the asymmetrical effect shown as A in Figure 5 (page 33) be produced; Lark & Bird (1965a) have presented data which they interpret as supporting this. Thus they labelled the DNA of cells growing in succinate (g = 70 min) during 80 minutes amino acid starvation, and then analysed the number of labelled fragments per cell by autoradiography. A mode of 2 fragments per cell was found in the control culture (ie. one labelled chromosome). This was increased to 4 by previous thymine starvation, whereas they predicted a bimodal distribution of 2 and 6 for the effect B in Figure 5. However, these modes are only compatible with their model of cells growing in succinate possess two alternately replicating chromosomes (as proposed by Lark & Lark, 1965), and if the majority of them do not divide during the period of amino acid starvation. Evidence against alternate replication has been produced by Koch & Pachler (1967) and Cooper & Helmstetter (1968). Even their own data are against them on this score: they record that 13% of the control cells are unlabelled, whereas, their model predicts that no cells should be unlabelled. Although Lark & Bird (1965a) do not state what fraction of the cells divided during starvation, Lark & Lark, (1965) found 23% did so under similar conditions, and these would thus form 46/123 = 37% of the final population.

Taking these two points into account, it can be shown that (a) the pattern predicted by Cooper & Helmstetter (1968) for this

growth rate is compatible with Lark & Bird's data, and then that (b) using this pattern, the distribution of labelled fragments reported by them is compatible with effect B (Figure 5) after thymine starvation. (A complex distribution is predicted, with modes at 2,4,6 and 12, with 4 being the most frequent class). A third possible effect of thymine starvation, not mentioned previously, that two thirds of the cells reinitiate fully, while the other third fail to replicate their DNA at all, seems unlikely as Lark & Bird (1965a) found that only 0.3% of the thymine starved population contain no labelled fragments.

Although not designed for this purpose, experiments in Chapter I have been used to obtain an estimate of the value of C in 15T⁻(555-7). The data suggest that this is the same as in B/r. An estimate of D can also be obtained, from the cell division that occurs when nalidixate is added to a culture: according to the studies of Helmstetter & Pierucci (1968) on B/r, the cells which divide under these circumstances (or when DNA synthesis is inhibited with mitomycin C or ultraviolet irradiation) are those which had terminated a cycle of replication. They therefore concluded that completion of a cycle of replication is a necessary and sufficient condition for cell division to occur. The increment in Coulter counts in a nalidixate-treated culture of 15T⁻(555-7) (Figure 8, page 44, and consistently observed many times) is compatible with this view. The fact that cell division ceases after about 20 minutes

and an increment of about 40% is observed, both lead to D being estimated as a little over 20 minutes, ie. also the same as the estimate for B/r by Cooper & Helmstetter (1968).

The conformity of these estimates of C+D in $15T^-$(555-7) to those in B/r is satisfying, but leads to a puzzle as to why, then, the former strain should have a greater DNA content than the latter, as discussed in the Introduction (p.17). It is possible that our and Lark's strain of $15T^-$(555-7) are no longer the same. It seems more likely, however, to be due to a difference in the growth conditions used: a high thymine concentration, could, by restricting the size of the DNA precursor pool (Chapter III), reduce the rate of DNA synthesis, ie. increase C/g, which according to Cooper & Helmstetter's model will lead to an increased DNA content. Further work is necessary to resolve this puzzle.

In disagreement with Helmstetter & Pierucci's conclusion, Donachie, Hobbs & Masters, (1968) observed no increase in cell number after removal of thymine from a culture of JG151 growing exponentially with g = 60 min. (Helmstetter's model predicts that about 30% should do so, assuming C = 41min and D = 22min). This disparity could perhaps be due to a peculiarity of the strain JG151 (see Chapter II), but it seems more likely to be due to the thymine starvation: perhaps thymine is necessary for septum formation. This may be an important clue to understanding the lesser effect of thymine starvation on reinitiation discussed above. In glucose medium, about half a bacterial population

will be involved in septum formation. If this group failed to reinitiate in the absence of thymine, this would be quantatively compatible with the data obtained, and explain why thymine starvation prevents nalidixate producing its full effect.

There is another observation that this postulate could explain, viz the plateau in the stimulation of DNA synthesis caused by nalidixate (Figure 9, page 47). According to the postulates above, a third cycle of replication cannot be initiated in the absence of DNA synthesis, because septum formation is first necessary, and this is prevented as the prerequisite termination of the second cycle obviously cannot take place. Perhaps it should be pointed out that the second cycle can be initiated in all the cells according to this scheme, because at the growth rate used (g = 40 min), about half the population (aged 0 to 20 min) have no intervening septum formation stage before the point of initiation is reached, while the other half (aged 20 to 40 min) have already started septum formation (which is not stopped by nalidixate treatment).

This speculation, and Pritchard's model, although compatible with the data, obviously require further work to be verified or disproved. Further work is also necessary to confirm the proposal that nalidixate interacts with DNA in vivo. The compound, oxolinic acid (1-ethyl-1,4-dihydro-6,7-methylenedioxy-4-oxo-3-quinolinecarboxylic acid) has a very similar molecular structure to nalidixate (their right-hand halves, as drawn on page 22, are identical) and

seems to have very similar properties to nalidixate (Pianotti, Mohan & Schwartz, 1968).

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METHODS

(1) Bacterial growth

Bacteria were cultured aerobically at 37° in M9 synthetic medium (Na_2HPO_4, 0.7%; KH_2PO_4, 0.3%; NaCl, 0.05%; NH_4Cl, 0.1%; $CaCl_2$, 0.002%; $MgSO_4$, 0.02%; pH 7 to 7.2) in a New Brunswick gyrotory shaker. Glucose (10-20 mM) was used as carbon source unless otherwise stated. The medium was inoculated (about a 1% inoculum) with a fresh overnight culture, grown in the same medium without aeration. (This was itself inoculated with an isolated colony of the strain).

Cultures of *E.coli* 15T⁻(555-7) (*met*⁻ *arg*⁻ *try*⁻ *thy*⁻ *tlr*⁻) were supplemented with 350 µM methionine, 250 µM arginine, 250 µM tryptophan and (unless otherwise stated) 20 µM thymine, those of 15TAU⁻ (*pyrA*⁻ *thy*⁻ *tlr*⁻), with 100 µM arginine, 100 µM uracil and 20 µM thymine, and those of JG151 (*thy*⁻ *tlr*⁻) solely with 20 µM thymine. Cultures of C600 (*thr*⁻ *leu*⁻ *thi*⁻), P152 (*leu*⁻ *thi*⁻ *thy*⁻ *drm*⁻) and CR34 (*thr*⁻ *leu*⁻ *thi*⁻ *thy*⁻ *dra*⁻) were supplemented with 200 µM thronine, 300 µM leucine, 5 µM thiamine and 40 µM thymine; cultures of KMBL90 (*thr*⁻ *leu*⁻ *thi*⁻ *pyr*⁻ *thy*⁻ *tlr*⁻) in addition to the above, were also supplemented with 130 µM uracil and 0.3% casamino acids.

The *S.typhimurium* strains used (kindly made available by Dr A.Eisenstark) were: KSU 2793 (*thy*⁺ *tlr*⁺); KSU 2006 (*thy*⁻ *tlr*⁺); KSU 2454 (*thy*⁻ *tlr*⁻ *thr*⁻); KSU 2456 (*thy*⁺ *tlr*⁻); KSU 2462 (*thy*⁻

tlr$^+$); KSU 2001 (*thy*$^-$ *tlr*$^-$ *leu*$^-$); KSU 2477 (*thy*$^+$ *tlr*$^-$ *leu*$^-$). The *thy*$^-$ mutation in 2006 was induced by N-methyl-N-nitroso-N'-nitroguanidine. 2454 was derived from 2006 by selection on a low thymine concentration (25/µM). It had also become *thr*$^-$. 2456 and 2462 are respectively *thy*$^+$ and *tlr*$^+$ revertants of 2454. Both had become *thr*$^+$ spontaneously. 2477 is a *thy*$^+$ revertant of 2001. Supplementation of the culture medium, where necessary, was with 200 µM threonine, 300 µM leucine and 20 µM or 300 µM thymine (for *tlr*$^-$ and *tlr*$^+$ strains respectively).

Cell growth was generally measured as particle titre using a Coulter counter. The absorbancy of cultures (usually at 600 mµ) was measured with a Unicam SP 500 spectrophotometer. The number of viable cells was measured on samples suitably diluted in phosphate buffer (pH 7). These were spread on plates containing the usual growth-medium, solidified with agar (1.5%).

(2) Changes of media

Bacteria were transferred from one medium to another by collecting the cells on a membrane filter (0.45 µ pore diameter - Millipore, Oxoid or Schleicher & Schuell), washing them on the filter with pre-warmed medium (basic M9, or the new medium) and resuspending them in the new medium by rapid shaking. The procedure was carried out in a 37° hot room, and generally took 1-2 minutes. Recovery of cells from the Oxoid filters was the highest, being close to 100%.

(3) Density-gradient centrifugation analysis

10 ml samples of the cultures (the medium containing 100 μM 5-bromouracil in place of thymine) were pipetted into centrifuge tubes containing a few grams of crushed frozen M9 medium at -45°, and kept frozen overnight. They were thawed and centrifugedat 20,000g for 10 min. The pellet was washed with a few ml of 0.1 M tris-hydroxymethyl-aminomethane (tris) - 0.01 M ethylene diamine tetracetic acid (EDTA) solution (pH 8.0) and then resuspended in 1 ml of this. 0.1 ml lysozyme, dissolved in the tris-EDTA (1 mg/ml), was added to this suspension, which was incubated with gentle shaking for 30 min at 37°. 0.1 ml pronase (1 mg/ml tris-EDTA) was added and the incubation continued for a further 30 min.

3.92 gm of CsCl was weighed into an ultracentrifuge tube. The extract and washings (water), to a total volume of 3.025 ml, were added and the CsCl dissolved, giving a density (ρ) of 1.7 gm/ml. (These quantities were calculated from an equation of Vinograd & Hearst (1962): weight % ($CsCl : H_2O$) = $137.48 - \frac{138.11}{\rho}$ at 25°). A 0.05 ml sample was taken from this solution to measure the total radioactivity. The solution was centrifuged in the SW39 rotor of a Spinco ultracentrifuge at 33,000 rev/min, for not less than 48 hours, or at 31,410 rev/min, for not less than 56 hours.

The tubes were punctured and 3-drop fractions (about 0.025 ml per drop) were collected on 1.8 cm squares of glass-fibre paper (Whatman - GF 83). CsCl was removed by soaking the squares in

75% ethanol. After drying, the squares were put into vials and covered with 4-5 ml of scintillation fluid (5 g of 2,5-diphenyl-oxazole and 30 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per litre of toluene). These were counted in a Packard Tricarb scintillation spectrometer. ^{3}H and ^{14}C were counted in different channels, correction being made to the ^{3}H counts for overlap of the ^{14}C energy spectrum. The slope of the density gradient was calculated from the refractive indices (μ) measured on the first and last fractions collected. Density is related to μ by the equation: $\rho = 10.8601\ \mu - 13.4974$ at 25° (taken from Vinograd & Hearst, 1962).

This technique resulted in about 80% of the total counts in the centrifuge tube forming light and hybrid density bands.

(4) Incorporation of radioactive thymine into DNA

Samples (usually 1 ml) were taken from the culture and added to an equal volume of cold 10% trichloracetic acid (TCA). After being kept in the cold for about half an hour, they were filtered through a small membrane filter, which was then washed through with about 5 ml distilled water (which had washed out the sample tube). The filters were dried and put into 5 ml scintillation fluid and the radioactivity assayed in a Packard counter.

To eliminate any incorporation into RNA, 1 ml samples were incubated at 37° with 0.1 ml 6N NaOH for $4\frac{1}{2}$ hours (Roodyn & Mandel, 1960), neutralised with 0.1 ml 6N HCl and then treated with 1.2 ml cold 10% TCA. These were filtered etc. as above.

(5) Release of acid-soluble radioactivity from prelabelled DNA

Samples (usually 0.5 ml) were taken from the culture, added to 0.1 ml cold 30% TCA and kept cold for more than half an hour. They were then passed through a membrane filter, the filtrate being collected. The filter funnel was washed with water and ethanol, and dried between each filtration. An aliquot (about 0.1 ml) of the filtrate was transferred to an 18 mm square of glass paper (GF83 - Whatmans) which was dried and assayed as above. The acid-insoluble material left on the filter could also be assayed for loss of radioactivity (as in the previous section). This loss was equal to the gain of radioactivity in the filtrate (after correction for different counting efficiencies).

The sample taken for the total radioactivity incorporated by the culture was hydrolysed with an equal volume of 10% TCA at 90° for 40 minutes. An aliquot was transferred to a square of glass paper, dried and counted as above. The counting efficiency of this sample was thus made the same as that of the filtrates.

(6) Isolation of DNA from E.coli

Cells from an exponential-phase culture were harvested on a membrane filter and washed with a buffered EDTA solution (0.1 N NaCl, 10 mM EDTA and 10 mM tris, adjusted to pH 8.0 with HCl). They were resuspended in the same solution at 2-5 x 10^{10} cells/ml. Solutions of lysozyme and RNase were added to give concentrations of 100 and 50 µg/ml respectively. After 30 minutes incubation at

37°, the solution was supplemented with pronase to give 50 µg/ml and incubated for further 30 minutes. This solution was cooled and an equal volume of 2-ethoxyethanol gently layered on top. At the interface, the DNA can be twisted into a glass rod, the liquids being gradually mixed to precipitate all the DNA. The spooled DNA was dissolved in 0.15 N NaCl, 0.015 M trisodium citrate pH 7.0.

(7) Ultraviolet-light irradiation

An exponential-phase culture (at a titre of about 2×10^8 cells/ml) was filtered, washed with unsupplemented M9 medium, and resuspended at the same titre in this medium. 10 ml of this suspension was irradiated in a 9 cm Petri dish with ultraviolet light, while being agitated by hand. (The energy emission of the ultraviolet lamp was measured with a photoelectric probe as 7.35 erg/mm^2/sec, at a distance of 46 cm. About 10% of this output was transmissible through a glass Petri dish lid).

For the measurement of viability after irradiation, appropriate dilutions in buffer, plating (as above) and subsequent growth were carried out, as far as possible, in the absence of light.

(8) Measurement of deoxyribose in culture medium

Samples were withdrawn from the exponentially-growing culture and immediately filtered through a membrane filter (0.45 µ pore diameter) into cold test tubes. 1 ml aliquots were added to 2 ml diphenylamine reagent (Burton, 1956) and incubated for

16-20 hours at 30°. The absorbancy at 600 mμ was read.

(9) Enzyme assays

Cell extract preparation. Osmotic shock extracts were prepared according to Neu & Heppel (1965), and toluenized extracts by thoroughly shaking washed cells, resuspended in the appropriate buffer, with a drop of toluene. Ultrasonication was performed for 1 min (100 watt MSE disintegrator) on cells, washed and resuspended at 5 x 10^9/ml, in 10 mM tris-HCl buffer (pH 7.0).

Deoxyriboaldolase The reaction mixture of 0.5 ml contained 2 mM dR-5-P and 50 mM arsenate-succinate buffer (pH 6.0) or tris-HCl buffer (pH 7.3). 0.05 ml samples were added to 0.95 ml 0.5 N $HClO_4$, and incubated with 2 ml diphenylamine reagent according to Burton (1956).

Deoxyribomutase The assay mixture of 0.5 ml contained 1 mM dR-1-P, 10 mM NaF, ultrasonicate and (probably unnecessarily) 50 μM ATP and 5 mM $MgCl_2$. Samples of 0.05 ml were taken into an equal volume of 1.0 N $HClO_4$ and (a) brought to pH 4.0 with 0.3 ml 0.2 N Na acetate, and assayed for phosphate (Lowry & Lopez, 1946), or (b) neutralized after 10 min with 0.3 ml 0.2 N Na acetate and treated with 0.1 ml 0.3M $ZnSO_4$ and 0.2 ml 0.15M $Ba(OH)_2$. The precipitate was centrifuged and the supernatant assayed for dR (Burton, 1956). The precipitate was treated with 1 ml 0.5 N $HClO_4$, recentrifuged and the supernatant assayed for dR. 0.05 ml samples from the assay mixture were also taken into 0.95 ml

0.5 N $HClO_4$ and (d) assayed for total dR.

Thymidine phosphorylase was assayed by the phosphorolysis or arsenolysis of thymidine. The assay mixture of 0.5ml contained 10 mM thymidine and 10 mM phosphate buffer (pH 6.8) or 50 mM arsenate-succinate buffer (pH 6.0). 0.05 ml samples were taken into 0.45 ml 0.1 N NaOH and their $A_{300\ m\mu}$ read (Hotchkiss, 1948).

Purine phosphorylase was assayed by the arsenolysis of deoxyguanosine according to Tsuboi and Hudson (1957). The assay mixture of 0.5 ml contained 5 mM gua-dR, 10 mM arsenate and 10 mM tris-HCl buffer (pH 7.3). Samples of 0.05 ml were taken into 0.35 ml 2% $NaCO_3$ in 0.1 N NaOH and 0.05 ml Folin reagent (diluted by half with water) added. Their $A_{600\ m\mu}$ was measured after about 3 hours (at room temperature).

Appendix 1: Kinetics of incorporation into, or excretion from, an exponential phase culture

Consider the exponential increase of a component A during the growth of a bacterium, such that the amount of A doubles each mean generation time, then:

$$A_t = A_o 2^{t/g}$$

t = time

g = mean generation time

Now consider a substance S which has a constant rate of incorporation into, or excretion from, each unit of A ie:

$$\frac{dS}{dt} = kA$$

Then during exponential growth:

$$\frac{dS}{dt} = kA_t = kA_o 2^{t/g}$$

Integrating:

$$S_2 - S_1 = kA_o \int_{t1}^{t2} 2^{t/g}\, dt$$

$$= \frac{kA_o\, g}{\ln 2} (2^{t2/g} - 2^{t1/g})$$

Putting $S_1 = 0$ when $t_1 = 0$

$$S = \frac{kA_o\, g}{\ln 2} (2^{t/g} - 1)$$

ie $\underline{\underline{S \propto 2^{t/g} - 1}}$

Thus plotting S against $2^{t/g} - 1$ will give a straight line with a gradient proportional to k.

Appendix 2: Derivation of the expected kinetics of replication of a DNA region immediately following the origin in a (random) exponentially-growing culture

The total DNA of the culture will be replicated according to the equation derived in Appendix 1 (accepting the assumption made that the rate of replication per fork is constant - as evidenced in the Introduction),

$$\text{ie. } S = K(2^{t/g} - 1)$$

Now, putting S = fraction of total DNA (ie. ^{14}C) that has been replicated, gives:

$$\underline{S = 2^{t/g} - 1} \qquad (\text{as } S = 1 \text{ when } t = g)$$

If the rate of replication of the chromosome is linear with time at each replication fork, a particular position on it, randomly distributed with respect to the replication forks, will also be replicated linearly with time. Let P = the replicated fraction of a pulse-labelled section of DNA located at the origin (ie. ^{3}H), then:

$$P = {}^{t}/_{g} \qquad (\text{as } P = 1 \text{ when } t = g)$$

But, $$S + 1 = 2^{t/g}$$

ie. $$\log_2 (S + 1) = {}^{t}/_{g}$$

therefore, $$\underline{\underline{P = \log_2 (S + 1)}}$$

The equation is used to plot the theoretical line in Figure 4.

Appendix 3: The predicted kinetics of reinitiation during the inhibition of DNA synthesis and the subsequent kinetics of DNA synthesis

The fundamental assumption will be that the initiation of replication is dependent entirely on the mass of a cell (see Discussion): a cell that has just initiated will reinitiate when its mass (or that of its daughters) has precisely doubled, whether or not DNA synthesis has been permitted. In an exponentially-growing culture, therefore, where the mass m_t, at time t, is related to the mass m_o, at time 0, by:

$$m_t = m_o \, 2^{t/g} \qquad (g = \text{generation time})$$

then during balanced growth, the number of replication forks R_t, at this time will be related to the number at time 0, by the same function:

$$R_t = R_o \, 2^{t/g} \qquad \text{- - - - (1)}$$

R_t is the resultant of the addition of newly initiated forks: R_a, and the loss of forks by termination: R_b

$$\text{ie. } R_t = R_o + R_a - R_b$$

$$\text{or} \qquad = R_o \, (1 + a - b) \qquad \text{- - - - (2)}$$

(where a and b are initiation and completion factors respectively).

Therefore from (1) and (2) $a - b = 2^{t/g} - 1$

The model of Cooper & Helmstetter (1968) predicts that

$$\frac{a}{b} = 2^{C/g}$$

Substituting: $b2^{C/g} - b = 2^{t/g} - 1$

therefore, $b = \frac{2^{t/g} - 1}{2^{C/g} - 1}$ and $a = \frac{2^{C/g}}{2^{C/g} - 1}(2^{t/g} - 1)$

Consider now the effect of inhibiting DNA synthesis for time = ti. Figure 8 shows that mass continues increasing exponentially at an unchanged rate for 50-60 minutes when nalidixate is added. According to the first assumption, therefore, initiation will continue at an unchanged rate up to this time. Termination is prevented; equation (2) therefore becomes:

$$R_{ti} = R_o(1 + a)$$

$$= R_o \left[1 + \frac{2^{C/g}}{2^{C/g} - 1} (2^{ti/g} - 1)\right]$$

$$= R_o \left[\frac{2^{ti/g} \times 2^{C/g} - 1}{2^{C/g} - 1}\right] \quad ----- (3)$$

On restoration of DNA synthesis at t = 0

$$R_t = R_{ti} + R_a - R_b$$

in which, for a subsequent period = C, termination of the old replication forks will give: $R_b = R_o \frac{(2^{t/g} - 1)}{2^{C/g} - 1}$

while, if mass continues increasing exponentially

$$R_a = R_o 2^{ti/g} \times \frac{2^{C/g}}{2^{C/g} - 1} (2^{t/g} - 1)$$

Substituting the three terms gives:

$$R_t = R_o\left[\frac{2^{ti/g} \times 2^{C/g} - 1}{2^{C/g} - 1}\right] + R_o 2^{ti/g} \times \frac{2^{C/g}}{2^{C/g} - 1}\,(2^{t/g} - 1) - R_o\left(\frac{2^{t/g} - 1}{2^{C/g} - 1}\right)$$

$$R_t = R_o\left[\frac{2^{t/g}\, 2^{ti/g} \times 2^{C/g} - 1}{2^{C/g} - 1}\right] \qquad - - - - (4)$$

Equations (1) and (4) give the number of replication forks with respect to time t, in the control and pretreated cultures respectively (during a period = C in the case of the pretreated culture). The total amount of label S, incorporated into DNA is obtained by integration as in Appendix 1:

From (1) $$S = \frac{kR_o\, g}{\ln 2}\,(2^{t/g} - 1)$$

From (4) $$S = \frac{kR_o\, g}{\ln 2}\left[\frac{2^{ti/g} \times 2^{C/g} - 1}{2^{C/g} - 1}\right](2^{t/g} - 1)$$

$$- - - - (5)$$

Thus both equations predict linear kinetics of incorporation of S when plotted against $2^{t/g} - 1$.

Also from such a plot, the ratio

$$\frac{\text{gradient of pretreated culture}}{\text{gradient of control}} = \frac{2^{ti/g} \times 2^{C/g} - 1}{2^{C/g} - 1} \qquad - - - - (6)$$

Appendix 4: The increment of DNA synthesized in the absence of required amino acids

The assumptions will be: 1. all cycles of replication in progress at the time of amino acids withdrawal are completed and no new ones are initiated, and 2. the rate of DNA synthesis per replication fork is constant, (the time taken to replicate the entire length of a chromosome = C).

Taking G as the number of completed genomes per cell after starvation, and $\bar{G}$ as the mean number of genomes per cell during exponential growth, then the DNA increment = $G - \bar{G}$.

Using the age distribution function of an ideal exponentially-growing culture given by Sueoka & Yoshikawa (1965), Cooper & Helmstetter (1968) showed that in a culture with a mean generation time = g,

$$\bar{G} = \frac{g}{C \ln 2} \left(2^{C+D/g} - 2^{D/g} \right) \quad ---- (1)$$

Where D is the time between completion of a cycle of replication and cell division. The value of D is irrelevant to the DNA increment since the time of cell division has no effect on the number of replication forks per chromosome, which depends only on the ratio C/g. To simplify the calculation, D is given a value such that initiation takes place at the start of the division cycle, causing each cell in the population to have the same number of completed chromosomes after starvation:

ie. let $D = ng - C$ (where n is an integer giving D a positive value),

then $G = 2^n$

Substituting D in equation (1) gives:

$$\bar{G} = \frac{g}{C \ln 2} \left(2^{(C+ng-C)/g} - 2^{(ng-C)/g} \right)$$

$$= \frac{2^n g}{C \ln 2} \left(1 - 2^{-C/g} \right)$$

The DNA increment expressed as a fraction of the DNA at the start of starvation

$$= \frac{G - \bar{G}}{\bar{G}} = \frac{G}{\bar{G}} - 1$$

Substituting:

$$= \frac{2^n}{\frac{2^n g}{C \ln 2} \left(1 - 2^{-C/g} \right)} - 1$$

$$= \frac{C \ln 2}{g(1-2^{-C/g})} - 1 \qquad \text{- - - - (2)}$$

Consider now a process causing the initiation of extra replication cycles before the onset of amino acid starvation. This will increase the value of G, initiation at all the available origins giving:

$$G = 2 \times 2^n$$

therefore fractional DNA increment

$$= \frac{2 C \ln 2}{g(1-2^{-C/g})} - 1 \qquad \text{- - - - (3)}$$

R E F E R E N C E S

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