The effect of 5-bromo-2'-deoxyuridine on growth and sporulation in *Bacillus subtilis*

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Declaration

This thesis is the original work of the author

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To Jacki and Christopher

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SUMMARY

The aims of the work were to elucidate the manner in which the thymidine (TdR) analogue, 5-bromo-2'-deoxyuridine (BUdR) interferes with sporulation in Bacillus subtilis, to use BUdR as a density-label to analyse chromosome replication required for sporulation, and to assess the basis by which BUdR-tolerant strains can grow in medium containing the analogue.

BUdR inhibited the growth of B. subtilis (thy A, thy B) when supplied in place of TdR, which is required for growth of this strain. An isogenic BUdR-tolerant derivative, but-32, grew normally with a BUdR to TdR ratio of between 7.5 : 1 and 15 : 1, but only between 1 and 5% of the cells, compared to control cells using TdR alone, were able to form spores.

Three other BUdR-tolerant, TdR-requiring strains were independently isolated, and the BUdR-tolerance mutations transferred by DNA-mediated transformation back into the thy A, thy B genetic background. Two mutations conferring tolerance to bromouracil (BU), the free base form of BUdR, were also separately transferred into the thy A, thy B background. Initial characterisation of these five strains, plus but-32, suggested that classes of BUdR-tolerance exist, and that the mutation conferring tolerance to BU also confers some resistance to BUdR, in medium containing TdR.

The basis of the tolerance phenotype of strain but-32 seems to lie in the preferential uptake of TdR over BUdR into the cell, when the analogue partially replaces TdR in the medium. The results of incorporation experiments involving radiolabelled TdR and BUdR, CsCl density gradient analysis of BUdR-substituted DNA, and marker-frequency analysis of DNA replication indicated that BUdR had a markedly greater inhibitory effect on the rate of DNA replication in the BUdR-sensitive parent strain than in strain but-32, and that the level of substitution of BUdR for TdR in DNA was greater in the sensitive strain than in but-32.

The use of 6-(p-hydroxyphenylazo)uracil (HPUra), a specific inhibitor of DNA synthesis, indicated that successful sporulation only occured on the completion of chromosome replication. Strain but-32 began to escape the inhibitory effect of HPUra on sporulation between 60 and 75 min after initiation of the process in starvation medium, the escape correlating with termination of chromosome replication, measured by marker-frequency analysis. Escape from BUdR inhibition of sporulation occured prior to, but in parallel with, escape from HPUra inhibition, indicating that BUdR inhibition was limited to the period of DNA replication which occurs at the onset of sporulation. Slowing down the rate of DNA synthesis with a subinhibitory concentration of HPUra delayed the escape of these cells from HPUra and BUdR inhibition of sporulation, and also inhibited sporulation, but to a lesser extent than BUdR. In addition, cells

grown and resuspended in BUdR experienced delayed escape from HPUra inhibition of sporulation, suggesting that BUdR delayed completion of chromosome replication during sporulation. Prolonged DNA replication in sporulation medium with BUdR was confirmed by a) direct measurement of the rate of incorporation of $[^{14}C]$- TdR into acid-insoluble material, b) density gradient analysis of BUdR-substituted DNA of strain but-32 during sporulation, and, c) by marker-frequency analysis of synchronised DNA replication during sporulation of Ts134, a TdR-requiring mutant temperature-sensitive for initiation of DNA replication.

CsCl density gradient analysis of DNA prepared from cells of strain but-32 grown with TdR, but initiated to sporulate with BUdR, showed that by 4h after initiation, 17% of the DNA was in the completely heavy form, 66% in a hybrid density form, and 17% in the completely light form. By 7h after initiation (by which time replication had been completed in cells given TdR alone) replication had not been completed, and 42% of the DNA was in the completely heavy form, 41% in a hybrid density form, and 17% in the completely light form. The presence of completely heavy DNA indicated that initiation of chromosome replication from the origin had occured in starvation medium. The low number of mature spores formed in BUdR medium were collected 20h after initiation, freed from cellular material, and the DNA extracted. The DNA appeared entirely of hybrid density, which suggested

either that incorporation of BUdR during replication into both strands of DNA prevented sporulation, or that continued initiation of DNA replication, promoted by BUdR, was the basis of the inhibition of sporulation. The absence of light density DNA in spores indicated that a full round of DNA replication must be initiated in starvation medium in those cells capable of forming mature spores.

When cells were grown with BUdR until the maximum level of BUdR substitution for TdR, 20%, was obtained, and the cells then initiated to sporulate with TdR, a normal level of sporulation was achieved. 30% of the DNA from these spores was in the hybrid density form, and 70% in the completely light form. The apparent absence of completely heavy form DNA suggested that at least one complete round of DNA replication probably occured during sporulation, under these conditions, for a spore to be made. The relatively low percentage of hybrid density DNA, compared to light density DNA, in these spores could not merely be accounted for by one complete round of DNA replication occuring in a starvation medium in addition to completion of the rounds of replication already in progress at the time of initiation of sporulation. It is proposed that the normal level of sporulation was achieved either via preferential incorporation into the spore of the chromosome with the least amount of DNA of vegetative origin, or as a result of two full rounds of DNA replication under starvation conditions. In both cases the expected composition of spore DNA agreed with that obtained by experiment.

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Abbreviations

BU	5-bromouracil
BUdR	5-bromo-2'-deoxyuridine
CDP	cytidine diphosphate
dCDP	deoxycytidine diphosphate
CH	casein hydrolysate
dCyd	deoxycytidine
DPA	dipicolinic acid (pyridine-2,6-dicarboxylic acid)
EMS	ethyl methanesulphonate
FU	5-fluorouracil
FUdR	5-fluoro-2'-deoxyuridine
GTP	guanosine triphosphate
HH	heavy-heavy
HL	heavy-light
LL	light-light
HPUra	6-(p-hydroxyphenylazo)uracil
IUdR	5-iodo-2'-deoxyuridine
MM	minimal medium
NAL	nalidixic acid
SM	sporulation medium
Spo^+	sporogenous
Spo^-	asporogenous
TCA	trichloroacetic acid
TdR	thymidine
ts	temperature-sensitive
Urd	uridine

INTRODUCTION

I. ENDOSPORE FORMATION

Cellular differentiation in eukaryotes involves a morphological transition from the growing cell to one specialised to carry out a specific function or functions. Examples of cell differentiation range from the morphological transitions between diploid vegetative cells and spores of Saccharomyces cerevisiae and the development of amoeboid cells of the slime mould Dictyostelium discoideum to stalk and spore cells, to the relatively complex differentiation of fertilised eggs into complex life forms in higher eukaryotes. However, an analysis of the temporal and spatial control mechanisms involved in the differentiation process in complex systems is difficult, and some workers, using the "reductionist" theory, have argued that information gained from the study of one simple differentiation system can serve as a model for comparison with more complex systems. With regard to this approach, bacterial sporulation has been proposed as a model for development in higher organisms (Freese, 1972).

A. The sporulation process

Bacterial sporulation is initiated at the end of the logarithmic phase of growth, when certain nutrients in the growth medium are depleted. The vegetative cells of bacterial genera, notably aerobic Bacillus and anaerobic Clostridium, are able to form endospores through an ordered sequence of morphological

and biochemical steps. The endospore, formed within the mother cell, is dormant and possesses a morphology and biochemistry that distinguishes it clearly from the vegetative cell (Murrell, 1967). Unlike vegetative cells, mature spores are resistant to high temperatures and pressure, dessication and radiation. These properties, together with the pathogenic activity of some sporulating species, have been of interest to both the medical field and the food industry.

Two experimental procedures can be used for initiation of sporulation in a species of the Bacillus genus, Bacillus subtilis, most commonly used for studies on sporulation. Both have the advantage that the vast majority of cells in a population can be induced to sporulate fairly synchronously and in a relatively short time, although there are apparently no cell-to-cell interactions. In liquid media, sporulation is usually triggered by starvation for a carbon or nitrogen source, and sometimes by phosphate starvation. Using the exhaustion method, the culture is allowed to grow in a rich broth medium until some nutrient becomes limiting, and the time at which growth ceases to be exponential in nature is taken as the time of induction, t_{zero}. Hourly periods after this are denoted by t_1, t_2, t_3, etc. (Schaeffer et al, 1963). With B. subtilis at 37°C, mature spores appear some 7 to 8h after the point of induction.

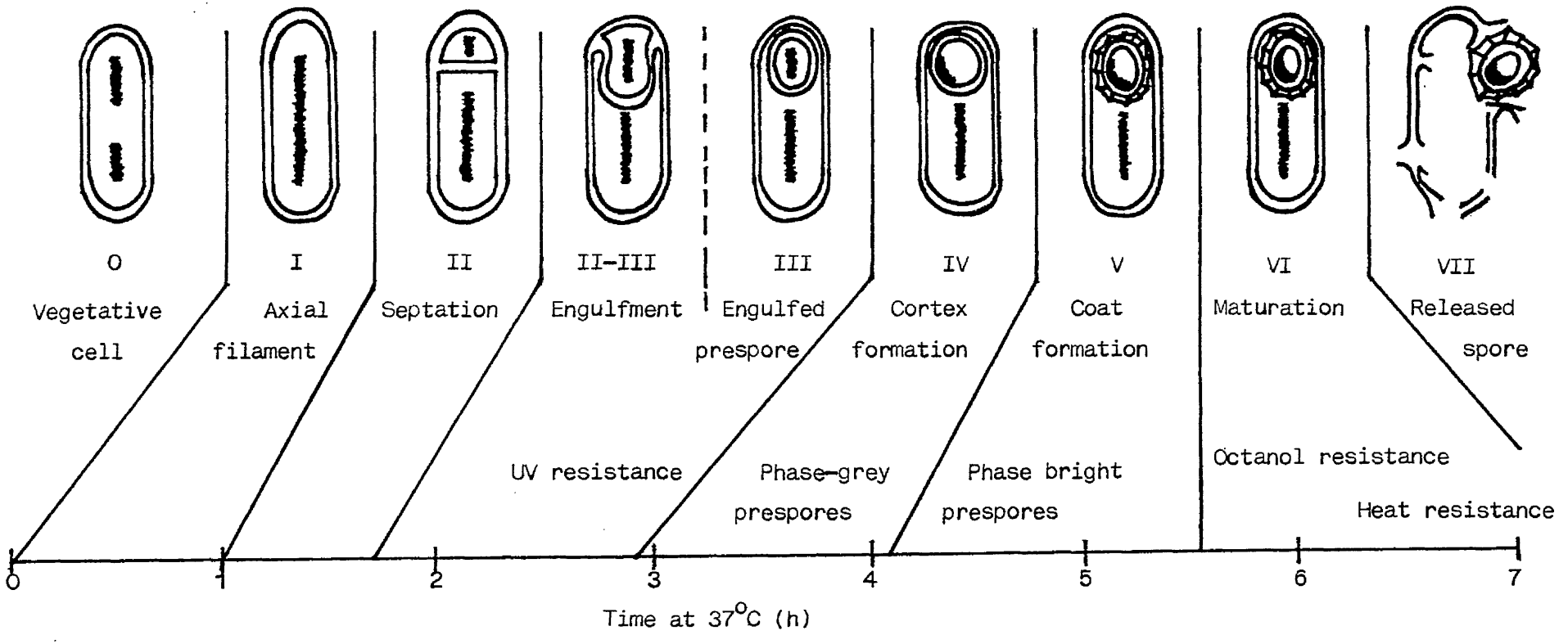
The exhaustion method has the advantage of simplicity, but usually t_{zero} is ill-defined, and vegetative growth may continue slowly for a time. In addition, the efficiency of sporulation and the degree of synchrony obtained depend on the medium used. Also, when the cells do sporulate they are in a complex chemical environment which is continuously changing, even when the initial growth is in a defined medium, due to accumulation of metabolic products. This complicates the measurement and interpretation of biochemical changes accompanying sporulation. Therefore, to avoid some of these difficulties a nutrient replacement or resuspension technique was introduced by Sterlini and Mandelstam (1969), whereby bacteria that are growing exponentially in a rich medium are harvested by centrifugation, and transferred to an equal volume of resuspension medium containing a suitably poor carbon source, such as glutamate, and an adequate supply of ammonia. Since carryover of the growth medium is small, even in unwashed cells, this method ensures the absence of metabolites whose identities and concentrations are unknown. This shift-down procedure gives a more clearly-defined starting point and an improved synchrony of sporulation than the exhaustion procedure. In addition, it is more suitable for experiments involving radioactive labelling as it is a chemically - defined medium. On the other hand, the exhaustion procedure is more convenient for large - scale experiments, and it is more comparable to the process as it occurs on solid media. For most purposes

the general order of sporulation events is the same using the two procedures.

The sequence of morphological events resulting in the formation of a spore has been mainly investigated by electron microscopy, and is similar for all species of Bacillus and Clostridium that have been examined (Fitz-James and Young, 1969). The process is continuous, but it has been convenient to divide it into stages. Fig. 1 is a diagrammatic representation of the sequence of events with the stages represented by Roman numerals. The durations of the stages refer specifically to B. subtilis at 37°C, and are approximate. There is a reasonably close, but not exact, correlation between the stage number and the hour after t_{zero}. Stage 0 refers to the vegetative cell.

In B. subtilis the vegetative cell (approximate dimensions 2.5 μm X 0.6 μm) contains two or more replicating copies of the chromosome. During growth the cell increases in length and septa are produced in a central position (Sargent, 1978). After transfer of the cells to resuspension medium the nuclei condense to form a single axial filament. This event is conventionally described as stage I. However, as no mutants have been isolated which are specifically unable to form axial filaments (Mandelstam et al, 1975), and similar filaments have been observed in electronmicrographs of Escherichia coli and other non-sporulating

Figure 1. Schematic diagram of morphological stages of sporulation in Bacillus species, as seen by electron microscopy. The biochemical events are common to most aerobic spore-forming bacteria. Some are essential for sporulation, some inessential, but appear to be integrally associated, and some are vegetative responses of the cells following the nutritional step-down that initiates sporulation (see text). The times at which the events occur are slightly variable and depend on the type of organism and on the experimental conditions. Taken from Young and Mandelstam (1979).


0
Vegetative cell
I
Axial filament
II
Septation
II–III
Engulfment
III
Engulfed prespore
IV
Cortex formation
V
Coat formation
VI
Maturation
VII
Released spore
UV resistance
Phase–grey prespores
Phase bright prespores
Octanol resistance
Heat resistance
0
1
2
3
4
5
6
7
Time at 37°C (h)
Serine protease
Coat protein synthesis
Coat protein assembly
Metalloprotease
Ribonuclease
Antibiotic
Cytochrome synthesis
Diaminopimelate ligase
Alkaline phosphatase
Cortex synthesis
DNAase
Glucose dehydrogenase
Heat-resistant catalase
Ribosidase
Dipicolinic acid
Ca2+ uptake
Alanine racemase
NADH oxidase
Sulpholactic acid
Alanine dehydrogenase
Adenosine deaminase
∝Amylase
Aconitase
Dipicolinate synthase
Arginase
Spore storage protein

bacteria, formation of this structure may be a non-specific response of DNA to transfer of cells to a poor medium. However, the usage has been retained by most investigators.

The chromosomes subsequently separate, one of them migrating to a position near one pole of the cell (Mandelstam et al, 1975). Stage II is characterised by formation of a membranous septum in a sub-polar position. This occurs by membrane invagination and growth in a manner resembling that which normally occurs during cell division, although it differs in that its position is sub-polar rather than central . Cell wall material is present as protrusions, or spikes, at the site of membrane invagination (Yamamoto and Balassa, 1969). However, there is little cell wall material visible in electron micrographs of the spore septum itself, although inhibitors of cell wall synthesis, such as penicillin, prevent formation of the spore septum (Hitchins and Slepecky, 1969b). Therefore, some peptidoglycan synthesis may be necessary at this time to give direction to membrane synthesis as the septum forms (Freese, 1972). However, the peptidoglycan is subsequently digested away (Guinand et al, 1974) and the process continues to stage III, which is the formation of a protoplast free within the mother cell.

At the end of stage II there are two cells of unequal size, and the larger one proceeds to engulf the smaller one by

movement of the points of attachment of the ends of the septum to the pole of the mother cell. This engulfment culminates in membrane fusion at the pole, and the whole structure becomes detached from the mother cell membrane to give a spore protoplast, or forespore. This process results in the two membranes of the spore protoplast being oriented in opposition i.e. with adjacent outer membrane surfaces.

Stage IV involves deposition of the primordial germ cell wall and formation of the cortex between the inner and outer forespore membranes. The primordial germ cell wall is the layer of vegetative cell-type peptidoglycan laid down closest to the forespore inner membrane, and there is evidence that this becomes the cell wall of the germinating spore (Fitz-James and Young, 1969). The spore-specific peptidoglyan, or cortex, is deposited as a layer external to the primordial germ cell wall. Most vegetative peptidoglycans are based upon glycan chains of alternating residues of N-acetylglucosamine and N-acetylmuramic acid, with each muramic acid residue substituted with a peptide. However, in B. subtilis cortex, about half of the muramic acid residues are present as the spore-specific muramic acid lactam. In addition, the cross-linking of spore peptidoglycan is considerably less than that of the vegetative polymer (Ellar, 1978). Although the exact function of the cortex has not been definitely demonstrated, there is evidence to suggest that it may act as an osmoregulatory organelle functioning

either to dehydrate the spore core (Gould and Dring,1975) or to maintain the dehydrated state (Ellar, 1978).

Also during stage IV the forespore begins to accumulate calcium and dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid). This latter compound is unique to sporulation and its synthesis continues in parallel with Ca^{2+} accumulation. In spores of most species DPA and Ca^{2+} are present in approximately 1:1 molar ratios (Murrell, 1969). It has been proposed that active transport of Ca^{2+} by the mother cell results in elevated levels of mother cell Ca^{2+}, which can enter the forespore by facilitated diffusion, where it complexes with DPA, which is confined to the forespore compartment (Ellar and Posgate, 1974). In this way the effective free Ca^{2+} concentration in the forespore is lowered, allowing further uptake by diffusion (Ellar, 1978). The Ca^{2+}/DPA complex is probably involved in the stabilisation of spore enzymes and nucleic acids in the protoplast (Murrell, 1981). Mutants have been described that lack DPA, but which do form heat-resistant spores (Zytkovicz and Halvorson, 1972). However, these do not retain their dormancy and are defective in germination, so DPA must be required for the latter purpose in addition to being a sporulation requirement.

During stage IV the spore can be viewed by phase microscopy as a grey body at one end of the cell. Deposition of layers of protein, the spore coats, around the cortex defines stage V,

and at this stage the spore appears phase-bright. Stage VI is the "maturation" of the spore, when it shrinks, and becomes resistant to high temperature, various organic solvents, and other adverse conditions. It is a widely held view that the extreme resistance and dormancy of bacterial endospores results mainly from a low water content in the central core (Gould, 1975).

The final "stage", stage VII, involves lysis of the mother cell, but is not strictly part of the sporulation process, as lysis of the cell will occur anyway under conditions of nutrient deprivation.

The biochemical events that occur during sporulation can be divided into several functional classes, depending on their degree of relevance to sporulation, and some are listed in Fig. 1. Firstly, there are those events which are essential and sporulation-specific, such as synthesis of serine protease in B. subtilis (Leighton et al, 1972; Dancer and Mandelstam, 1975a). In fact, the production of proteolytic activity is one of the first events to occur after the induction of sporulation, and many species produce several distinct enzymes. It is a general assumption that at least one of these enzymes participates in extensive protein turnover that occurs during sporulation in many species. Other examples in this category include the appearance of DPA at stage V, the enzyme diaminopimelate ligase, which is involved in cortex biosynthesis in Bacillus sphaericus (Tipper and Pratt, 1970), and the spore coat proteins.

Second, there are events that occur during sporulation which are not required for normal development, e.g. production of metalloprotease in B. subtilis (Hageman and Carlton, 1973). Although events in this category are not essential for spore formation their times of appearance are precisely controlled and serve as useful marker events in experiments. The third category includes those events required for sporulation but which may also be expressed during vegetative growth, such as synthesis of the citric acid cycle enzymes, which are required for energy production during sporulation (Freese, 1972). Finally, the fourth category includes certain events which are apparently a result of the nutritional "step-down" conditions achieved by the replacement method of initiating sporulation, but which are otherwise unrelated to the process, e.g. arginine synthesis (Schaeffer, 1969). It is not clear into which category many known events should be placed, since mutants with specific defects in these functions have not been reported. However, many events have been placed into one of the above categories by the study of sporulation - defective mutants.

Mutations in unnecessary events should not affect sporulation, whereas mutations in necessary effects should, and if the event is sporulation - specific, should not affect vegetative growth. In addition, biochemical events involved in sporulation should not be expressed in sporulation - defective mutants. In

practice, such mutants are readily isolated since, on a variety of solid media, colonies of the sporogenous (Spo^+) type produce a brown pigment, whereas colonies of asporogenous (Spo^-) mutants are poorly pigmented (Iichinska, 1960). However, sporulation - defective mutants can be further categorised into asporogenous mutants, which produce no spores at all, and oligosporogenous mutants, which produce spores at a reduced frequency, most of the cells being blocked at a defined stage. The frequency of sporulation in oligosporogenous mutants varies from about 50% to $1:10^8$, but for a particular mutant the frequency is constant and reproducible under standard conditions. Also, when a spore produced by an oligosporogenous mutant is used to establish a new population of cells, sporulation will occur at the same frequency as it did in the original population.

In a study of oligosporogenous mutants, Coote (1972b) obtained evidence that mutations causing oligosporogeny may lie very close to those causing asporogeny, suggesting that the two phenotypes may be the expression of different mutations in the same gene (Piggot and Coote, 1976). It is probable, therefore, that oligosporogenous mutants are "leaky" mutants in which a defective protein is produced in normal amounts.

Many biochemical activities associated with sporulation increase in activity at particular times during the process.

(Warren, 1968; Waites et al, 1970). For example, an increase in exoprotease activity is associated with the earliest stage (0-I), an increase in alkaline phosphatase activity with stage II, an increase in glucose dehydrogenase activity with stage III and the synthesis of dipicolinic acid with stage IV-V. (Waites et al, 1970; Coote, 1972a). The morphological stage during sporulation at which many Spo$^-$ mutants became blocked was determined by electron microscopy and the activity of various biochemical markers was also assayed in these strains (Waites et al, 1970). For any one mutant examined, the structural changes and biochemical activities would proceed as normal up to the block, but when the differentiation process stopped as a result of the mutation, the increase in biochemical activities normally associated with later events did not occur, indicating that the process could be regarded as a dependent sequence of events, where later steps depended on the successful completion of earlier ones.

In addition, when a mutation which affected an early event in sporulation was introduced into a strain containing a mutation which affected a later stage, it was the early mutation which determined the phenotype of the double mutant (Coote and Mandelstam,1973). However, when mutations affecting stages after stage III were used to construct these double mutants, the phenotypes of the double mutants in some cases were different from either of the parent strains. To explain this, it was suggested that two or more pathways might run in parallel, especially

during the later stages (Coote and Mandelstam, 1973).

The study of conditional temperature - sensitive (ts) mutants has provided additional information on the time of expression of spo genes, but the most significant observation appears to have been that spo loci vary enormously in the time during which they are expressed. For example, a ts spo mutation which affected serine protease activity was in a locus expressed for at least the first 3h of the process. (Leighton et al, 1972), whereas another ts spo mutation, which affected a stage II marker event, was in a locus whose expression was only required for 15 minutes (Young, 1976). In addition, the complexity of the developmental process has been accentuated by the observation that spore coat proteins covering the forespore at stage V are synthesised as early as stage II (Aronson and Fitz-James, 1976).

B. Initiation of sporulation

As the inverted membrane configuration around the developing forespore no longer allows normal active transport of metabolites (Ellar, 1978), the metabolism of the forespore must differ from that of the mother cell, which might allow synthesis of spore-specific proteins to be derepressed. Whereas the asymmetric septation usually depends on a nutritional signal, the subsequent forespore development depends only on the genetic constitution of the organism.

The nature of the signal which the cell recognizes when the concentration of one of the several nutrients required for growth decreases has been intensively studied. When Schaeffer et al (1965) continuously diluted cultures of B. subtilis into the same medium, they found that cells could sporulate during exponential growth with a probability that increased greatly when the growth rate was low because the nitrogen or carbon source could only be slowly metabolized. They concluded that sporulation was repressed by a carbon - and nitrogen - containing metabolite which might control the synthesis of an enzyme required for sporulation. Similar results were obtained in chemostat cultures by Dawes and Mandelstam (1970). Therefore, because sporulation of B. subtilis remains suppressed in the presence of ammonium ions, phosphate, and carbohydrates such as glucose, and the synthesis of many catabolic enzymes similarly remains suppressed under these conditions, a phenomenon known as catabolite repression, it was suggested that a simple mechanism controls both sporulation and catabolite repression (Schaeffer et al, 1965).

The analogy with catabolite repression inspired a search for the catabolite effector, which still continues. One of the favoured compounds, cyclic AMP, has however, not been found in significant quantities in Bacillus (Setlow, 1973). Elmerich and Aubert (1973) proposed that the trigger molecule for induction of sporulation in Bacillus megaterium is glutamine, or alternatively, an intermediate

in purine biosynthesis whose nitrogen atoms are derived from the amide group of glutamine, but more recent experiments indicated that the enzyme glutamine synthetase itself may be involved (Reysset and Aubert, 1975). However, in B. subtilis most of the mutants that are defective in the glutamine synthetase structural gene will sporulate normally in the presence of added glutamine (Fisher and Sonenshein, 1977).

Other candidates proposed as effector molecules are the highly-phosphorylated nucleotides (HPNI to HPNIV) appearing at the on set of sporulation. They are mainly phosphorylated adenosine (p_3Ap_3) derivatives, apparently associated with the onset of sporulation under carbon or phosphate depletion. They are not formed by a Spo^- mutant blocked at stage 0 (Rhaese et al, 1977), but their exact relationship to initiation remains to be established.

More recently, it has been shown that sporulation can be initiated in the presence of excess glucose, ammonia, phosphate and other nutrients by conditions causing a partial deprivation in the synthesis of purine, and in particular, guanine nucleotides (Freese et al, 1979). Also, in the presence of excess glucose, a decrease of guanine nucleotides in B. subtilis initiated sporulation, but did not prevent catabolite repression of three inducible enzymes (Lopez et al, 1980). It was concluded therefore, that the ultimate mechanism repressing enzyme synthesis differs from that

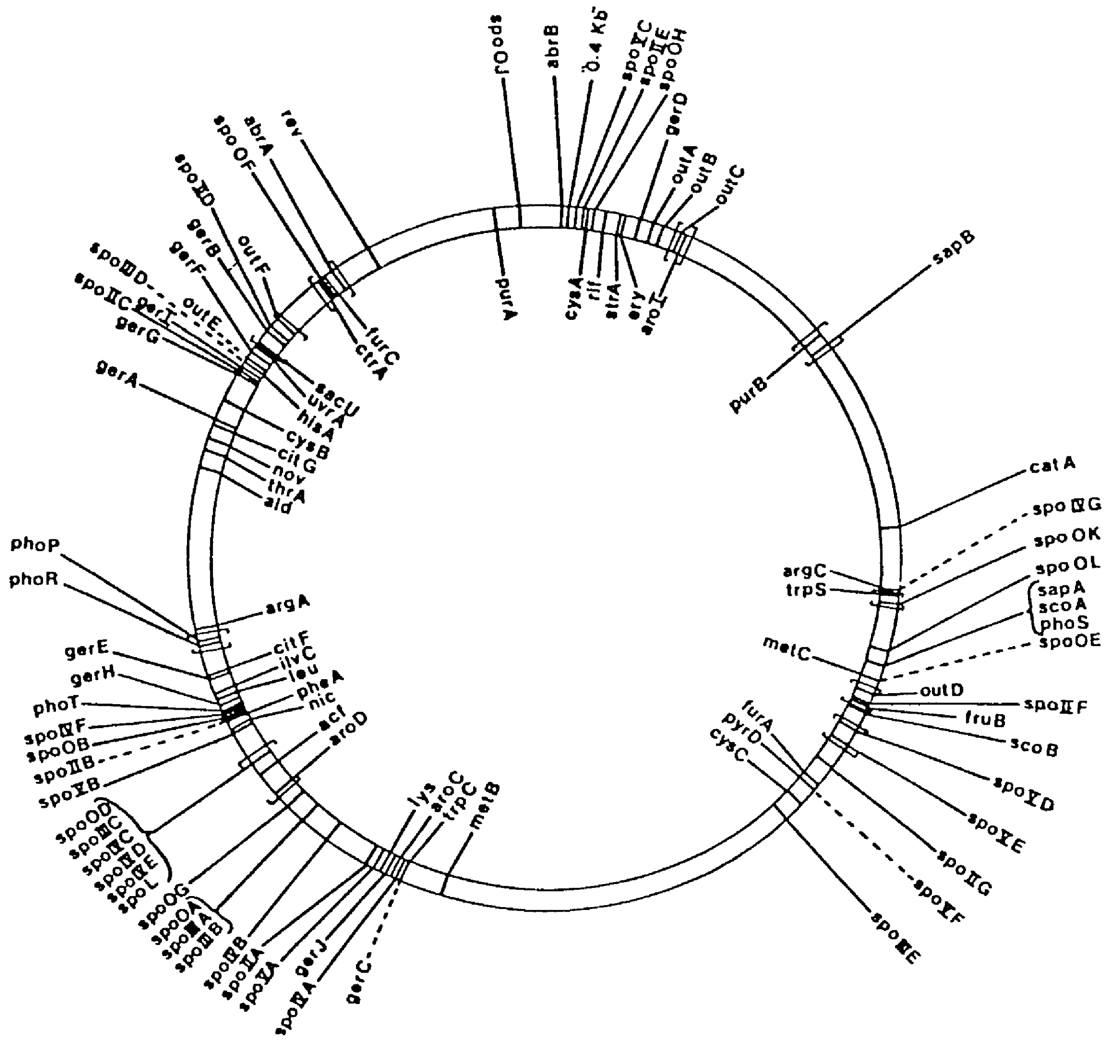
suppressing sporulation.

In addition, Freese (1981) reported that all nutritional conditions examined so far which cause the initiation of sporulation also resulted in a decrease of guanosine triphosphate (GTP), whereas the other nucleotides increased in some and decreased in other cases, and he suggested that GTP may suppress sporulation directly, in combination with a protein, or indirectly by enabling the synthesis of another metabolite.

Therefore, there is still some uncertainty about which molecule(s) bring about the induction of sporulation, but this is hardly surprising, due to the complex nature of the intermediate metabolism of the bacterial cell. Until more is discovered about the control of sporulation and the functioning of the first operon concerned in the process, it will be difficult to identify the effector molecule. However, whatever the true initiating event is, there is presumably some interaction between it and the genetic loci involved in the initiation of the process, or the gene products of these loci. There are at least 10 of these loci, mutations in which cause cells to become blocked at the earliest stage of sporulation. They are stage 0 or spo 0 genes, and are located at widely scattered sites on the B. subtilis chromosome (see Fig. 2). A mutation in any one of these genes blocks sporulation prior to completion of any of the morphological stages

Figure 2. Genetic map of B. subtilis 168, showing differentiation-associated loci. The distribution of genetic markers is based on that of Piggot et al (1981). Groups of loci whose orientation relative to each other has not been determined are shown as mapping at a single site. Where no orientation is known and linkage is uncertain, this is indicated by a dotted line. Map positions placed in parentheses have not been ordered relative to outside markers and may not have been ordered relative to each other.

Fig. 2



spoOJ
abrB
purA
cysA
strA
ery
outA
outB
outC
gerD
sapB
purB
catA
argC
trpS
spoOK
spoOL
sapA
scoA
phoS
spoOE
metC
outD
fruB
scoB
furA
pyrD
cysC
metB
lys
aroC
trpC
gerJ
gerC
spoOG
acf
aroD
phoP
phoR
argA
gerE
gerH
phoT
citF
ilvC
leu
pheA
nic
spoOB
gerA
cysB
citG
nov
thrA
ald
sacU
uvrA
hisA
gerG
outE
gerB
gerF
outF
spoOF
abrA
rev
furC
ctrA

characteristic of early spore development, and they are therefore thought to be regulatory cistrons whose products are directly involved in the initiation stage. Little is known about the nature and function of their gene products, although at least some, if not all of them are thought to be expressed in vegetative cells (Losick, 1981). Until recently no spo0 gene products had been definitely identified, but Rhaese et al (1981) have presented evidence that the spo0F gene codes for the p_3Ap_3 synthetase enzyme responsible for the production of the highly-phosphorylated adenosine nucleotides observed at the onset of sporulation. Other spo0 genes have also been isolated by gene cloning methods (Dubnau et al, 1981; Kobayashi et al, 1981), and it should not be long before the roles of these loci will be elucidated.

C. The genetic analysis of sporulation

Although our knowledge of the regulation of sporulation has been enhanced by examination of the morphological and biochemical properties of asporogenous mutants, it has been supplemented by information gained from genetic studies using mainly techniques for genetic exchange in B. subtilis, such as transduction and transformation. Mapping of spo mutations using these techniques can establish the position of any spo mutation on the chromosome. The generalised transducing phage PBS1 transfers about 5% of the donor chromosome (Lepesant - Kejzlarová et al, 1975) and is used for

rough mapping data, by determining cotransduction frequencies of spo mutations with auxotrophic or other markers of known location. Fine structure mapping can be achieved by DNA-mediated transformation, which spans approximately 20 to 30 genes (Bodmer and Ganesan, 1964).

The system of nomenclature in general use identifies the spo loci by the morphological stages which they affect (Piggot and Coote, 1976). Thus stage 0 mutants represent the appearance in electron microscopy of vegetative cells. The loci containing mutations producing this phenotype specifically, with no apparent effect on any vegetative functions, are denoted spo 0A, spo 0B, etc. To date, some 35 spo loci have been described (Piggot et al, 1981), and the position of these, relative to non-sporulation loci, are shown in Fig. 2.

The primary biochemical lesion is not definitely known for any of the spo loci, but is presumed to be in a sporulation-specific function, since the mutants are not affected in vegetative growth. Mutations were placed in separate loci if they were a) separated by a non-sporulation genetic marker, b) unlinked by transformation, and c) blocked at different stages in the sporulation process (Piggot, 1973). The list of spo loci is probably incomplete, as some mutations have not yet been mapped, but the genetic map of spo loci (Fig. 2) shows that they are not clustered into one area of the chromosome. Loci affecting totally different stages in the process can often be found grouped together. This

observation is important when a model for the overall control of the process is considered. There is much evidence favouring sequentially dependent induction of gene transcription during spore formation (Halvorson, 1965; Mandelstam, 1976; Piggot, 1979), and Piggot *et al* (1981) have presented a simple model focussing on a short sequence of operon inductions (see later).

There is recent evidence suggesting that different genes are expressed in the prespore and mother cell of sporulating organisms, an example of *compartmentation*. de Lencastre and Piggot (1979) described a method for identifying which *spo* loci are expressed in each cell type. Asporogenous mutants were transformed with *spo*$^+$ DNA during sporulation, allowing spore formation with a minimum of DNA replication between transformation and spore formation. After allowing adequate time for sporulation of any organisms that had "rescued" the relevant *spo*$^+$ gene from the transforming DNA, the cultures were heat-treated to kill vegetative cells, and the cells plated on minimal medium to allow germination. It was found that strains bearing mutations at certain loci gave rise to Spo$^+$ colonies whereas others gave rise to Spo$^-$ colonies. Under the conditions used, it was likely that only one of the two copies of the chromosome in the sporangium was transformed to *spo*$^+$. The assumption underlying the experiments was that the sporulating organism, a two-celled structure, had one wild-type and one mutant allele for a sporulation gene. If the *spo*$^+$ chromosome segregated into the mother cell, and expression there was sufficient for spore

formation, then the mutant chromosome would segregate into the forespore, and the resulting heat-resistant spores would be genetically asporogenous. Alternatively, if the spo$^+$ chromosome segregated into the forespore and expression there was sufficient, the resulting spore would be genetically spo$^+$.

Using this method, it was found that one locus, spo VA, had to be expressed in the forespore, whereas for 8 other loci expression in the mother cell was sufficient for spore formation, suggesting that the forespore chromosome is relatively inactive, possibly due to a change in the micro-environment of the DNA in the forespore as it becomes dormant (Baillie et al, 1974).

Dancer and Mandelstam (1981), using a similar approach, induced late-blocked Spo$^-$ mutants to form spores by fusing protoplasts of mutants and Spo$^+$ strains at stage III of sporulation. Many of the spores formed retained the spo genotype of the mutant, and fusions between spo IVC mutants revealed at least two complementing groups, or cistrons, in this locus. It can be seen therefore, that there are probably many more genes than loci controlling sporulation.

It is also signigicant that many of the substances produced during sporulation are found exclusively in one or other of the two cells of the developing sporangium, although it is not possible to infer the site of synthesis of a compound from its distribution.

For example, dipicolinic acid is found only in the prespore (Ellar and Posgate, 1974), whereas the enzyme concerned with its synthesis, dipicolinate synthetase, is found only in the mother cell (Andreoli et al, 1975).

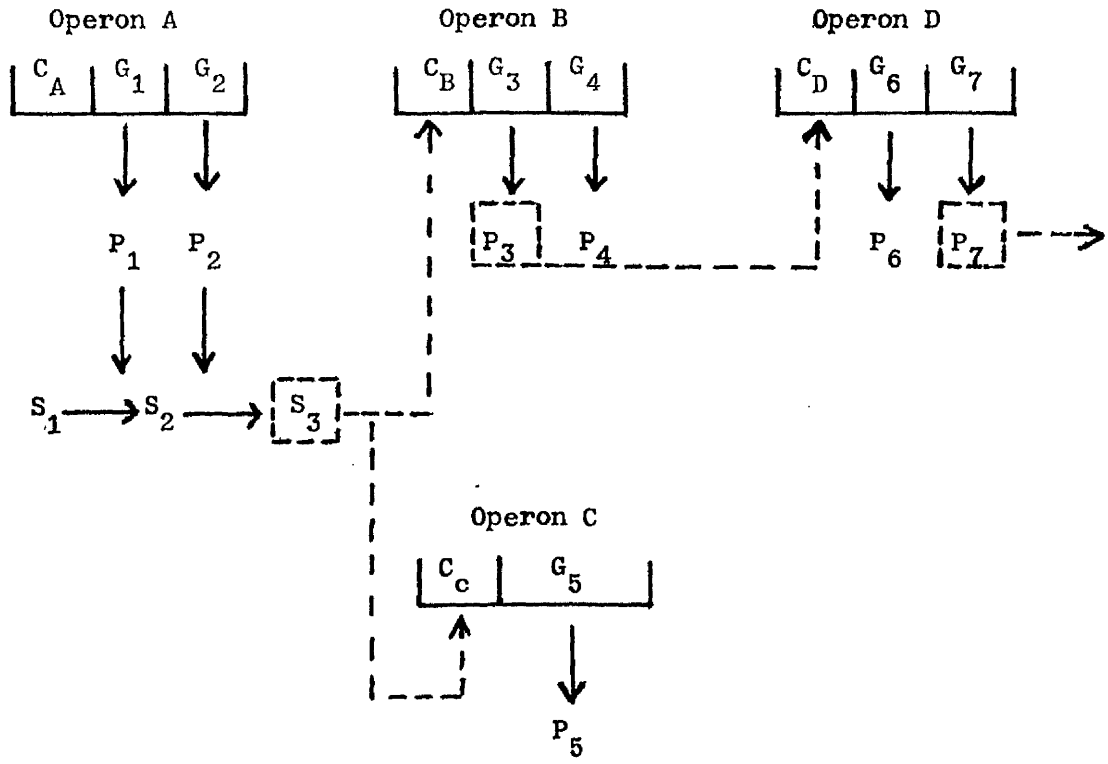
D. Regulation of sporulation

Piggot et al (1981) recently presented a simplistic model of regulation of sporulation genes, focussing on a short sequence of operon inductions (Fig. 3), but admitted that the actual system is likely to be more complex. It was presumed that there is an initiating locus whose transcription is blocked during growth by repressing compounds, and starvation leads to a lowering of their concentrations, resulting in initiation of the process by expression of the first locus.

In this model, the "switching on" of successive operons via elements C_A, C_B, C_C, etc. could be mediated by low molecular weight products of enzyme action (as S_3 on C_B or C_C) or by protein (P_3 on C_D). It can be seen that point mutations that inactivate different gene products might have different consequences. For example, in operon A, mutation in G1 or G2 would prevent expression of operon B and subsequent operons. In operon B, mutation in G_3 would prevent expression of subsequent operons. Since operons A and B together represent only a small part of the overall sporulation sequence, the phenotype resulting from a G_3 mutation might be

Figure 3. Schematic representation of a sequence of operon induction during sporulation (Piggot et al, 1981). There are four operons, A, B, C and D. Each contains structural genes G_1, G_2, G_3, etc., coding for proteins P_1, P_2, P_3, etc. Where the proteins are enzymes, they act on a substrate S: P_1 on S_1, P_2 on S_2. Each operon also contains a control element (C_A in operon A, C_B in operon B, etc.) controlling its transcription. Compound S_3 acts on C_B and C_C to switch on operons B and C, respectively. Protein P_3 acts on C_D to switch on operon D.

Fig. 3


Operon A
Operon B
Operon D
C_A G_1 G_2
C_B G_3 G_4
C_D G_6 G_7
P_1 P_2
P_3 P_4
P_6 P_7
S_1 S_2 S_3
Operon C
C_c G_5
P_5

difficult to distinguish from that resulting from a mutation in G_1 or G_2. Thus, mutations producing very similar or identical phenotypes may map separately. Alternatively, mutation in G_4 of operon B could not prevent the expression of subsequent operons. Mutation in G_3 would be a typical pleiotropic spo mutation, but if P_4 had no regulatory role and functioned in the final assembly of a mature spore, then mutation in G_4 would give an incomplete spore, and could be represented by a late spo mutation.

A consequence of this type of model is that each separately located spo locus must have its own control sections of DNA, hence the use of the term "operon" rather than "locus". This model also assumes that sequential transcription of the relevant loci is the primary control mechanism for the changes that occur after initiation of sporulation, and there is strong evidence, from DNA-RNA hybridisation experiments, that sporulating bacteria produce new species of messenger RNA (mRNA) (for example, Sumida - Yasumoto and Doi, 1974; Pero et al, 1975).

However, during sporulation, vegetative mRNA continues to be synthesised, and many vegetative functions continue to be expressed (Kornberg et al, 1968). The mRNA studies indicated that sporulation is a much more complex process than growth, since the sequential expression of sporulation genes is superimposed on the expression of vegetative genes. The pattern of mRNA present in sporulating cells also indicates that two types of transcriptional apparatus may exist

simultaneously, one type presumably transcribing vegetative genes and the other capable of transcribing sporulation - specific genes. The basis for this difference has been the subject of intensive research over the last few years, and nearly all the work on the mechanism of transcriptional control has concentrated on the possible role of RNA polymerase. The isolation of rifampicin-resistant mutants which are also affected in the capacity to sporulate, but blocked at different stages during the process, has established that this enzyme is necessary for sporulation. In addition, their existence has been used to support the theory that RNA polymerase undergoes sequential changes during sporulation that could modulate transcriptional specificity during development of a spore (Doi, 1977).

Therefore, much effort has been put into the isolation and characterisation of RNA polymerase and its associated factors, in an attempt to monitor changes in enzyme specificity during spore formation. It has been shown that core RNA polymerase from sporulating cells is identical to that extracted from vegetative cells (Linn et al, 1973), and more recently, that there is interference with the binding of vegetative sigma (σ) factor to RNA polymerase during sporulation, although the σ factor itself does not disappear (Tijan and Losick, 1974). It is generally accepted that promoter recognition by bacterial RNA polymerases is mediated by this subunit. The principal form of the complete RNA polymerase in B. subtilis during growth contains a sigma subunit of 55 kilodaltons, known as σ^{55}. Studies on the transcription in vitro of cloned sporulation

genes have indicated that sporulation can be correlated with the appearance of sigma-like regulatory proteins that bind to and modify the promoter recognition specificity of core RNA polymerase. σ^{37} is present in growing cells, but has only a minor role in transcription events, whereas it directs the transcription of at least two cloned sporulation genes, known as "0.4 kb" and spoVC (Haldnenwang and Losick, 1980). Neither of these genes is recognized by the principal form of the B. subtilis holoenzyme containing σ^{55}. The 0.4 kb gene is known to be under sporulation control as its transcription is severely restricted in mutants blocked at the earliest stage in development (Segall and Losick, 1977; Ollington et al, 1981). Rosenbluh et al (1981) recently constructed in vitro a deletion mutation within the 0.4 kb sequence and inserted this mutation into the B. subtilis chromosome by transformation with cloned DNA. Cells harbouring this mutation were oligosporogenous, and electron microscopy showed that this mutation, designated spoVG, impaired sporulation at about the fifth stage. This therefore identified the 0.4 kb gene as a new sporulation locus and also showed that its product functions at a late stage. A mutation in the spoVC gene also causes a block at a late stage (IV to V) in sporulation (Piggot and Coote, 1976).

It has been suggested, therefore, that because it is present in vegetative cells, before the onset of development, as well as in early sporulating cells, and as it directs transcription of the 0.4 kb and spoVC genes, σ^{37} could be involved in the initiation of sporulation (Haldenwang and Losick, 1979).

At a later stage, σ^{55} or σ^{37} are replaced on the polymerase by a third transcriptional modifier known as σ^{29} (Haldenwang et al, 1981). The induction of σ^{29} is itself sporulation-specific, as it was absent in holoenzyme from an early blocked sporulation mutant, spo 0A (Halenwang et al, 1981). σ^{29} then directs the continued transcription of the 0.4 kb and spoVC genes, as well as at least one further gene not recognized by either σ^{55} or σ^{37}. Therefore, σ^{29} could be a regulatory protein that controls developmental gene expression at an early to intermediate stage of sporulation. Incidentally, σ^{37} and σ^{29} are unlikely to be the only sigma-like regulatory proteins controlling sporulation RNA synthesis, and other sporulation - associated components of RNA polymerase have still to be characterised. There is now evidence that each sigma factor dictates the recognition of specific nucleotide sequences on the promoter regions of particular sporulation genes (Lee et al, 1980; Moran et al, 1981).

However, RNA polymerase modification, although important, may not be the exclusive controlling mechanism, and other, more general types of transcriptional control, such as altered DNA conformation (Baillie et al, 1974), or modification and restriction of DNA (Holliday and Pugh, 1975) may operate. In addition, a finer control over the process may involve interactions at the translational and post-translational levels. If control mechanisms exist at these levels they could function by i) regulation of

translation of particular mRNA molecules, ii) directed changes in the composition of ribosomes of sporulating cells, or iii) directed alterations in transfer RNA (tRNA) or tRNA synthetases.

With regard to the first possible control mechanism, it was originally proposed that mRNA concerned with sporulation might have a longer half-life than that of the vegetative cell. Once sporulation was established in Bacillus cereus, the rest of the process was resistant to actinomycin D, an inhibitor of transcription (Aronson and del Valle, 1964). In addition, Sterlini and Mandelstam (1969) showed that sporulation - associated biochemical events continued for periods of up to an hour after the addition of actinomycin D. However, these findings were contradicted by the results of other workers, including Szulmajster et al (1963), using higher concentrations of the antibiotic. It is therefore difficult to make a definite conclusion about the stability of mRNA during sporulation from the results of experiments using this antibiotic since, when a low concentration was used inhibition of RNA synthesis may have been incomplete, leaving enough residual synthesis of mRNA to allow sporulation to continue. Conversely, a high concentration of the drug, which completely blocks transcription, may also damage the cells so badly that sporulation is impossible. However, the existence of some individual mRNA molecules with intermediate or long half-lives, such as the 0.4 kb transcript of the 0.4 kb gene (Segall and Losick, 1977), or the dipicolinate synthetase

mRNA in B. subtilis (Chasin and Szulmajster, 1969), tends to favour the idea that the production of more stable mRNA species during sporulation may be a controlling influence during sporulation.

In addition, several investigators, including Fortnagel et al (1975), have reported that the protein composition of the ribosomes of sporulating cells and spores differs from that of vegetative cells. It is also apparent that ribosomes from sporulating cells have an altered specificity compared with those from vegetative cells when tested in in vitro translation systems (Chambliss and LeGault-Demare, 1977). The existence of pleiotropic mutants, both resistant to antibiotics that inhibit protein synthesis and exhibiting modified sporulation phenotypes, suggests a possible involvement of translational control in the regulation of gene expression during sporulation.

Finally, there have been reports of altered tRNA species in sporulating cells such as the modification of tyrosine tRNA (Menichi and Heyman, 1976), and of in vivo modification to a tRNA synthetase enzyme (Steinberg, 1975). Therefore, post-translational control mechanisms may also exist during sporulation.

E. DNA replication during growth and sporulation

There is evidence that initiation of sporulation is tied to the cell cycle (Dawes and Mandelstam, 1970), a relationship also exhibited during differentiation of eukaryotic cells

(Holtzer et al, 1972). When B. subtilis was grown in a chemostat with the carbon or nitrogen source as a limiting nutrient, a mixed population was established consisting of growing and sporulating cells, and spores (Dawes and Mandelstam, 1970), with the relative proportions of each dependent on the growth rate. An increase in the level of sporulation correlated with a decrease in the rate of growth. When rapidly growing cells were subjected to repeated short periods of starvation by decreasing the dilution rate, only a proportion of the cell population were induced to sporulate, and therefore sensitive to the starvation stimulus. It was therefore concluded that initiation of sporulation is probably restricted to a particular point in the cell division cycle.

Further information was obtained from an examination of the behaviour of sister cells during sporulation (Dawes et al, 1971). When growing cells of B. subtilis are transferred to a sporulation - inducing medium the sister cells tend to remain attached, and either proceed through a cycle of vegetative growth or sporulate in synchrony, suggesting that the induction stimulus is effective only during a critical period of the cell cycle. It is also apparent that sporulation can only occur while DNA is being replicated. This conclusion was first suggested by the results of experiments using B. subtilis Ts134, a thymine or thymidine (TdR)-requiring derivative of B. subtilis 168 which is temperature - sensitive (ts) for initiation of DNA replication (Dawes et al, 1971). At the restrictive

temperature (45°C), chromosome replication is completed and there is no initiation of further rounds of replication. At the permissive temperature (35°C) chromosome replication proceeds normally (Mendelson and Gross, 1967). A preliminary experiment was carried out in which the cells were grown to mid-exponential phase at 35°C in a rich medium containing TdR. The culture was transferred to 45°C for 45 min to allow DNA synthesis to be completed, then centrifuged, and the cells resuspended in sporulation medium (SM) (Sterlini and Mandelstam, 1969). In two portions of the suspension incubated at 35°C, one with and one without TdR, the resulting level of sporulation was very poor. This was not due to cell damage caused by the exposure of cells to the high temperature as there was no effect on viability. The possibility was therefore considered that sporulation has to be induced in a sporulation medium before chromosome replication is complete, and that if it is not, the cells are committed to another cycle of vegetative growth. To test this hypothesis the cells were again grown at 35°C, the culture centrifuged and the cells resuspended in two portions, the first in fresh rich growth medium, and the second in SM. Both media contained TdR, and again the chromosomes were allowed to complete replication at 45°C for 45 min. At this point both suspensions were centrifuged and the cells in each induced to sporulate in SM without TdR. After 10h those cells which had completed their chromosomes in rich medium produced no spores, whereas of those that had completed their chromosomes in poor medium, 10-20% sporulated. This value is low compared to

the wild-type strain (B. subtilis 168), and was similar to the value for the control culture not subjected to the higher temperature. No reason was given to account for the relatively poor sporulation of this strain, but it may be due to partial inactivation of the ts gene product, even at a temperature of 35°C.

In addition, after 45 min at the restrictive temperature, in those cells which had completed DNA synthesis in rich medium, the chromosomes appeared in electron micrographs as tightly-packed nucleoids resembling chromosomes seen in exponentially growing cells at 35°C. However, when replication had been completed in poor medium, the DNA appeared as a diffuse filament extending along the length of the cell. This is the appearance normally described as stage I of sporulation.

Therefore, these workers suggested that these results supported the idea that, for sporulation to be induced, the starvation stimulus has to be applied while the DNA is still replicating, and that once replication has gone to completion in a rich medium the cells are committed to a further cycle of vegetative growth.

A similar conclusion was reached by Mandelstam and Higgs (1974), who used the same ts strain to synchronize chromosome replication. During growth in a rich medium at 45°C the chromosomes were "lined up" by completion of rounds of chromosome replication

already initiated during growth at 35°C. The culture was then diluted back to 35°C, and incubation continued, to initiate DNA replication in a synchronous manner. At intervals after the dilution, samples were transferred to a starvation medium at 35°C and the level of sporulation determined by a direct count of cells containing phase-bright spores 6h after the transfer. It was found that, whereas those cells transferred immediately after completing their chromosomes sporulated poorly, the ability to sporulate rose rapidly, and reached a maximum value (about 10% of the total number of cells) 15 min after reinitiation of DNA synthesis. It was therefore suggested that sporulation can be induced only when the chromosome replication fork is passing through a particular region of the chromosome, some 15 min from the origin of replication.

When chromosome replication during sporulation of a TdR-requiring mutant of B. subtilis 168 was prevented by TdR starvation, additional blocks on the production of serine protease and other sporulation marker events, which could only be relieved by addition of TdR, were observed (Dancer and Mandelstam, 1975b). Therefore, the susceptible period of DNA replication lies before and is necessary for the occurence of one of the earliest sporulation events. However, prevention of DNA synthesis in a TdR-requiring strain by TdR starvation did not prevent the induction of enzymes such as sucrase and histidase (Coote, 1974), suggesting that even though repression of sporulation and enzyme induction may involve some

common catabolite repressor, a fundamental difference exists between the two processes. The requirement for DNA replication clearly distinguishes sporulation from other catabolite-repressed functions of B. subtilis. In fact, the initial stages of sporulation can be regarded more as a modification of cell division, with the starvation conditions promoting an unbalanced metabolism, one result of which is the production of an asymmetrically - located spore septum (reviewed by Hitchins and Slepecky, 1969a). Therefore, at this stage it is important to summarise some of the results of investigations into chromosome replication and cell division during growth.

(1) DNA replication during growth

Under normal conditions of bacterial growth chromosome replication is coordinated with cell division, but the two processes can be uncoupled. Donachie et al (1971) achieved this by blocking DNA synthesis of B. subtilis with nalidixic acid (NAL), or removal of thymine from thymine - requiring strains. Under both these conditions DNA synthesis stopped immediately, but growth and division continued for several hours, resulting in the production of some cells without DNA. The control of cell division in B. subtilis differs from that of Escherichia coli since, in the latter bacterium, division can continue if DNA synthesis is inhibited, but only until each cell contains a single chromosome (Barner and Cohen, 1958). Sargent (1975) also observed the production of anucleate cells in B. subtilis Ts134 during growth at the restrictive temperature for

a period greater than one generation time, after termination of the existing rounds of chromosome replication at the time of transfer to the higher temperature.

The rate of DNA replication apparently depends on the frequency of initiation and not on changes in the rate at which DNA is synthesised (James, 1978). Rapidly-growing cells can initiate a new round of DNA replication before the previous one has been completed, a phenomenon termed multiforked, or dichotomous, replication (Oishi et al, 1964). Initiation of replication is an important point of regulation in cell division, but it is as yet unclear exactly how it is regulated, although it may involve control via effector or repressor molecules which reach a critical concentration at a critical cell mass, termed the initiation mass (see James 1978).

Wake (1972) presented autoradiographic evidence that the chromosome of B. subtilis is circular, of which at least 50% is replicated from the origin in a bidirectional manner (Gyurasits and Wake, 1973). There is also evidence that both replication forks approach the terminus simultaneously (Wake, 1974). In addition, Hye et al (1976), using gene frequency analysis of DNA replication, provided confirmation that complete bidirectional replication occurs from a specific origin, with both replication forks approaching the terminus simultaneously.

Once a cell has replicated its genome the sister chromosomes

segregate, so that, at division, each cell contains a complete copy. Chromosome segregation is thought to require the participation of the cell envelope, in particular the cell membrane (Jacob et al, 1963; Sargent, 1974). It isapparent that both the origin and terminus of the chromosome are membrane-bound in B. subtilis (Sueoka and Hammers, 1974; Yamaguchi and Yoshikawa, 1975) and in E. coli (Parker and Glaser, 1974). In addition, there is an association between the DNA around the replication fork and the cell membrane in B. subtilis (Hye et al, 1976). Also, restriction enzyme analysis of the radioactively-labelled terminus region of the B. subtilis chromosome has shown that, like initiation, termination of replication occurs at a relatively specific site, rather than an arbitrary meeting place of both replication forks (Weiss et al, 1981). The same conclusion was reached by Sargent (1980c), who used a similar approach to label the chromosome terminus of B. subtilis, but his method of identifying the region was to detect DNA sequences homologous to bacteriophages SPβ and Ø3T, which integrate into the terminal region of the chromosome.

Further evidence for a specific termination site came from an analysis of the replication order of genetic markers in a merodiploid strain containing a large non-tandem duplication on one side of the chromosome (O'Sullivan and Anagnostopoulos, 1982). The results showed that a B. subtilis terminal marker, gltA, normally situated symmetrically opposite the origin, remains a terminal

marker in the merodiploid, where it is not located symmetrically opposite the origin. In the merodiploid strain the gltA marker was shown to replicate after another marker situated symmetrically opposite the origin.

The temporary association of the origin and the terminus has been implicated in the regulation of replication initiation and termination (e.g. Jones and Donachie, 1974), but most of the available evidence supports the theory that membrane-chromosome association occurs throughout the entire chromosome replication cycle (Ryder and Smith, 1975; Harmon and Taber, 1976; Beeson and Sueoka, 1979). In addition, Doyle et al (1980) found that cell walls also contain segments of DNA enriched for genetic markers near the replication origin and terminus of the B. subtilis chromosome, suggesting that the binding of DNA-membrane complexes to the rigid cell wall, and the replication of the wall could be a mechanism by which segregation of growing chromosomes occurs.

Therefore, the available evidence strongly suggests that the replication origin, replication fork and replication terminus are three important control points in chromosome replication, and their membrane association may play an important role in the organization and control of the process.

It is known that termination of DNA replication in E. coli

is a prerequisite for cell division and appears to provide a signal that determines its timing (Meacock and Pritchard, 1975). However, there exist strains of B. subtilis which apparently grow normally with large deletions in the terminal region (Hoch and Matthews, 1972), suggesting that termination may not be a crucial step in cell division.

The uncoupling of chromosome replication and cell division, by continued growth and division in the absence of DNA synthesis (see earlier), frequently leads to the production of cells without DNA, cut off by a septum located to one side of the condensed chromosome. When a single round of DNA replication, following germination and outgrowth of spores of two temperature-sensitive DNA initiation mutants of B. subtilis 168, was allowed to proceed to completion at the restrictive temperature, a central division septum formed between the segregated daughter chromosomes (Callister and Wake, 1977). However, at levels of thymine which prevented progress of the round to termination, central septation did not occur, and asymmetrical septation occured at a relatively low frequency. It was therefore suggested by these workers that formation of the central septum is coupled to termination of DNA replication, and possibly triggered by it. Furthermore, when the first round of DNA replication following B. subtilis spore germination was blocked by a potent and specific inhibitor of DNA replication in B. subtilis, 6-(p-hydroxyphenylazo)uracil (HPUra), at a stage before termination

(when the chromosome was 70% replicated), the central septum still formed (McGinness and Wake, 1979). In these circumstances DNA was present on both sides of the septum, which presumably closed down on a partially replicated chromosome. It was concluded that whereas termination of chromosome replication was not obligatory for the formation of the division septum with which it is normally coupled during growth, it may be primarily the position of the septum rather than its formation that is coupled to chromosome replication during growth. It was presumed that in the experiment mentioned earlier (Callister and Wake (1977)) central septation did not occur at a low level of thymine, which reduced DNA replication to less than 10% of its normal rate, because the necessary minimum amount of replication could not be achieved within the time available. Sargent (1975) suggested that the dnaB gene product in one of these ts initiation mutants (Ts134) at the permissive temperature may function as a repressor of septation. Furthermore, McGinness and Wake (1979) postulated that in the normal situation, termination of replication could act to derepress the dnaB gene product, and so trigger the formation of a septum. The idea of production of specific termination proteins necessary for chromosome release and subsequent cell division is not new, and was also proposed by Jones and Donachie (1974) for E. coli, and Winston and Matsushita (1976) for B. subtilis.

(2) DNA replication during sporulation

The first evidence that spores of B. subtilis contain completely replicated chromosomes was obtained by Oishi et al (1964).

These workers inferred from the order of replication of genes in outgrowing spores and vegetative cells that spores contain completely replicated chromosomes. Later, it was suggested that spores of thymine - and TdR - requiring strains may also contain incomplete chromosomes, as they appeared to have anomalous ratios of origin to terminus markers, when DNA-mediated transformation was used (Ephrati - Elizur and Borenstein, 1971; Gillin and Ganesan, 1975). The number of genomes per spore has also been examined by autoradiography of outgrowing spores (Callister and Wake, 1976). The results of this work suggested that B. subtilis spores contain one complete genome. The apparent anomalous gene frequencies found in spores of TdR - requiring strains are probably incorrect, as the efficiency of integration of one of the markers, met B, varies depending on its source. Two mutations, thy A and thy B, are required for the thymidine - requiring phenotype (Neuhard et al, 1978), and these originated from B. subtilis W23 and were transformed into B. subtilis 168, possibly with other genes from the terminus of W23 (Callister and Wake, 1974).

Studies with a DNA initiation mutant and the wild-type strain of B. subtilis 168 have indicated that, for sporulation to be induced, the biochemical stimulus resulting from the transfer of exponentially growing cells to a starvation medium had to be applied at a specific stage of chromosome replication (Mandelstam and Higgs, 1974; Keynan et al, 1976). Synchronous chromosome replication was

obtained in a culture of B. subtilis Ts134 at the restrictive temperature (Mandelstam and Higgs, 1974; see earlier). At intervals after reinitiation of DNA replication at the permissive temperature, samples were transferred to sporulation medium, and the level of sporulation determined at a later time. It was found that the capacity for induced sporulation reached a peak about 15 min after chromosome replication had begun.

However, as it was thought that the use of ts mutants to impose synchrony on DNA replication might have produced artefacts, Keynan et al (1976) used an alternative system, i.e., the resporulation of outgrowing B. subtilis 168 spores, to examine the relationship between the induction of sporulation and chromosome replication. It had already been shown that there was synchrony of chromosome replication during outgrowth (Oishi et al, 1964). Spores were germinated in outgrowth medium, and samples removed to SM at intervals to test the capacity of cells to sporulate. In this system subsequent sporulation capacity reached a peak when the cells were transferred after about 110 min in outgrowth medium, at which time it was proposed that the cells were well into their second round of DNA replication (see later).

The results of these two experimental approaches have been interpreted by two different models for the initiation of sporulation. One model predicts that a certain gene required to trigger asymmetric

septation can be activated only while it is being duplicated, and the cell has to be under step-down conditions for this activation (Dawes et al, 1971; Young and Mandelstam, 1979). The other model postulates that the membrane-attached origins of replication have to migrate towards the cell poles and initiate on one cell end only the growth of an asymmetric septum before duplication of the chromosome terminus enables symmetric septation to occur. The slowing down of cell expansion is essential for the proper movement of the origin towards the cell pole and the triggering of asymmetric septation (Freese, 1976).

Mandelstam et al (1971) found that when a TdR-requiring mutant of B. subtilis was induced to sporulate by the replacement technique, TdR had to be present for at least the first 90 min in the sporulation medium to obtain maximum sporulation, consistent with a requirement for termination of replication. This was later confirmed by Dunn et al (1978), who used HPUra to inhibit DNA replication of B. subtilis 168. Their results indicated that sporulating cells terminate their final round of DNA replication at the same time as they escape from the inhibitory effect of HPUra on sporulation. If added at the point of initiation of sporulation, HPUra prevented sporulation, and the time after resuspension at which DNA replication ceased to be essential for the subsequent sporulation of this strain was determined by removing cells from the sporulation medium at intervals after t_{zero}, adding HPUra (at a concentration

normally inhibitory to sporulation when added at t_{zero}), and continuing incubation of all samples until mature spores were formed in control cultures (t_{20}). At this point the level of sporulation was determined. Using this procedure it was found that the cells began to escape the subsequent effect of HPUra about 35 min after t_{zero}, and that escape was essentially complete 2h after initiation. A similar escape effect was observed by Shibano et al (1978), who found that HPUra also inhibited sporulation induced by the nutrient exhaustion method.

Now, since the chromosome replication time of the wild-type strain in this medium is approximately 55 min (Dunn et al,1978; Ephrati-Elizur and Borenstein, 1971), it was concluded that those cells which escaped the effect of HPUra on subsequent sporulation at 35 min after initiation must have already begun their final round of DNA replication at the time when they were transferred to sporulation medium, when they were probably about 20 min into their final round of replication. This result therefore provided evidence that there is a critical time for induction of sporulation, and that it occurs some 20 min after chromosome replication is initiated. Using a different approach, Mandelstam and Higgs (1974) obtained a value of 15 min, in experiments using B. subtilis Ts134 (see earlier). They concluded that the biochemical stimulus initiating sporulation has to be applied when the replication fork is moving through a particular segment of the chromosome, and tentatively suggested that this segment contains one of the operons concerned with the

transition from stage 0 of sporulation to the next stage.

The results of an analysis of the sporulation potential of outgrowing B. subtilis spores also suggested that induction of sporulation is linked to a specific stage in the cell cycle (Keynan et al, 1976). These workers also concluded that sporulation could only be induced in cells under starvation conditions at a specific stage of chromosome replication. The experimental procedure used in this work was similar to that used to induce "microcycle" sporulation in other Bacillus species. This was defined by Vintner and Slepecky (1965) as the "direct transition of outgrowing bacterial spores to new sporangia without intermediate cell division." However, spores of B. megaterium and B. cereus apparently contain amounts of DNA equivalent to two chromosome complements (Fitz-James and Young, 1959), whereas there is evidence that B. subtilis spores contain only one chromosome equivalent (Callister and Wake, 1976; Wake, 1976). At intervals during outgrowth of germinated spores of B. subtilis samples were transferred to SM. Sporulation potential was shown to reach a peak at about 110 min, and the result was interpreted as follows. Outgrowing spores transferred to SM from outgrowth medium before the start of DNA synthesis (before 45 min) could not be expected to sporulate as they only had 1 chromosome each, which had not yet started to replicate. Those cells transferred after the beginning of the first replication cycle (between 45 and 75 min approximately) had one replicating chromosome and, after completion of this replication,

each cell divided into two daughter cells,each containing only a single chromosome (The occurence of cell division in this experiment means that the term microcycle sporulation cannot be applied to the process). It was suggested that the cells could not sporulate due to the lack of a second chromosome. However, outgrowing spores transferred after about 75 min have initiated the second or third rounds of DNA replication, but had also lost some degree of synchrony by this time. These cells had divided to give 2 or 4 daughter cells, each of which contained two chromosomes and will therefore sporulate.

Therefore, if this method of inducing sporulation in B. subtilis was similar to that used for normal vegetative cells during exponential growth, it would be expected that the cells transferred later than 110 min in outgrowth medium to SM would have a larger sporulation potential, as each should have had more than one replication fork at the time of transfer. In fact, the sporulation potential decreased after 110 min. Therefore, it may be that the conclusions derived from these results are questionable, because of this major difference between the sporulation potential of outgrowing cells and normal exponentially growing cells.

In the same report (Keynan et al, 1976), it was shown that nalidixic acid (NAL), an inhibitor of DNA synthesis, greatly reduced DNA synthesis of cells in the outgrowth medium, but did not prevent the development of sporulation potential when the cells were

transferred to SM lacking NAL. This suggested that DNA synthesis in the outgrowth medium was not an essential prerequisite for the development of sporulation potential. To test if cells could initiate new DNA replication sites (in the presence of NAL) which could have become functional on transfer of cells to SM, germinated spores were incubated in outgrowth medium with NAL. Periodically, samples were removed and the cells incubated for 90 min in outgrowth medium without NAL but with chloramphenicol, and the total DNA measured. This procedure allowed the completion of rounds of replication that had been initiated in the presence of NAL but prevented the initiation of new rounds (Yoshikawa, 1965). By the end of the experiment, the cells treated in this manner had accumulated a four-fold increase in final DNA, suggesting that new initiation sites must have been generated in the presence of NAL. In control cells (without NAL), the same procedure resulted in a 15-fold increase in DNA. It was suggested that the fact that sporulation potential developed in NAL supports the theory that the replication fork should pass through the "sensitive" point of the chromosome while the cells are in a step-down condition. However, the development of sporulation potential during outgrowth in NAL was unlike that obtained in control cells since the potential, once developed, did not then decline. In both cases the final spore yield at t_{20} began to increase for cells transferred to SM after 60-80 min in outgrowth medium. However, as it was admitted that by this time the cells were probably becoming asynchronous, there still remained the possibility that the cells only

had to initiate and undergo a full round of chromosome replication in SM, instead of merely completing the round already initiated before the transfer.

Further experiments, in which low concentrations of HPUra were used to slow down the rate of DNA replication but not inhibit sporulation (subinhibitory concentrations), showed that the subsequent escape of cells from a fully inhibitory concentration of HPUra depended on the duration of chromosome replication (Dunn et al, 1978). When the rate of DNA replication was reduced, the escape of cells from the fully -inhibitory concentration of HPUra was delayed. This was confirmed by Young and Jeffs (1978), who also showed that delayed termination of replication had no effect on the time of appearance of certain sporulation events, such as serine protease, alkaline phosphatase and heat-resistance. It was concluded that the dependent sequence of sporulation events was actually independent of the time of termination of chromosome replication. Dancer and Mandelstam (1975b) also showed that in a TdR-requiring strain, the production of serine protease, one of the earliest events required for spore formation, was prevented by TdR starvation. Therefore, it appears that initiation of DNA replication is required for the appearance of serine protease. Furthermore, Clarke and Mandelstam (1980) found that the induction of serine protease and ribonuclease (RNAase), another very early sporulation event, occured equally well, regardless of the time at which cells were exposed to step-down conditions during the replication

cycle of synchronized populations of B. subtilis Ts134. They suggested that a "watershed" operon, i.e. the operon whose activation under starvation conditions diverts cells into the sporulation pathway, is one of the stage 0-1 events occuring later than the expression of protease. However, this conflicted with the results of Young and Jeffs (1978), where slowing down the rate of DNA replication had no effect on the time of appearance of serine protease, and Dancer and Mandelstam (1975b), who found that inhibition of DNA synthesis by TdR starvation prevented the induction of serine protease. These latter two findings suggest that an event such as initiation of DNA replication in a SM might be the critical point for induction of sporulation, rather than replication of a segment of DNA some distance from the chromosome origin.

In addition, if the only cells that successfully sporulate are those in which replication of a particular operon occurs at the time of transfer to sporulation medium, the proportion of cells which form mature spores would be relatively low. Yet at least 80% of the cells present in a resuspended culture of B. subtilis 168 normally form spores. Therefore, initiation of DNA replication must occur in many cells in a starvation medium, and this is consistent with the late escape (60-120 min after t_{zero}) of many cells from HPUra inhibition of sporulation (Dunn et al, 1978). This contradicts the earlier finding that if chromosome replication in a ts mutant is completed in a rich medium, new rounds are not subsequently initiated in the

poor medium (Mandelstam and Higgs, 1974). To test whether this was also the case with the wild-type strain, and not just an artefact of the ts strain, Dunn et al (1978) used chloramphenicol to inhibit further initiation of DNA replication during growth of B. subtilis 168. The cells were then transferred to resuspension medium when, after an initial lag period, there was an observed accumulation of DNA equivalent to one round of DNA replication. In addition, HPUra still prevented sporulation when added during this time, indicating that initiation of DNA replication in this medium was a necessary prerequisite for sporulation. Presumably, therefore, cells in which the critical point has been passed at the time of resuspension are unable to sporulate without undergoing another complete round of DNA replication. In this way it can be seen that most cells transferred to sporulation medium do eventually sporulate successfully.

Sargent (1980b) described a method for determining the rate of termination of chromosomes destined to be packaged into spores, by specifically labelling the terminus. When a sporulating culture of a TdR-requiring derivative of the wild-type strain was pulse-labelled with [^{3}H]-TdR, and then treated with HPUra to inhibit DNA synthesis, the only labelled spores formed were those that had completed replication during the pulse period. DNA-mediated transformation was used to show that the DNA of spores formed in the presence of HPUra had the same ratio of origin to terminus markers as DNA from untreated spores. Also, spores formed in the presence of HPUra had

the same DNA content as untreated spores, and presumably contained completed chromosomes. The rate of termination of chromosomes destined to be packaged into spores, as determined by this method, reached a broad peak 2h after initiation of sporulation, but still continued at a considerable rate 4h after t_{zero}. This result differed from that obtained in the study of HPUra inhibition of sporulation in the wild-type strain, in which escape from HPUra inhibition was essentially complete 2h after t_{zero} (Dunn et al, 1978). This difference was not merely due to the use of a TdR-requiring strain in the experiment of Sargent (1980b), since he reported that essentially similar results were obtained with the wild-type strain. In addition, the amount of spore DNA synthesised in resuspension medium was close to half the amount of DNA present in mature spores (Sargent, 1980b), suggesting firstly, that chromosomes destined to be packaged into spores are replicated from close to the origin and possibly initiated in the sporulation medium, and second, that regardless of the time at which replication of the spore chromosome is completed, the template strand is always of vegetative origin. However, in this experimental procedure the cells were initiated to sporulate after growth to about 600 µg (dry weight) per ml ($E_{540\ nm}$ of 3.0), followed by resuspension at 200 µg (dry weight) per ml in starvation medium. In the original paper describing the nutrient replacement method for initiating sporulation (Sterlini and Mandelstam, 1969), the bacteria were grown to a density of 250 µg (dry weight) per ml, and resuspended in starvation medium at the same density. There remains the possibility therefore that cells resuspended in starvation medium at a higher density

(Sargent, 1980b) were already under conditions of nutrient deprivation. Thus, sporulation might have already been induced in at least some of the cells, so that on resuspension in a starvation medium the behaviour of the replicating chromosomes may have been different to that normally occuring when sporulation is initiated during mid-exponential growth.

The hypothesis that there may be a requirement for a complete round of replication initiated from the origin under sporulation conditions differs from the model proposed by Mandelstam and Higgs (1974), i.e. that only those cells in which a replication fork was passing through a particular segment of the chromosome, some distance from the origin, under step-down conditions, would be induced to sporulate. If this latter theory is correct, then a proportion of cells in a growing population, with replication forks lying before the critical point, would only require to complete, in sporulation medium, the round of replication initiated in growth medium to successfully sporulate.

One obvious experimental approach which could be used to determine which of the two theories outlined above was the most likely would be to use the TdR analogue, 5-bromo-2'-deoxyuridine (BUdR), to label DNA during growth of a TdR-requiring strain. The amount of DNA replication occuring during sporulation could then be monitored by density gradient analysis, by noting the progressive loss, both

during and after completion of sporulation in medium lacking BUdR, of the heavy BUdR label initially present in both strands of DNA in cells at the time of initiation of sporulation. Such an experiment might go some way towards solving the following problem. Is initiation of chromosome replication from the origin required for sporulation, or do growing cells merely have to terminate in sporulation medium a round of replication already initiated at the point of induction of sporulation, when the replication fork lies before a critical point some distance from the origin? However, this experiment has proved impossible to perform because of the toxicity of the analogue for TdR-requiring strains (see section II). This problem could be overcome by the isolation of a BUdR-tolerant strain which could incorporate BUdR instead of TdR into DNA, without any effect on cell viability or DNA replication. Several bromouracil (BU)-tolerant strains of B. subtilis W23 (thy, his) have already been isolated (Bishop and Sueoka, 1972), as has a BUdR-tolerant derivative of B. subtilis 168 (thy, but-32) (Coote, 1977). However, as yet these strains have not been used successfully in an analysis of chromosome replication during sporulation. Adams and Wake (1980) attempted to density-label the chromosomal terminus of strain but-32 with BU, but found that all chromosomes labelled with BU in starvation medium were excluded from mature spores.

Finally, Sargent (1980b) postulated that the wide distribution of times of initiation of replication of the chromosome destined

to be packaged into a spore observed in his experiment might be scheduled by a clocklike process which is a normal feature of vegetative growth and in some way related to cell age, and that when initiations occur after the onset of starvation, the chromosomes are modified by a special mechanism, as yet unknown, so that they participate in sporogenesis.

In summary, therefore, studies with a TdR-requiring strain ts for initiation of chromosome replication, and the wild-type strain, have indicated that, for sporulation to be induced, cells in sporulation medium must be replicating DNA, and furthermore, a replication fork must be positioned before a critical segment of the chromosome. If the replication fork has passed this point the existing round of replication is terminated and the cells undergo another round of DNA replication before sporulation can continue. In addition, termination of chromosome replication is a requirement for successful sporulation. The results of experiments involving an analysis of synchronized DNA replication, either in a ts initiation mutant, or in outgrowing spores of the wild-type strain, have suggested that the critical point for initiation may lie some 20 min after the chromosome origin. The escape time of the wild-type strain from HPUra inhibition of sporulation is consistent with this theory. However, when wild-type cells with completed chromosomes, obtained by exposure of growing cells to chloramphenicol, were transferred to starvation medium, chromosome replication was initiated and spores were obtained

(Dunn et al, 1978). This suggested that initiation of chromosome replication may be a requirement for sporulation. Furthermore, Sargent (1980b) has also presented evidence that initiation of chromosome replication from the origin may be required for sporulation. However, unlike the known requirement for termination of chromosome replication, there remains an as yet unresolved question about the necessity for initiation of chromosome replication during sporulation.

II. The effect of BUdR on growth and differentiation

The halogenated TdR analogue, 5-bromo-2'-deoxyuridine (BUdR) is, where required, readily incorporated in place of TdR into DNA by most cellular and in vitro DNA synthesising systems. This results in an increased buoyant density in CsCl equilibrium density gradients compared to the normal unsubstituted DNA, a property which has been widely exploited in experiments designed to discriminate between newly-synthesised and previously-synthesised DNA during chromosome replication in both eukaryotic and prokaryotic cells (Braun and Wili, 1962; Mueller and Kajiwara, 1966; Smith et al, 1970). However, its usefulness in this respect has been limited by different effects on a variety of cells. Firstly, BUdR has been reported to induce the synthesis of viral antigens and viral replication in certain virus-negative cells (Hampar et al, 1971; Sugawara et al, 1972). In addition, a cytotoxic and mutagenic effect of BUdR has been reported for cells

grown over a long period of time in the presence of the analogue, or when its concentration is high (Roy-Burman, 1970). Also, Kato (1974) reported induction of sister-chromatid exchanges by BUdR in mammalian cells, a property correlating well with the mutagenic effect of the analogue (Davidson et al, 1980).

However, it is the specific effect of BUdR on differentiating cells which has been most extensively reported. BUdR apparently affects systems as dissimilar as chick embryo muscle cells (Bischoff and Holtzer, 1970), cultured mammary tissue of pregnant mice (Turkington et al, 1971) and malignant cells (Schubert and Jacob, 1970). The first observed effect of BUdR on differentiation was inhibition of development of presumptive myoblasts (Stockdale et al, 1964) and in most eukaryotic systems investigated, BUdR prevents the expression of differentiated functions (Rutter et al, 1973). In some cases, however, it has been shown that BUdR has the opposite effect and induces or activates the expression of certain differentiated functions (Schubert and Jacob, 1970; Koyama and Ono, 1972). A selection of the effect of BUdR on various systems is listed in Table 1. These observations led many workers to suggest that BUdR could be used as a tool for investigating, at a molecular level, the basic regulatory mechanisms underlying development (Maclean, 1976).

A. Toxicity and mutagenicity of BUdR

The molecular structures of TdR and BUdR are given in Fig. 4, the only structural difference between the two being

TABLE 1. The effects of BUdR on various cell systems.

System	Feature measured or studied
Repression	
Chondrocytes	Chondroitin sulphate synthesis (Mayne *et al*, 1973)
Chondrocytes	Cell shape (Abbot and Holtzer, 1968; Coleman *et al*., 1970)
Myoblasts	Myogenesis (Stockdale *et al*., 1964; Rogers *et al*., 1975; Bischoff and Holtzer, 1970; Lough and Bischoff, 1973)
Chick erythropoiesis	Haemoglobin synthesis (Weintraub *et al*., 1972)
Mouse leukaemia cells	Haemoglobin synthesis (Ostertag *et al*., 1973)
Mouse leukaemia cells	Thymidylate synthetase activity (de Clerq *et al*., 1981)
Melanoma cells	Pigmentation and tumourigenicity (Silagi and Bruce, 1970; Silagi *et al*., 1977)

TABLE 1. (contd.)

Melonoma cells	Adhesion (Evans et al., 1977)
Melanoma cells	Fluorescence polarisation (Rosenthal et al., 1978)
λ coliphage transcription	Photosensitisation (Jones and Dove, 1972)
Limb-bud mesenchyme	Histology (Levitt and Dorfman, 1972)
Tetrahymena pyriformis	Cell division (Lykkesfeldt and Andersen, 1974)
Tetrahymena pyriformis RNA synthesis	Ribosomal DNA transcription (Lykkesfeldt and Andersen, 1977)
Amnion cells	Mucopolysaccharide synthesis (Mayne et al., 1971)
Mouse fibroblasts	Ribonucleotide reductase activity (Meuth and Green, 1974)
Mouse fibroblasts	Chromatin transcription (Hill et al., 1974)
Dimethylsulphoxide (DMSO)-stimulated Friend leukaemia cells	Globin mRNA synthesis (Priesler et al., 1973)
Cultured tobacco cells	Chloroplast differentiation (Seyer and Lescure, 1977)

TABLE 1. (contd.)

Rat Hepatoma cells	DNA methylation (Singer et al., 1977)
Rat Hepatoma cells	Tyrosine aminotransferase (Stellwagen and Tomkins, 1971)
Human sternal marrow cells	Resistance to radiation (Djordjevic and Szybalski, 1960)
Nicotinia glauca	Pith tissue formation (Durante et al., 1977)
Hela cells	Cloning efficiency (Kajiwara and Mueller, 1964)
Dictyostelium discoideum	Sporulation (Kay et al., 1978)
Physarum polycephalum	Sporulation (A. Chapman, pers. comm.)
Frog embryos	Processing of nuclear RNA (Shepherd and Flickinger, 1979)
Mammary gland cells	Differentiation (Turkington et al., 1971)
Exocrine pancreas cells	Differentiation (Walther et al., 1974)
Rat odontogenesis	Odontogenic epithelial - mesenchymal interactions (Schwartz and Snead, 1982)

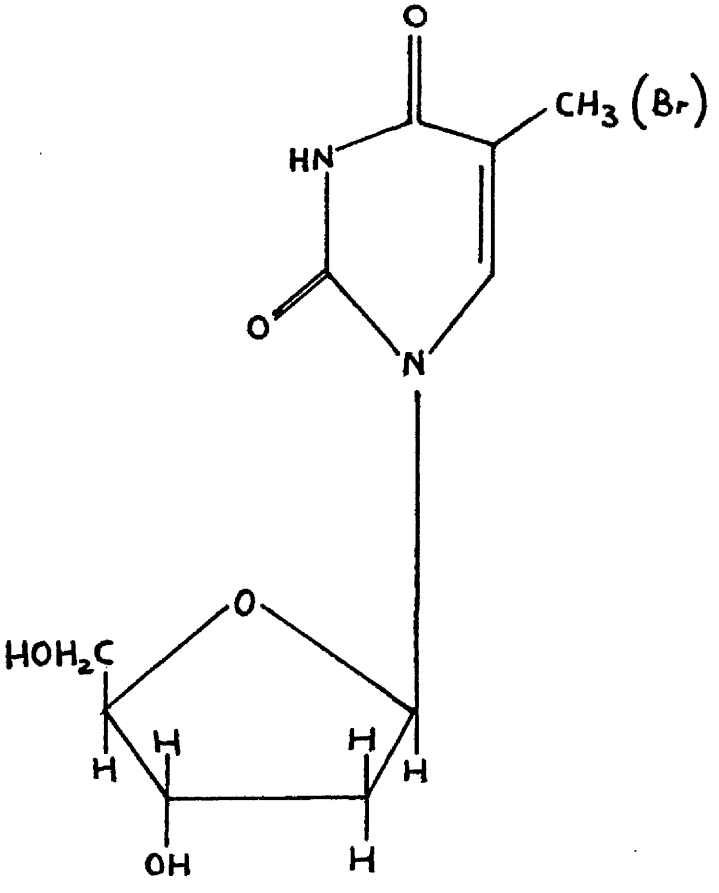
TABLE 1. (contd.)

Induction	
Rat pituitary tumour cells	Prolactin synthesis (Biswas et al., 1977)
Avian cells	Expression of a tumour-specific antigen (Biquard and Aupoix, 1978)
Lymphoid cells (Burkitt lymphoma clones)	Epstein-Barr virus (Sugawara et al., 1972; Hampar et al., 1971)
Hybrid mouse - Chinese hamster cells	Alkaline phosphatase (Koyama and Ono, 1972)
HeLa cells	Alkaline phosphatase (Goz, 1974)
Glial tumour cells	Phosphodiesterase activity (Schwartz et al., 1973)
Neuroblastoma cells (mouse)	Neurite formation (Schubert and Jacob, 1970)
Neuroblastoma cells (human)	Tyrosine hydroxylase (Prasad et al., 1973)

Figure 4. The structure of thymidine (TdR)

Substitution of the methyl group at position 5 of the pyrimidine ring by bromine forms 5-bromo-2'-deoxyuridine (BUdR).

Fig. 4



the replacement of the methyl group at position 5 of the pyrimidine ring of TdR by bromine in BUdR. The pyrimidine analogue 5-bromouracil (BU) also readily replaces thymine bases in DNA. BUdR or BU are usually incorporated in the -keto form, which does not cause mispairing of bases, but they more readily undergo tautomeric shifts to the -enol form than TdR that enables them to base-pair with guanine. Thus BU may be incorporated as a pairing mate to adenine, and then produce a G-C substitution for the original A-T, or it may occasionally be incorporated in the -enol form as a pairing-mate with guanine, and then revert to its -keto form to produce an A-T substitution for the original G-C. A "missense" point mutation usually results from mispairing of bases, leading to the production of an altered protein via transcription and translation. Replication of the DNA is necessary for the errors produced by tautomeric shifts to appear in DNA molecules.

In addition to the mutagenic properties of BU and BUdR extensive substitution in DNA of thymine by BU is lethal for most thymine-requiring E. coli and B. subtilis strains. To decrease this toxicity, Oishi and Sueoka (1965) suggested adding thymine to BU-containing media. Using this approach, Laird and Bodmer (1967) assayed BU incorporation using CsCl density gradient centrifugation of DNA extracted from a TdR-requiring mutant of B. subtilis 168 grown with various mixtures of thymine and BU, or TdR and BUdR. A TdR-requiring strain was required as the wild-

type strain does not take up BUdR from the medium. It was found that the buoyant densities of the DNA preparations were considerably less than would be expected if BUdR and TdR (or BU and thymine) were utilised at random. There was a marked preference for thymine incorporation compared to BU incorporation, which was greater than the preference for TdR compared to BUdR. They suggested that this discrimination was possible due to the large number of opportunities for selectivity during the utilisation of pyrimidine intermediates in the synthesis of DNA. Pyrimidine metabolism is a complex series of energy-dependent pathways, culminating in the formation of ribonucleoside triphosphates (ribonucleotides) for RNA synthesis, and deoxyribonucleoside triphosphates (deoxyribonucleotides) for DNA synthesis. Figure 5 is a summary of the major pyrimidine deoxyribonucleotide interconversions, showing the steps catalysed by four enzymes. It has been shown that 5-halogenated deoxyuridine compounds, as well as TdR, can act as substrates for thymidine kinase in E. coli, and that deoxycytidine di- or tri-phosphate (dCDP; dCTP) activate this enzyme (Okazaki and Kornberg, 1964).

In an earlier study on the effects of thymine and thymidine analogues on thymine-requiring strains of E. coli, Cohen and Barner (1956) found that 3 μgml^{-1} of BU alone allowed a 5 to 6-fold increase in culture turbidity, during which time the viable count only doubled, and then declined, as in "thymineless"

Figure 5. Summary of the major pyrimidine deoxyribonucleotide interconversions (from White et al, 1978).

Enzymes catalysing reactions:-

(1) Thymidine phosphorylase

(2) Thymidine kinase

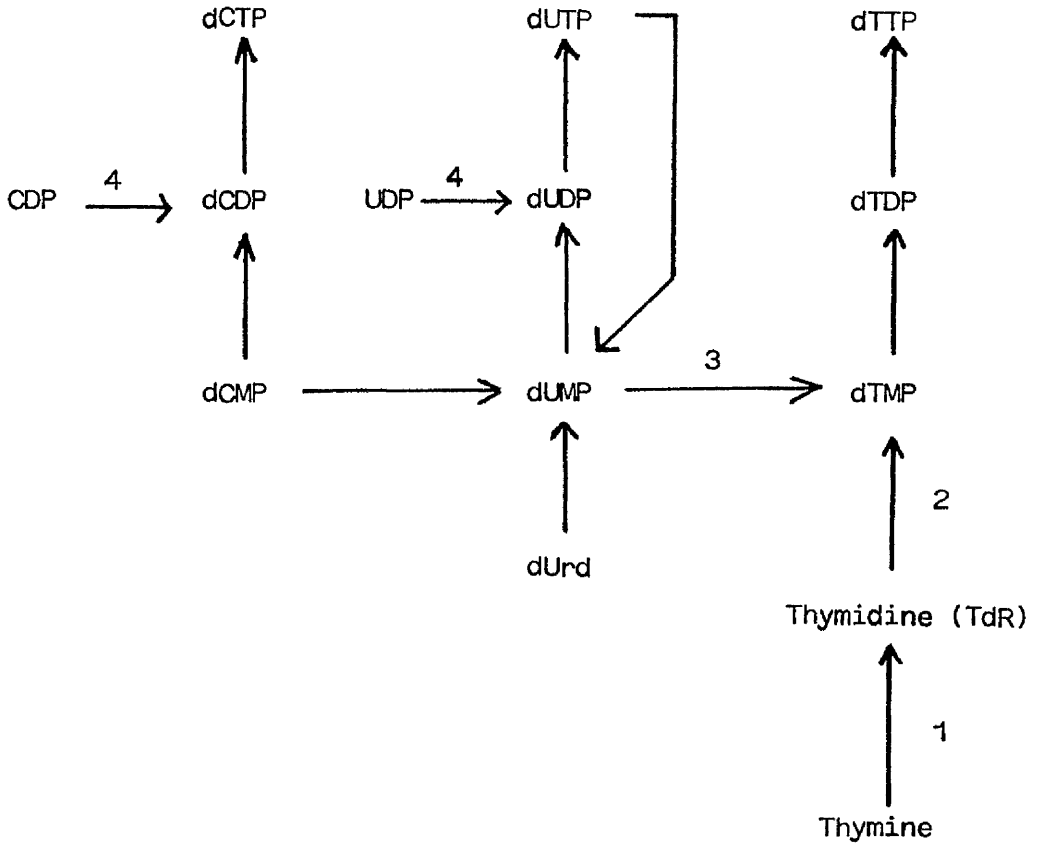
(3) Thymidylate synthetase

(4) Ribonucleotide reductase

Abbreviations not used in text:-

(d)CDP	-	cytidine diphosphate (deoxycytidine diphosphate)
UDP	-	uridine diphosphate
dUrd	-	deoxyuridine
dTMP	-	thymidine monophosphate

Fig. 5


dCTP
dUTP
dTTP
CDP
4
dCDP
UDP
4
dUDP
dTDP
dCMP
dUMP
3
dTMP
2
dUrd
Thymidine (TdR)
1
Thymine

death. Also, in the presence of thymine, BU provoked cell death, even though the turbidity increase was extensive, and examination of the morphology of the cells revealed unusually long bacteria. At a weight ratio of BU to thymine of 10:1 $\mu g ml^{-1}$, the bacteria were killed after a single division, and in order to completely overcome the inhibitory action of BU at 10 $\mu g ml^{-1}$ it was necessary to add 200 µg of thymine per ml of culture. However, BU had no effect on the division time of the thymine-independent parent strain (which would grow in the absence of thymine), even at a concentration of 500 $\mu g ml^{-1}$. It was also noted that BU was apparently less effective than BUdR at killing bacteria in the presence of TdR due to the preferential incorporation of pyrimidine nucleosides over the free pyrimidine bases. This was confirmed when, in the presence of the free base thymine, BUdR was much more effective at inducing cell death than BU.

The enzyme thymidylate synthetase (see Fig. 5) has been proposed as the target enzyme for the inhibitory activity of 5-substituted 2'-deoxyuridine compounds, including BUdR, on growth of mouse leukaemia cells, although the inhibitory effect of a related compound, 5-fluoro-2'-deoxyuridine (FUdR) was much stronger than that of BUdR (de Clerq et al, 1981). Incidentally, some thymidylate synthetase inhibitors such as FUdR (or fluorouracil, its free base form) have for several years been utilised as anti-tumour drugs in cancer chemotherapy. FUdR is a precursor of

5-fluoro-2'-deoxyuridine monophosphate (FdUMP), a potent inhibitor of thymidylate synthetase in E. coli (Cohen et al, 1958), and therefore an inhibitor of de novo thymidine nucleotide synthesis. In addition, whereas 5-fluoruracil (FU) is excluded from E. coli DNA (Warner and Rockstroh, 1980), BU is not (Lindahl et al, 1977).

However, it is unlikely that BUdR toxicity during growth of TdR-requiring strains of B. subtilis 168 is due to inhibition of thymidylate synthetase, as these strains lack this enzyme activity, and require exogenous TdR or thymine to allow growth (Wilson et al, 1966). Bishop and Sueoka (1972) isolated two BU-tolerant strains of B. subtilis which were considerably less-sensitive to BU than the parental strain from which they were derived, although not completely resistant to its inhibitory action on growth. It was suggested that these strains could be used in experiments requiring specific density labelling of DNA, with minimal interference with DNA metabolism. In this respect the germination of thymine-requiring spores of B. subtilis in BU media provides a system for genetic mapping, since the order of transfer of genetic markers from light-light to heavy-light DNA reflects their relative position on the chromosome (Oishi et al, 1964). The success of this technique depends on the maintenance of synchrony of chromosome replication, but the breakdown of synchrony observed when spores of a BU-sensitive strain are germinated in BU medium (Nagley and Wake, 1969) limited the usefulness of BU as a density label

for this type of mapping until the isolation of BU-tolerant strains.

Thymine-requiring B. subtilis spores germinated in an unenriched medium containing BU and thymine, at a molar ratio of 10:1, apparently initiated a second round of DNA replication before the first round was complete, whereas in thymine alone initiation of a second round only occured when the first round was completed (Nagley and Wake, 1969). The percentage replication of both rounds in BU medium was calculated from the results of analytical equilibrium density gradient centrifugation in Cs_2SO_4. Second-round replication was recognized by the appearance of heavy-heavy DNA, whereas first-round replication was shown by the appearance of heavy-light DNA. However, in thymine alone an alternative to density gradient analysis of DNA replication was required. This was achieved by calculation of the time taken for the first round, from a knowledge of the relative rates of DNA replication in BU - or thymine medium, measured by incorporation of $[^3H]$-TdR into trichloroacetic acid (TCA) -insoluble material, and a correction made for the percentage of BU for thymine residues in DNA. The calculated time for the first round of replication in thymine alone was 62 min, about 40% of the value for BU-treated cells. The effect of BU in causing such dichotomous replication was explained solely by its ability to slow down the rate of individual rounds of replication, leaving unaltered the rates of RNA and protein synthesis. Compared to a culture with thymine alone spores germinating in BU

medium started to lose viability after the time (150 min) when the second round of DNA replication was initiated, and at 450 min they were 20% viable. However, the optical densities of the two cultures increased in exactly the same manner over this period. In addition, it was found that thymine-starvation for 110 or 130 min had no effect on the relative rates of first and second rounds of DNA replication when thymine was added to the medium. Therefore, the timing of initiation was maintained even when DNA replication was prevented. Yoshikawa and Haas (1968) also proposed that BUdR slows down the rate of DNA replication, resulting in dichotomous replication. These results are consistent with the assumption that what controls initiation of DNA replication is the accumulation of a regulatory material (repressor or activator) which acts when its level reaches a critical value, since the timing of initiation of DNA replication was maintained irrespective of the rate or extent of DNA replication. It was further suggested that, if the time taken for a round of replication at a particular temperature is fixed and largely independent of the growth rate, it might be possible to improve synchrony of initiation of rounds of replication using a richer medium, in which the proposed regulatory material could reach a critical level amongst the cell population over a shorter period of time (Nagley and Wake, 1969). Oishi et al (1966), using a strongly supplemented BU germination medium, obtained a better synchrony of initiation of the first round of replication.

In addition, an increased rate of DNA synthesis was reported for BU-tolerant strains of B. subtilis in BU medium compared to the rate for a BU-sensitive strain (Bishop and Sueoka, 1972). These mutants also transferred genetic markers from light-light to heavy-light DNA more rapidly and completely than the sensitive strain during spore germination in the presence of BU (One of these mutants, But-1, was used by Quinn and Sueoka (1970) to show the symmetrical nature of dichotomous replication of the B. subtilis chromosome). Furthermore, during DNA synthesis of the BU-sensitive strain thymine was selectively incorporated in preference to BU, confirming the conclusion of Laird and Bodmer (1967) (see earlier), but this preference was significantly reduced in the BU-tolerant strains.

Coote (1977), using a selection procedure similar to that of Bishop and Sueoka (1972), isolated a BUdR-tolerant mutant of B. subtilis 168 (thyA, thyB, trpC). Like the BU-tolerant strains, this strain was unable to grow for more than a few generations with BUdR alone, and a small amount of the natural nucleoside had to be present to allow growth. However, the basis of the increased resistance to analogue toxicity had not been further investigated in either case. The tolerance phenotype may be a result of increased preferential uptake of TdR over BUdR, although Bishop and Sueoka (1972) presented evidence that BU-tolerant strains exhibit a decreased preferential uptake of thymine over BU. Alternatively, an increased number of replication origins may be initiated in the

BUdR-tolerant strain to offset a possible decrease in the overall rate of replication induced by BUdR. Another possibility is that BUdR tolerance is a result of lost, or defective, thymidine kinase activity, an enzyme catalysing phosphorylation of TdR and its analogues (see Fig. 5). Mandelbaum-Shevit and Kisluik (1978) proposed that the decrease in the activity of this enzyme may be the basis of resistance of some Pediococcus cerevisiae strains to FUdR. Also, Ostertag et al (1973) observed that thymidine kinase-deficient mouse leukaemia cells were resistant to BUdR. Furthermore, Lunn et al (1977) isolated 24 BUdR-resistant mutants of Physarum polycephalum. All except one had low thymidine kinase activity, and the strains were categorised into 2 complementation groups, one containing 23 mutants with low thymidine kinase levels, the other containing the resistant mutant with normal enzyme activity. However, it is unlikely that BUdR or BU resistance in the TdR-requiring, BUdR-tolerant strains of B. subtilis is due to loss of activity of thymidine kinase, as these strains grow normally in the presence of TdR (Bishop and Sueoka, 1972; Coote, 1977).

In a detailed analysis of BUdR resistance (Bradley et al, 1982), it was found that full BUdR resistance (i.e. over a range of BUdR concentrations) in Chinese hamster ovary (CHO) cells occurs via three discrete steps. Whereas the second and third steps (i.e. resistance to two higher BUdR concentrations) were correlated with partial and complete reduction, respectively, in the specific

activity of thymidine kinase, the first step (i.e. growth at the lowest BUdR concentration tested) appeared to result from a mutation in the gene for ribonucleotide reductase producing an altered enzyme with decreased BUdR-triphosphate sensitivity. This enzyme controls deoxyribonucleotide triphosphate pool sizes (see Fig.5), and both dTTP and its analogue BUdR-triphosphate exert strong negative-feedback control on the conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) in mouse cells (Meuth and Green, 1974). At sufficiently high concentrations, these effectors induce a "deoxycytidineless" state and cell death. In addition, extensive studies on the mechanism of BUdR toxicity and mutagenicity in mammalian cells (Davidson and Kaufman, 1978; Kaufman and Davidson, 1978; Kaufman and Davidson, 1979; Ashman and Davidson, 1981) have shown that perturbations in the dCTP pool size, rather than incorporation of the analogue into DNA play a key role, again suggesting an important effect of BUdR on the reductase enzyme. Mutant cell lines resistant to hydroxyurea, an inhibitor of ribonucleotide reductase (Bazill and Karamata, 1972; Rima and Takahashi, 1978), also showed increased resistance to the toxic and mutagenic effects of BUdR (Davidson and Kaufman, 1979). Therefore, BUdR-tolerance in B. subtilis may be associated with resistance to possible inhibition of ribonucleotide reductase by BUdR-triphosphate.

In summary, therefore, BUdR toxicity has limited its use as a density label in the analysis of DNA replication. Thymine-

requiring strains of B. subtilis apparently exhibit a marked preference for thymine and TdR incorporation compared to BU and BUdR incorporation. The preference for BU over thymine may be decreased in BU-tolerant strains of B. subtilis. Thymidylate synthetase has been proposed as the target enzyme for the inhibitory action of BUdR, but this is unlikely to be the basis of BUdR toxicity during growth of TdR-requiring strains of B. subtilis. BU apparently slows down the rate of DNA replication in a TdR-requiring strain of B. subtilis, resulting in multiforked replication. BU-tolerant strains may overcome this effect by increasing the number of replication origins to offset a decrease in the overall rate. It is unlikely that resistance to BUdR or BU in tolerant strains of B. subtilis is due to the loss of thymidine kinase activity. Finally, there remains the possibility which has not yet been investigated that tolerance is a result of resistance to inhibition of ribonucleotide reductase by BUdR-triphosphate.

B. The effect of BUdR on differentiation

(1) Incorporation of BUdR into DNA

In general, because BUdR is most apparent inside cells as a component of DNA, most of the molecular mechanisms proposed for the selective action of BUdR on differentiation in eukaryotes are consistent with a DNA-mediated effect. For most cell types, inhibition of differentiation by BUdR is readily reversible, and there-

fore not a consequence of mutagenicity.

Although a positive correlation between DNA synthesis in the presence of BUdR and suppression of differentiation has been demonstrated in hepatoma cells (Stellwagen and Tomkins, 1971), chick chondrocytes (Mayne et al, 1973), mouse leukaemia cells (Ostertag et al, 1973), chloroplast differentiation in cultured tobacco cells (Seyer and Lescure, 1977), and differentiation of pith tissue in the plant Nicotinia glauca (Durante et al, 1977), there are no results which conclusively demonstrate a causal relationship. Indeed, Schubert and Jacob (1970) found that BUdR induced differentiation of mouse neuroblastoma cells in the absence of DNA synthesis, and proposed that this effect of BUdR was not due to its incorporation into DNA. However, Biswas et al (1977) noted that BUdR-induced prolactin synthesis in rat pituitary tumour cells could be overcome by addition of TdR, and attributed this effect to a reduction in the level of BUdR incorporation into DNA. Likewise, it appears that in many eukaryotic systems the inhibitory action of BUdR on differentiation could be reversed by further cell multiplication in its absence (Rutter et al, 1973), although in some experiments, particularly those using cartilage cells, recovery from BUdR suppression of differentiation has not been uniformly obtained (Chako et al, 1969; Levitt and Dorfman, 1972).

It was also apparent that deoxycytidine could reverse the suppression of differentiation of melanoma cells by BUdR (Horn and Davidson, 1976), and also overcome its toxicity (Rogers et al, 1975) and mutagenicity (Davidson and Kaufman, 1978; Ashman and Davidson, 1981). If, as Kaufman and Davidson (1978) suggested, the reversal of BUdR inhibition of differentiation by deoxycytidine is due, at least in part, to the intracellular conversion of exogenously supplied deoxycytidine to thymidine nucleotides, the addition of deoxycytidine should decrease the amount of BUdR incorporated into DNA, consistent with a DNA-mediated mechanism of action for BUdR. This has been shown for melanogenesis in hamster cells (Horn and Davidson, 1976). Conversely, addition of deoxycytidine did not reverse BUdR inhibition of differentiation of embryonic rat pancreas (Walther et al, 1974), although addition of thymidine prevented the inhibition, correlating with a decreased incorporation of the analogue into DNA. Similar effects were observed for BUdR inhibition of differentiation of chick embryo skeletal muscle cells, and specialised synthesis by sternal cartilage and pigmented retina cells in culture, at BUdR concentrations not affecting cell proliferation (Coleman et al, 1970). All effects were reversible in the presence of TdR alone, but addition of deoxycytidine in the presence of BUdR had no effect on inhibition of differentiation.

Furthermore, Davidson and Kaufman (1977) found that, for BUdR inhibition of pigmentation im melanoma cells, over a range

of deoxycytidine concentrations the degree of BUdR incorporation concommitantly decreased, but then levelled off, so that there was no signigicant decrease in BUdR incorporation with further increases in the concentration of deoxycytidine. It was during this plateau, however, that deoxycytidine exerted its greatest effect in reversing suppression of pigmentation caused by BUdR. Reff and Davidson (1979) also showed that this reversal occured without a change in the distribution of BUdR in DNA. Therefore, these latter results suggested that DNA might not be the primary site of action for BUdR during differentiation. Further evidence for this theory came form the results of experiments using rat hepatoma cells in which changes in gene expression (measured by changes in methylation of DNA) caused by BUdR were not associated with selective distribution of the analogue in DNA (Singer et al, 1977). However, preferential incorporation of BUdR into eukaryotic "middle-repetitive" DNA (mrDNA) has been observed when normal rat embryo cells, developing chick cartilage cells, and the slime mould Dictyostelium discoideum were present in media containing low concentrations of BUdR (Schwartz et al, 1975; Schwartz and Snead, 1982; Strom and Dorfman, 1976; Monier et al, 1977). These findings are significant, in that it has also been proposed that mrDNA contains sites of regulation of transcription of genes located in "unique" DNA (Davidson et al, 1977).

CsCl gradient centrifugation analysis of DNA extracted from cultured tobacco cells grown in the presence of BUdR,

which inhibits differentiation of chloroplasts, indicated that the analogue was not incorporated at random into total cellular DNA, and that a particular fraction, possibly chloroplast DNA, was more heavily labelled (Seyer and Lescure, 1977). In addition, irreversible arrest of odontogenic differentiation in vitro was achieved when only a small amount of BUdR was substituted within a single strand of the duplex DNA (Schwartz and Snead, 1982). Souleil and Panijel (1970) also presented evidence that in antigen-primed lymphoid cells BUdR caused incorporation of DNA precursors in part into a more rapidly-sedimenting DNA fraction of lighter density, compared to native thymine-containing lymphoid cell DNA. This was attributed to the presence of lipid materials in the fraction obtained from BUdR-containing cells, and they concluded that the presence of BUdR may have caused an increase in the stability of DNA-membrane complexes. Therefore, the possibility of a DNA-mediated effect on differentiation cannot be ruled out, especially when the timing of the effect of BUdR on differentiation is considered.

(2) Cell-cycle dependency of BUdR action

Sensitivity to the analogue usually occurs during a restricted period of development, when DNA replication is in progress. For example, BUdR incorporation into early-replicating DNA during the S-phase of synchronized myogenic cells resulted in a decline

in myoblast fusion, whereas incorporation into late-replicating DNA was without effect (Lough and Bischoff, 1976). Hampar et al (1973) also identified a critical period during the S-phase of human lymphoblastoid cells for activation of the Epstein-Barr virus by another halogenated pyrimidine nucleoside, 5-iododeoxyuridine (IUdR). This effect also required incorporation of the analogue into DNA. Similarly, haemoglobin production during erythropoiesis was only blocked when BUdR was incorporated into DNA of precursor erythroblasts during the previous cell cycle (Weintraub et al, 1972).

(3) The effect on transcription

It also appears that in some systems transcription is directly altered by BUdR. Jones and Dove (1972) demonstrated inhibition of the transcription rate of lambda (λ) coliphage genes in which the TdR in DNA was completely or partially-substituted. Hill et al (1974) also presented evidence that the RNA synthesised by chromatin and/or DNA of mouse fibroblasts grown in the presence of BUdR has an altered base composition, the effect being stronger with chromatin than with "naked" DNA. Simpson and Seale (1974), using spectral analysis, demonstrated that the conformation of BUdR-substituted DNA differed from that of normal chromatin. Lykkesfeldt and Andersen (1974) tested the effect of BUdR on DNA replication and cell division in Tetrahymena pyriformis, and found that after a full round of replication in the presence of BUdR cell

proliferation ceased and the replication rate decreased. They concluded that "heavy-heavy" DNA synthesised during the second round of replication in the presence of BUdR had a defective template activity for the transcriptional processes necessary for cell division. In another system, BUdR-substituted λ DNA had an increased affinity for highly-purified pea seedling histone proteins immobilised on nitro-cellulose filters (Lin et al, 1976). Gordon et al (1976) also found that when chromatin (from cultured hepatoma cells) containing BUdR in a single strand was chromatographed on hydroxylapatite (HAP), only the BUdR-containing strand was retarded, due to an increased affinity of BUdR-DNA for chromosomal proteins, which remained firmly adsorbed on the HAP. It was also shown that this effect was dependent primarily on histone fractions of chromosomal proteins. Since one function of histones is structural, controlling the relative condensation of DNA (Kornberg and Thomas, 1974), it was suggested that perturbations of histone-DNA interactions by BUdR incorporation changes the state of condensation of the chromosome and therefore the expression of genes.

Further evidence for this explanation came from work on the effect of BUdR on messenger RNA synthesis. Stellwagen and Tomkins (1971) showed that BUdR decreased the rate of tyrosine aminotransferase synthesis in cultured hepatoma cells by decreasing the rate of synthesis of mRNA for this enzyme, and suggested

that BUdR affects the promoter region of DNA, thus lowering the rate of initiation of transcription of the structural gene by RNA polymerase. In addition, Priesler et al (1973) found that the stimulation of Friend leukaemia cells to differentiate by dimethylsulphoxide (DMSO) was inhibited by BUdR. Molecular hybridisation with total cell RNA and $[^3H]$-DNA complementary to mouse globin mRNA showed that cells treated with BUdR and DMSO had 70% less globin mRNA than cells treated with DMSO alone.

Furthermore, Lykkesfeldt and Andersen (1977) established a general correlation between the degree of BUdR substitution in Tetrahymena pyriformis DNA and a reduction in the rate of total RNA synthesised. However, independent of the degree of BUdR substitution, the synthesis of 25 S and 17 S ribosomal RNA (rRNA) was preferentially inhibited. It was concluded that the various genes respond differently to BUdR substitution with regard to transcription. It is unlikely, however, that this explanation applies to those systems where BUdR induced an enhancement of the expression of differentiated functions. Enhancement of gene expression by BUdR was demonstrated by Koyama and Ono (1972), who found that both BUdR and IUdR increased alkaline phosphatase activity in a hybrid mouse - Chinese hamster cell line. The effect was reproduced in HeLa cells (Goz, 1974), and BUdR increased phosphodiesterase levels in glial tumour cells (Schwartz et al, 1973) and tyrosine hydroxylase levels in human neuroblastoma

cells (Prasad et al, 1973). Furthermore, Lin and Riggs (1976) showed that the catabolite gene activator protein in E. coli has a higher affinity for BUdR-substituted DNA than normal DNA, and suggested that the basic effect of the analogue might be an alteration of the binding of regulatory proteins to DNA. The same workers also showed that purified E. coli lac repressor binds more strongly to BUdR-substituted DNA than to normal DNA (Lin and Riggs, 1976). To account for the tighter binding of both activator and repressor proteins to BUdR-substituted DNA, these workers proposed that, since several bacterial systems (including the lac operon) are under both positive and negative control, and if both activator and repressor proteins function more effectively on BUdR-substituted DNA, the two effects may cancel one another out. This provides a possible explanation for the selectivity of BUdR action, since only those systems under exclusively positive or negative control, e.g. differentiating cells, would be significantly affected by the presence of the analogue.

Further evidence that BUdR alters the binding of regulatory proteins to DNA came from the results of David et al (1974), who found that chromatin containing BUdR-substituted DNA had an apparent increased thermal stability. They suggested that the effect of BUdR on differentiation of cultured rat embryo pancreatic and hepatoma cells was therefore due to an increased affinity of BUdR-containing DNA for regulatory proteins. Walther et al (1974)

also proposed a mechanism involving altered binding of regulatory proteins to BUdR-substituted DNA, with a consequent specific inhibition of transcription of certain genes, to account for the failure of deoxycytidine to reverse BUdR inhibition of exocrine pancreas differentiation.

In summary, therefore, there is much evidence favouring the hypothesis that an altered association of regulatory proteins to BUdR-substituted DNA may be the basis for the specific interference of the analogue with differentiation.

(4) Cell-surface alterations

It is also clear that BUdR can alter the surface properties of cells. Schubert and Jacob (1970) proposed that BUdR induced differentiation of neuroblastoma cells to cells resembling mature neurons by increasing the affinity between the cell and the substrated surface. Evans et al (1977) also showed variations in cell adhesion to the substrate using mouse melanoma cells grown in the presence or absence of BUdR. They also noted changes in the aqueous polymer partitioning behaviour of the cells, suggesting a basic alteration of the cell surface to account for the observed phenotypic changes.

In addition, BUdR inhibition of pigmentation in mouse melanoma cells in vitro was accompanied by flattening of the

cells into monolayers (Silagi and Bruce, 1970; Silagi et al, 1977). Cloned chondrocytes treated with BUdR also flattened, and had an increased surface area as they developed greater adhesiveness to culture dishes (Abbot and Holtzer, 1968). A similar effect, perhaps due to increased contact inhibition in cultured cells, was reported for chicken embryo skeletal muscle cells, sternal cartilage cells and pigmented retinal cells grown in the presence of BUdR (Coleman et al, 1970).

Furthermore, fluorescence polarization examination of BUdR-dependent Syrian hamster melanoma cells showed that cells grown in the presence of BUdR had an altered membrane lipid composition, compared to cells grown in the absence of the analogue (Rosenthal et al, 1978), although unlike BUdR-sensitive cells they tended to flatten in the absence of BUdR (Davidson and Bick, 1973).

Mayne et al (1971) demonstrated a correlation between BUdR incorporation into DNA of amnion cells and inhibition of mucopolysaccharide synthesis, and it is possible that cell surface alterations are a general consequence of BUdR incorporation into DNA. However, deoxycytidine reversal of BUdR inhibition of differentiation is not always accompanied by alterations in the BUdR content (Davidson and Kaufman, 1977) or its distribution in DNA (Reff and Davidson, 1979). Therefore, at

least in some systems a more direct effect of BUdR on membrane composition cannot be ruled out, such as an alteration in the nature or supply of sugar nucleotides involved in membrane synthesis. Since membrane alterations may be involved in the control of cellular differentiation (Lyman et al, 1976) it follows that BUdR-induced cell surface alterations might result in inhibition of the process.

However, Kay et al (1978) rejected the idea of a specific target for BUdR action, and suggested that there are many targets for BUdR, both during growth and development. They found that concentrations of BUdR that effectively inhibited sporulation of Dictyostelium discoideum also slightly inhibited growth. Apparently BUdR caused progressive inhibition of specific developmental functions, appearing at defined stages during the process, such as cell-associated cyclic AMP phosphodiesterase, UDP-glucose pyrophosphorylase and spore formation, giving values of 25%, 12% and 1.5% respectively of the maximum control values. These workers therefore preferred an explanation which assumed that instead of a single target for BUdR, there are many targets for BUdR during development, and at concentrations strongly inhibiting spore formation some of these earlier targets would be only partially inhibited. They suggested, therefore, that inhibition of differentiation by BUdR is a consequence of the nature of the developmental process, i.e. as a dependent sequence of events, rather than some unique specificity of the inhibitor.

In summary, therefore, in the majority of systems studied, when incorporated into the DNA of eukaryotic cells, BUdR selectively inhibited processes associated with development, without significantly altering cell growth or gross RNA and protein synthesis. Usually BUdR is only effective at a specific stage of the cell cycle, when DNA replication is in progress. There is much evidence suggesting an effect on specific transcription, perhaps by altered binding of regulatory proteins. However, BUdR can also alter the surface properties of cells. Whether cell-surface alterations are a direct effect of BUdR or a consequence of its incorporation into DNA is not clear.

Thus, it has been difficult to propose a general hypothesis to account for the range of BUdR effects, although most of the available evidence suggests a DNA-mediated mechanism.

C. The effect of BUdR on sporulation in B. subtilis

Preliminary experiments (Coote, 1977) indicated that incorporation of BUdR into the DNA of a TdR-requiring, BUdR-tolerant strain of B. subtilis, at a level not affecting growth, effectively prevented spore formation. In the presence of BUdR the level of mature spores was about 1% of that given in control cultures containing TdR alone, and examination of the bacteria by electron microscopy indicated that BUdR prevented most of the cells reaching the stage of spore septum formation (stage II). Most of the cells

were normal in length and had nuclear material in the form of an axial filament. Although the precise nature of the sporulation-associated events affected by BUdR was unknown, the presence of BUdR had the effect of increasing the activities of both exprotease and alkaline phosphatase over normal control values. The increase in the activity of the latter is normally associated with stage II (see Fig. 1), but in this case the increase occured without the accompanying morphological changes. The over-production of enzyme activity has also been noted in certain spo mutants (Piggot and Coote, 1976) and also during BUdR incorporation in eukaryotes (Lin et al, 1976; Lin and Riggs, 1976).

It is unlikely that BUdR incorporation had a general repressive effect on transcription, since this strain grew normally in a minimal resuspension medium supplemented with glucose, plus TdR and BUdR, but did not sporulate, whereas more than 50 % of the cells sporulated in a culture grown in the same medium with TdR alone. Also, when L-histidine was included in the normal resuspension medium, the rate of synthesis of the inducible enzyme histidase was the same whether the cells had incorporated BUdR or not.

The inhibitory effect of BUdR on sporulation only occured within an initial 2h after initiation, when DNA replication was in progress, and the effect was reversible on removal of the analogue. Sensitivity to BUdR during a restricted period

of development and reversibility of its effects by further DNA replication in its absence have been observed in many eukaryotic systems (see earlier). Therefore, the effect of BUdR on sporulation in B. subtilis seemed comparable to the specific inhibition of eukaryotic differentiation . Whether bacterial sporulation can be regarded as a model for cell differentiation depends on the similarity or otherwise of the control processes influencing cell differentiation in both eukaryotes and prokaryotes. Further analysis of the effect of BUdR on growth and sporulation in B. subtilis might go some way to seeking similarities between differentiation in both cell types.

OBJECTS OF RESEARCH

Endospore formation in B. subtilis can be regarded as a primitive form of cellular differentiation, and one of the outstanding problems in biology is an understanding of the regulatory mechanisms underlying development. In many eukaryotic systems the TdR analogue, BUdR, has dramatic effects on cells during differentiation, but not during growth, or after differentiation. Therefore, it was hoped to use BUdR as a tool in studying the basic regulatory mechanisms underlying development in B. subtilis. With this in mind, the aims of the work were as follows:

(1) To elucidate the manner in which BUdR interferes with sporulation in B. subtilis.

(2) To isolate a series of mutants of B. subtilis (thyA, thyB, trpC) which would tolerate BUdR concentrations that are normally inhibitory to growth of the parent strain.

(3) To assess the basis by which cells become tolerant to BUdR. It was also hoped to determine if more than one type of mutation gives rise to this phenotype.

(4) To analyse chromosome replication during sporulation of a BUdR-tolerant strain, using BUdR as a density-label.

MATERIALS AND METHODS

I. Strains of *B. subtilis*

The strains used are presented in Table 2. *B. subtilis* Marburg 168 (*trpC2*) is referred to as the wild-type strain, and requires indole or tryptophan for growth. A TdR-requiring derivative of the wild-type, *B. subtilis* (*thyA*, *thyB*, *trpC2*), referred to as Thy⁻A, or the parent strain, was made available by Dr. J. G. Coote. A BUdR-tolerant derivative of this strain, *B. subtilis* (*thyA*, *thyB*, *trpC2*, *but-32*), designated But(32), which grew normally in a rich casein hydrolysate medium supplemented with TdR (1 $\mu g ml^{-1}$) and BUdR (7.5 $\mu g ml^{-1}$), in which growth of the parent strain was inhibited (Coote, 1977), was also made available.

Two mutants of *B. subtilis* tolerant to bromouracil (BU) were provided by N. Sueoka (But 23.1) and H. de Lencastre (VUB112). The genotypes of these strains are given in Table 2. A derivative of the parent strain which is temperature-sensitive for initiation of DNA replication, designated *Ts134*, was also provided by Dr. J. G. Coote. Two BUdR-tolerant derivatives of this strain, designated *Ts134(but-1)* and *Ts134(but-11)* were isolated during the course of this work. Three other BUdR-tolerant derivatives of the parent strain, designated But(18), But(21) and But(24), were isolated. Isogenic derivatives of the parent strain containing BU or BUdR-tolerance mutations from But(32), But(18), But(21), But(24), But 23.1 and VUB112, were also constructed during the course of this work.

TABLE 2. Genotypes and description of strains of B. subtilis used.

Strain	Genotype	Description
Marburg 168	trpC2	Referred to as wild-type
Thy⁻A	thyA, thyB, trpC2	Referred to as the parent strain. Mutations in 2 unlinked loci resulting in a requirement for thymine or TdR. (Wilson et al., 1966)
Ts134	thyA, thyB, trpC2, dnaB(Ts)134	Derivative of the parent strain temperature - sensitive for initiation of DNA replication (Mendelson and Gross, 1967)
Mu8u5u16	purA16, leu-8, metB5	Derivative of the wild-type with requirements for adenine, leucine and methionine.
BD112	cysA14	Derivative of the wild-type with a requirement for cysteine, but not requiring tryptophan.
But(32)	thyA thyB, trpC2, but-32	Derivative of the parent strain; BUdR-tolerant.
But(18)	thyA, thyB, trpC2, but-18	Derivative of the parent strain, BUdR-tolerant.
But (21)	thyA, thyB, trpC2, but-21	Derivative of the parent strain; BUdR-tolerant.

TABLE 2. (contd.)

Strain	Genotype	Description
But(24)	*thyA*, *thyB*, *trpC2*, *but-24*	Derivative of the parent strain; BUdR-tolerant.
But(23.1)	*thy*$^-$, *his*$^-$, *but-23.1*	BU-tolerant strain with requirements for thymine and histidine. (from N. Sueoka)
VUB112	*thyA*, *thyB*, *trpC2*, *but-112*	BU-tolerant strain derived from the parent strain. (from H. deLencastre)
Thy$^-$A(*trp*$^+$)	*thyA*, *thyB*	Derivative of the parent strain, with no requirement for tryptophan.
but-32	*thyA*, *thyB*, *but-32*	Isogenic derivative of Thy$^-$A (*trp*$^+$), with mutation *but-32* from BUt(32).
but-18	*thyA*, *thyB*, *but-18*	Isogenic derivative of Thy$^-$A (*trp*$^+$) with mutation *but-18* from BUt(18)
but-21	*thyA*, *thyB*, *but-21*	Isogenic derivative of Thy$^-$A (*trp*$^+$), with mutation *but-21* from BUt(21)
but-24	*thyA*, *thyB*, *but-24*	Isogenic derivative of Thy$^-$A (*trp*$^+$), with mutation *but-24* from BUt(24)

TABLE 2. (contd.)

Strain	Genotype	Description
but-23.1	thyA, thyB, but-23.1	Isogenic derivative of Thy^-A (trp^+), with mutation but-23.1 from BUt(23.1)
but-112	thyA, thyB, but-112	Isogenic derivative of Thy^-A (trp^+), with mutation but-112 from VUB112.
Ts134(but-1)	thyA, thyB, trpC2, but-1, dnaB(Ts)134	BUdR- tolerant derivative of Ts134.
Ts134(but-11)	thyA, thyB, trpC2, but-11, dnaB(Ts)134	BUdR - tolerant derivative of Ts134.

A recipient strain for transformation, designated Mu8u5u16, was obtained from N. Sueoka. Strain BD112 was obtained from Dr. J. G. Coote.

II. Maintenance and Storage of B. subtilis

All strains used in this work were maintained on nutrient agar (Oxoid) at 4°C. All formed spores on this medium and remained viable for long periods. They were sub-cultured onto fresh nutrient agar at approximately 3 month intervals.

III. Growth of B. subtilis

A. Growth in liquid medium

(1) Inoculation procedure for growth in casein hydrolysate (CH) medium

In general a sample of an overnight culture of B. subtilis was prepared and used to inoculate fresh medium the following day. One loopful of stock culture was streaked on nutrient agar and grown for 7h at 35°C. At this point 2 loopfuls of cells were transferred to and suspended in 1.0 ml of sterile Penassay broth (PAB, Difco Antibiotic Medium No.3). One drop of this suspension was transferred to 10 ml PAB using a sterile Pasteur pipette, mixed, and one drop of this suspension added to another 10 ml PAB. After mixing, 2 drops of this suspension were used to inoculate a 100 ml sterile conical flask containing 20 ml CH

medium (see Appendix). Thymidine (TdR) was included at 8.5 $\mu g ml^{-1}$ for growth of TdR-requiring strains. A control flask minus TdR was also inoculated to check for contamination or reversion of one of the thy mutations. Cultures were incubated overnight (approximately 15h) at 35°C in a Gallenkamp orbital shaker, set at 125 rev. min^{-1}.

After overnight incubation a sample of the culture was filtered through a sterile cellulose acetate membrane filter (Oxoid, pore size 0.45 μm), the cells washed with sterile CH medium, and resuspended in fresh CH medium plus auxotrophic requirements. This step was included to ensure that no residual TdR in the overnight culture was transferred to the fresh media. The volume of the inoculum was 2% of the volume of CH medium to be used in any particular experiment. The volumes of growth media used during the course of this work ranged from 10 ml in 100 ml conical flasks to 300 ml in 2 litre dimpled flasks.

(2) Inoculation procedure for growth in minimal (MM) growth medium

The procedure was identical to that used for CH medium, except that the cells were washed with sterile MM (see Appendix) after filtration, and resuspended in fresh MM with necessary additions.

(3) Estimation of the rate of growth in liquid medium

Growth was monitored by measurement of the extinction at 600 nm (E_{600nm}) of aseptically-removed portions of a culture, using a Pye-Unicam SP500 Spectrophotometer and 1.0 cm silica micro-cuvettes, read against a distilled water blank. The mean generation time (doubling time) was calculated by reading from a plot of E_{600nm} (on a logarithmic scale) against time (on a linear scale) the time taken for the E_{600nm} to double during a period of exponential growth.

B. Growth on solid media

The phenotypes of all strains were confirmed by growth on selective minimal solid media (see Appendix). From the stock plate a fresh nutrient agar plate was inoculated, and incubated for approximately 7h at 35°C, or overnight at 30°C. A loopful of bacteria was then used to inoculate minimal plates containing various combinations of auxotrophic requirements. In general the plates were observed after 48h incubation at 35°C.

IV Sporulation in B. subtilis

A. Conditions for obtaining sporulation

(1) Replacement system

This procedure has been described previously (Sterlini and Mandelstam, 1969), and was used in all but one series of

experiments. Cells were grown with shaking at 35°C in CH medium plus auxotrophic requirements. When exponentially-growing cells had reached an E_{600nm} of 0.6 to 0.8 the culture was centrifuged in a MSE "Super Minor" centrifuge (MSE Scientific Instruments Ltd., Manor Royal, Crawley, West Sussex) at 2000xg for 15 min, and the pellets taken up in resuspension medium (SM, see Appendix) plus auxotrophic requirements, at the same density. The time at which resuspension occurred was taken as the time of initiation of sporulation, i.e. t_{zero}. Incubation was continued at 35°C and the extent of sporulation determined 20h after t_{zero}, i.e. at t_{20} (see below).

(2) Single medium exhaustion system

This procedure was used in one series of experiments where both growth rate and sporulation capacity in a single medium were measured. The cells were grown with shaking at 35°C in MM (see Appendix) with auxotrophic requirements until at least 20h after the first deviation from logarithmic growth, which is designated as t_{zero} in this system (Schaeffer, 1969), and the extent of sporulation was then determined (see below).

B. Determination of sporulation

Resistance to heat and organic chemicals is a characteristic property of mature spores, appearing during the final stages of sporulation. Resistance to heat above 80°C, which

kills vegetative cells, was used as a convenient assay for the detection of mature spores in a culture. In addition, spores become phase-bright when viewed under phase-contrast due to their low water content, and this can be used to estimate the degree of sporulation in a culture.

A portion (0.1 ml) of culture at t_{20} was transferred to 0.9 ml of basal salts medium (see Appendix) and heated at 80-85°C for 15 min in a water-bath (Grant Instruments Ltd., Barrington, Cambridge CB2 5Q2, England). Serial dilutions of the heated samples were made with sterile distilled water, and 0.05 ml aliquots of the appropriate dilutions spread on nutrient agar. After overnight incubation at 35°C the number of colonies appearing on the agar represented the number of heat-resistant cells at a particular dilution of the original culture.

Determination of the proportion of cells showing phase-bright spores in a sample of the original culture by phase microscopy (Vickers Instruments, Haxby Rd., York, England) was also used as an indication of the level of sporulation.

C. Total viable count during sporulation

This was determined in a similar manner to the heat-resistance assay, except that the heating step was omitted, and cultures were sampled during sporulation instead of at t_{20}.

V. Mutagenesis procedure

The procedure was essentially that adopted by Coote (1972a). The parent strain was grown at 35°C to an E_{600nm} of 0.6 in 2 parallel cultures of 20 ml CH plus TdR (8.5 $\mu g ml^{-1}$). One culture was divided into two 10 ml portions, and each transferred to a 100 ml conical flask. 0.1 ml of the mutagen ethyl methanesulphonate (EMS,Sigma) was added to each, and incubation continued at 35°C. The other culture was centrifuged at 2000 X g for 15 min in a MSE "Super Minor" centrifuge and the cells resuspended in 20 ml SM. This culture was divided into two portions, and 0.1 ml EMS added to each. Incubation was continued at 35°C.

Samples (0.1 ml) were withdrawn from both sets of cultures at 10 and 30 min after EMS addition, and transferred to separate 100 ml flasks containing 20 ml CH plus TdR (8.5 $\mu g ml^{-1}$). Incubation was continued for 6h at 35°C for post-mutational expression. After this time a 2 ml sample of each culture was centrifuged as above and each pellet taken up in 1 ml basal salts. Duplicate 0.1 ml samples were plated on selective media.

VI. Transformation in B. subtilis

A. Preparation of DNA for transformation

1. Extraction of DNA from cells in growth or resuspension medium

In general the method can be outlined as follows:
The cell were lysed with lysozyme at high concentration, treated

with ribonuclease and protease to remove RNA and proteins, and further deproteinized by phenol treatment, followed by precipitation of DNA with ethanol.

To a 10 ml sample of cells 0.1 ml of 0.1 M sodium azide was added, and the cells pelleted by centrifugation at 2000 X g for 15 min in a MSE "Super Minor" centrifuge. The tubes were well-drained, and the pellets taken up in 1.0 ml 0.15 M NaCl, 0.1 M EDTA (pH 8.0). At this point the cells were sometimes fast-frozen in dry ice/acetone, and stored at $-20^{o}C$, or were used immediately. EDTA, a chelating agent, was present to minimise DNA degradation by deoxyribonuclease.

Lysozyme (egg white muramidase, Sigma; 0.5% (w/v) in SSC, see Appendix) was added to 500 $\mu g ml^{-1}$ final concentration, and the cells incubated at $37^{o}C$ in a water-bath for 30 min. Bovine pancreas ribonuclease-A (RNase, Sigma; 0.1% (w/v) in 0.15M NaCl (pH 8)) was added to a final concentration of 50 $\mu g ml^{-1}$ (RNase was preincubated by heating to $80^{o}C$ for 10 min to denature contaminating DNase). Incubation was continued for a further 30 min, when protease (Streptomyces griseus, type V, Sigma; 0.1% (w/v) in 0.15 M NaCl, 0.1 M EDTA (pH 8) was added). After a further 30 min incubation at $37^{o}C$, the viscous lysate was mixed with an equal volume of buffer-saturated phenol (see Appendix), and centrifuged at 2000 X g for 10 min in a MSE "Super Minor" centrifuge.

The upper aqueous layer was removed with a Pasteur pipette and retreated with buffer-saturated phenol. After centrifugation the upper layer was taken, mixed with an equal volume of buffer-saturated ether and the mixture re-centrifuged as before. A second treatment with buffer-saturated ether was performed to ensure complete removal of phenol, which inhibits transformation.

After ether treatment, sodium acetate (3 M) was added to 0.3 M final concentration and 2 to 3 volumes of ice-cold 95% ethanol carefully poured into the test-tube. The resulting DNA precipitate was collected either by spooling on a sterile Pasteur pipette blocked off at the tip, or by centrifugation. The ethanol was drained, and the DNA dissolved in a small volume of standard saline citrate (SSC), usually 0.5 ml, and stored as 0.1 ml aliquots at -20^{o}C. The concentration of DNA was estimated by noting the exinction at 260 nm in a Pye-Unican spectrophotometer. An E_{260nm} unit of 1.0 was taken as equivalent to a DNA concentration of 50 $\mu g ml^{-1}$ (Mandel and Marmur, 1968).

2. Extraction of DNA from spores of B. subtilis

a) Preparation of spores

The method was very similar to that described by Sargent (1980a). Since a resuspension medium at t_{20} contains a mixture of mature spores and non-sporulating cells, the latter had to be removed completely before DNA could

be extracted specifically from spores.

Cultures containing mature spores were centrifuged at 10,000 X g for 10 min, the supernates discarded, and the pellets taken up in 0.01 M KH_2PO_4, 0.01 M $MgCl_2.6H_2O$ (pH 7.8) buffer containing lysozyme (500 $\mu g ml^{-1}$), using 1/10th of the original culture volume. The spores were incubated at 37°C for 1h in a water-bath, and solid sodium dodecyl sulphate (SDS) added to 1% (w/v) final concentration. The lysate was vigorously sheared using a Vortex mixer, and the purity of the spore suspension monitored by phase microscopy. If contaminating vegetative cells were still present the spores were harvested by centrifugation (see above) and further purified by centrifugation through a 2 ml layer of 45% Urografin (in 0.1% SDS), at 20,000 X g for 20 min in a 3 X 6.5 ml MSE titanium swing-out rotor. This procedure pelleted only spores under these conditions, and recovered at least 50% of the spores originally present.

Spores were washed with a volume of sterile distilled water corresponding to 1/10th of the original culture volume, and pelleted by centrifugation at 2000 X g for 15 min. Washing was repeated at least a further twice, and the spores heated at 80-85°C for 15 min to kill any remaining vegetative cells. After centrifugation the pellets were taken up in

1/3rd volume of sterile distilled water. The concentration of heat-resistant cells was determined by plating out a diluted sample of the concentrated spore suspension, which was then distributed into sterile bijou bottles and stored at -20°C before extraction of DNA.

b) Preparation of spore DNA

The conditions used to disrupt the spore coat extract sufficient coat protein so that detergents release DNA after lysozyme treatment.

After thawing the suspension, the spores were pelleted by centrifugation at top speed for 5 min in a Microfuge (Beckman - RIIC Ltd., Turnpike Rd., Cressex Industrial Estate, High Wycombe, HP12 3NR, Bucks., England), using 1.8 ml sealed tubes. The spores were resuspended at high concentration (approximately 10^9 spores ml^{-1}) in 1 ml of spore solution A, and incubated at 30°C overnight in a sealed tube (see Appendix for description of solutions). After this time the spores were washed with 1.5 ml spore solution B, sedimented using the Microfuge, washed in 1.5 ml spore solution C, re-centrifuged and suspended in 1.0 ml spore solution C containing 0.5 M sucrose and lysozyme at 1 $\mu g ml^{-1}$. After 1 h incubation with lysozyme at 50°C, the spores were harvested, washed in 1 ml spore

solution C, and frozen and thawed 5 times using a dry ice/ acetone mixture. At this stage the spores appeared swollen and phase-light. Solid Sodium lauryl sarcosinate (1%, w/v, final concentration) and protease (S. griseus, type V; 0.2% w/v, in spore solution C, 200 $\mu g ml^{-1}$ final concentration) were added, and incubation continued for 1h at $50^{o}C$. The resulting lysate was phenol - and ether - extracted by the method used for vegetative cell DNA, and the DNA dissolved in a minimum volume of 0.1 X SSC, and stored at $-20^{o}C$.

B. Procedures for transformation in B. subtilis

Two methods were used to prepare cells "competent" for transformation. "Competence" refers to the ability of a bacterial cell to irreversibly bind DNA in such a way that it becomes resistant to deoxyribonuclease (Bott and Wilson, 1968) and is the first major step in the transformation process. Later steps involve integration and recombination with the recipient genome, followed by replication and expression of the new genetic information. The ability of the Marburg strain of B. subtilis to be transformed was first shown by Spizizen (1958).

(1) Method 1

In this method (based on that of Ayad and Barker, 1969) competent cells were obtained after a "step-down" to a growth-

limiting medium after a few hours of normal growth. Competent recepient cells were then incubated with cells of a donor strain grown to a stage when they excrete DNA (Ephrati-Elizur, 1968).

The strain to be used as a recipient for transformation was inoculated from a stock culture onto nutrient agar, and incubated overnight at 30°C. In addition, one loop of the donor strain was inoculated from a stock culture into a 100 ml flask containing 20 ml PAB plus auxotrophic requirements at 35°C and shaken overnight.

One loop of recipient cells from an overnight culture was inoculated into 20 ml PTM (see Appendix), supplemented with auxotrophic requirements. The culture was aerated vigorously at 37°C for 4h, or less if the E_{600nm} was 1.2 before this time. 4 ml of this culture was transferred to a flask containing 16 ml pre-warmed TM (see Appendix) plus the appropriate requirements, but omitting the one being selected against (e.g. L-tryptophan was omitted from TM when transformation of the trpC marker was required).

The cell suspension was incubated with slow aeration for 45 min, at which point the cells should have reached maximum competence. Two 5 ml aliquots were removed from this culture

into separate 100 ml flasks, one of which contained 0.5 ml of the overnight culture of the donor strain. The 2 flasks were then shaken at 37°C for 1h, and the cells harvested by centrifugation at 2000 X g for 15 min, followed by resuspension in 1.0 ml SMM (see Appendix). The cells in 1 ml of the donor culture were also harvested by centrifugation, and resuspended in 1.0 ml SMM.

0.1 ml samples of cells in SMM were plated on minimal medium selecting against growth of both donor and untransformed recipient cells. Transformants were scored after 2 days incubation at 35°C.

(2) Method 2

A method of obtaining competent cells of B. subtilis in a semi-defined medium without any step-down conditions or culture manipulation was reported by Bott and Wilson (1967). In this medium the peak of competence occured 3h after the end of logarithmic growth. The same workers later reported an increased efficiency of transformation after formulating a defined medium (Wilson and Bott, 1968), supplemented with additional nitrogen in the form of ammonium ions, and using a modified buffer to control the pH (Bott and Wilson, 1968).

For the majority of transformations performed in this work, using strain Mu8u5u16 as a recipient, the original method of Bott and Wilson (1967) was followed.

The recipient strain was grown overnight as in method 1, and the cells inoculated in the medium at an E_{600nm} reading of approximately 0.1. The cells were grown at 35°C with shaking until about 3h after the end of logarithmic growth (generally 6h after inoculation), and 0.2 ml samples removed into sterile test-tubes. Donor DNA was added at a saturating concentration (at least 1 $\mu g ml^{-1}$) and the mixture agitated at 35°C in an orbital shaker for 30 min. One sample was incubated without DNA, as a control.

After the 30 min incubation 0.8 ml SMM was added to each, and the cells centrifuged at 2000 X g for 15 min. The pellets were taken up in 1.0 ml SMM, recentrifuged and taken up in 2 ml SMM. 0.05 ml aliquots were spread onto selective media (see Appendix), and the plates incubated for 48h at 35°C, when the number of transformants (normally at least 200) for each marker selected was scored. The sterility of the DNA preparations was monitored by streaking a loopful onto nutrient agar and incubating overnight at 37°C.

VII. Caesium chloride (CsCl) equilibrium density gradient analysis

A. Centrifugation method

Equilibrium density gradient centrifugation, introduced by Meselson et al (1957), is a versatile tool for the separation and purification of a variety of macromolecules, and this technique was used to estimate the degree of incorporation of BUdR

in place of TdR in B. subtilis DNA preparations.

Separation of DNA samples is based on their buoyant densities in a concentrated CsCl solution, the gradient being self-generated in a centrifugal field. BUdR has a higher molecular weight than TdR, and its presence in DNA in place of TdR increases the buoyant density in CsCl.

Centrifugation is continued until equilibrium is reached, i.e. until the DNA fractions reach their isodensity positions. At this point their buoyant densities correspond to the CsCl density at a defined level in the centrifuge tube. This technique belongs to the class of isopycnic centrifugation, in which the buoyant density of the solvent encompasses the density range of the solute.

The length of time it takes to form a gradient by centrifugation can be shortened (from about 48h to 16h) by partially preforming the gradient, using three different concentrations of CsCl to form three "steps" in the tube (Carpenter and Binkley, 1968). The preformed gradient used in this work consisted of three layers, each of 2 ml volume, of densities 1.65, 1.72 and 1.79 gml^{-1} of CsCl (BDH Analar Grade, 99.9-100%). The concentrations of CsCl required to make up these solutions were obtained from a calibration curve of CsCl density (gml^{-1}) against CsCl

concentration (gl^{-1} of solution) at 20°C (Fig. 6). In general 10 ml solutions were made, and the densities were checked by measurement of the refractive index at 20°C, using a refractometer (Bellingham and Stanley, Ltd.). The refractive indices were obtained from a calibration curve of density versus refractive index at 20°C (Fig. 7).

In addition, the time taken for the formation of a CsCl gradient can be reduced using the "short column" technique (Griffith, 1978). This consists of partially filling the tube so that a smaller total volume of CsCl is used, effectively subjecting the column to a higher average force field in a reduced path length. In this work 10 ml polyallomer centrifuge tubes (MSE) were partially filled with the three CsCl solutions (see above), with the DNA samples layered a top. The DNA was was radioactively-labelled with either [2-^{14}C] or [methyl-^{3}H]-thymidine (Amersham International), prepared as in part B (below). In general, 0.1 to 0.2 ml of each sample was applied to the column, and the tubes filled with liquid paraffin (BDH, specific gravity 0.865-0.890) to prevent tube collapse at high centrifugal force, and the caps tightly fitted.

Centrifugation was performed in a MSE "Superspeed 65" Ultracentrifuge at 40,000 rpm (110,000 X g) for 24h at 20°C, using a MSE 8 X 35 ml fixed-angle titanium rotor with 10 ml adaptors. A fixed-angle rotor is preferable to a swinging-bucket

Figure 6. Calibration curve of CsCl concentration (g litre^{-1}) as a function of density (g ml^{-1}). Drawn from data given by Dawson et al, 1969.

Fig. 6

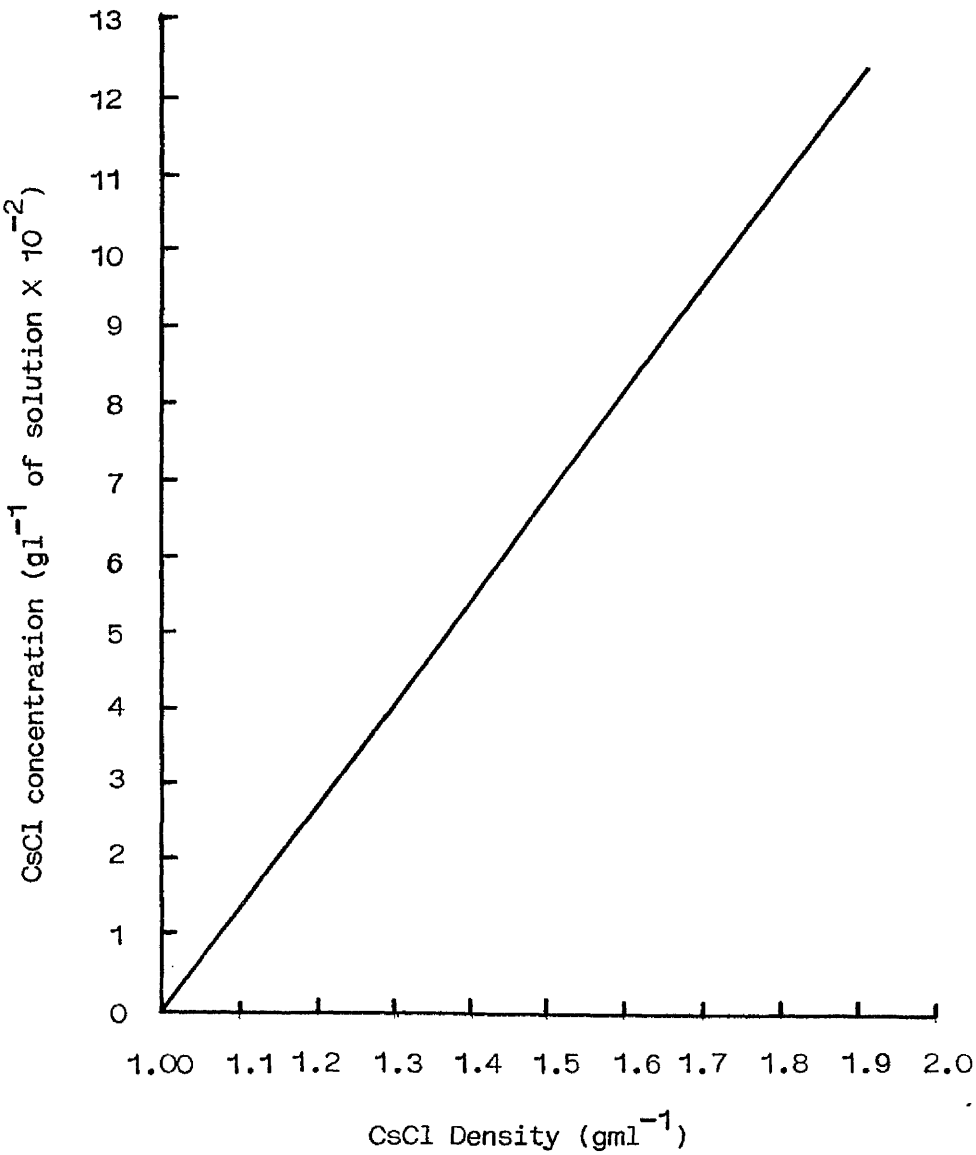
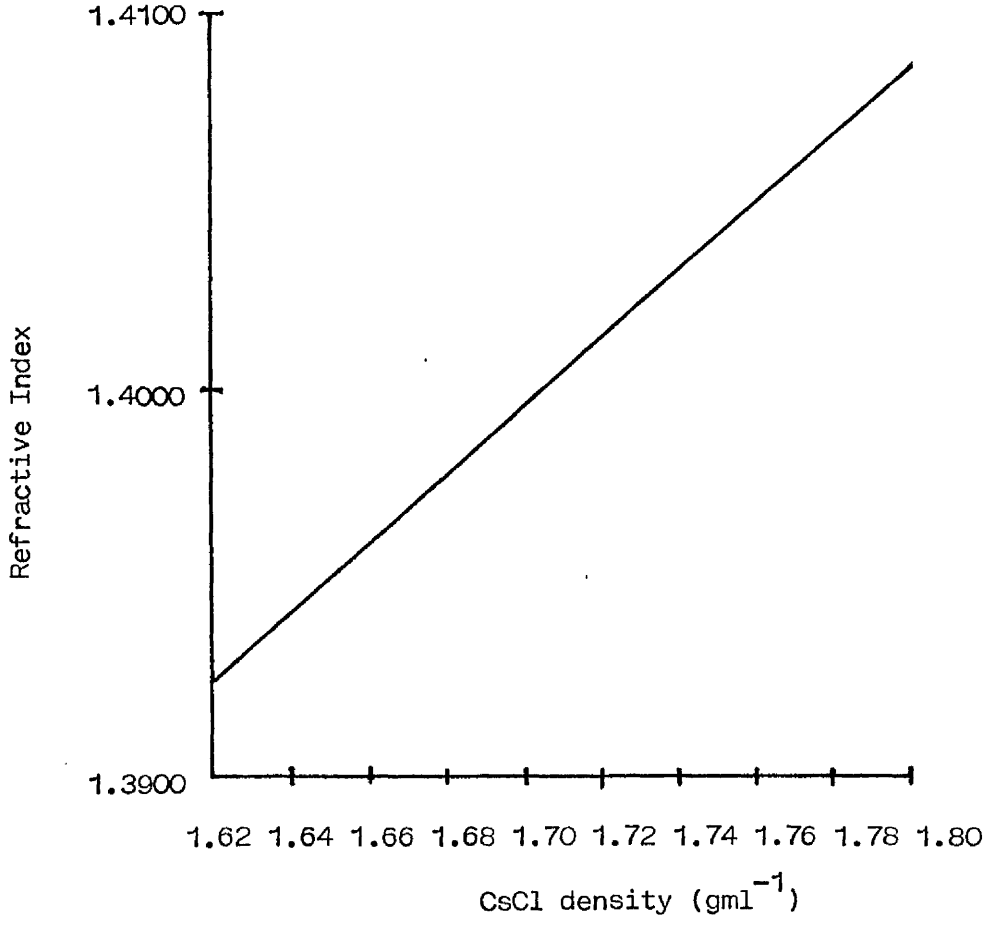

13
12
11
10
9
8
7
6
5
4
3
2
1
0
CsCl concentration (gl^{-1} of solution X 10^{-2})
1.00 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2.0
CsCl Density (gml^{-1})

Figure 7. Calibration curve of refractive index (relative to air for sodium yellow light) as a function of CsCl density (g ml^{-1}) at 20°C. Drawn from data presented by Weast, 1980.

Fig. 7


1.4100
1.4000
1.3900
Refractive Index
1.62 1.64 1.66 1.68 1.70 1.72 1.74 1.76 1.78 1.80
CsCl density (gml^{-1})

type for isopycnic centrifugation, as it provides greater resolution, due to the creation of a greater interband volume, the volume between samples of different buoyant density.

After centrifugation, the tubes were pierced using a MSE tube piercer and 0.1 ml fractions collected via a Hughes Hilo-flow pump (F. A. Hughes and Co., Ltd., Epsom, Surrey) in Packard scintillation vials (Packard Instruments Ltd., 13-17 Church Rd, Caversham, Berks.) containing smaller insert vials (Sterilin Ltd., Teddington, Middlesex). The linearity of the CsCl gradient was monitored by measurement of the refractive index at 20°C of every 10^{th} fraction. Samples were counted for radioactivity in a Packard Tri-Carb 300C scintillation counter (see Section IX). The buoyant density of a DNA peak was obtained from a calibration curve (Fig. 7) of refractive index versus CsCl density (gml^{-1}) at 20°C. The approximate percentages of total DNA present in light, intermediate or heavy forms were determined by cutting out and weighing the plotted peaks of radioactivity, assuming a normal distribution around the peak density point.

B. Preparation of DNA samples for centrifugation in a CsCl gradient

Radioactively-labelled DNA from cells obtained during growth or sporulation was prepared as in section VIA(1), but omitting phenol and ether treatments. Radioactively-labelled

spore DNA was prepared as in section VIA (2), but was stored at $4^{o}C$ over chloroform.

Reference DNA samples representing the two extremes of the buoyant density range into which the isodensity points of all test samples fell were prepared from strain but-32 as follows. DNA substituted with BUdR to the maximum level for this strain was prepared from cells grown overnight in 20 ml CH medium containing [methyl-^{3}H]TdR (0.5 $\mu Ciml^{-1}$, 47 $Cimmol^{-1}$) plus TdR and BUdR at 1 and 15 μgml^{-1}, respectively. The culture was inoculated as in section III, but four drops of the concentrated suspension of cells in 1 ml PAB were required for successful growth overnight in this medium. When the test sample was DNA prepared from growing cells, unsubstituted reference DNA was prepared from cells grown to mid-exponential phase in 20 ml CH medium containing [methyl-^{3}H]TdR (0.5 $\mu Ciml^{-1}$) plus TdR(8.5 μgml^{-1}). However, when the test sample was either spore DNA, or DNA prepared from cells in resuspension medium, the unsubstituted reference DNA was prepared from spores formed from cells grown and initiated to sporulate in 300 ml SM supplemented with TdR(8.5 μgml^{-1}), plus either [2-^{14}C]TdR (0.025 $\mu Ciml^{-1}$, 54m$Cimmol^{-1}$) or [methyl-^{3}H]TdR (0.5 $\mu Ciml^{-1}$).

VIII. Measurement of DNA, protein and RNA Synthesis

DNA synthesis was measured as the incorporation of either [methyl-^{3}H]TdR or [2-^{14}C]TdR into trichloroacetic acid (TCA)-

insoluble material. Protein synthesis and RNA synthesis were measured as incorporation of L- [2-^{3}H] methionine or [5-^{3}H] uridine respectively, into TCA-insoluble material.

A. DNA synthesis

Incorporation of radioactive TdR into cells during growth and sporulation was monitored. In general the label was added at the start of the sampling period, usually at the time of inoculation for growing cells, and at the time of resuspension for cells initiated to sporulate. [methyl-^{3}H] TdR was added to 0.5 μCiml^{-1} final concentration and [2-^{14}C] TdR to 0.025 μCiml^{-1}. Unlabelled TdR was always present in excess. Samples (0.1 ml) were precipitated in 1 ml ice-cold 10% (w/v) TCA containing TdR (100 μgml^{-1}). (In some experiments, 0.2 ml volumes were precipitated in 2 ml 10% (w/v) TCA + TdR). After mixing the contents, the tubes were left on ice for at least 20 min, remixed and filtered through GF/A glass-fibre filters (Whatman Lab Sales, Springfield Mill, Maidstone, Kent) pre-soaked in ice-cold 10% (w/v) TCA + TdR. A filtration manifold (Amicon Ltd., Amicon House, 2 Kingsway, Woking,Surrey, GU21 1UR) was used to simultaneously filter 12 samples. The filters were rinsed with 5-10 ml ice-cold 10% (w/v)TCA, washed with 95% ethanol, placed in scintillation vials and dried under an infra-red lamp. The filters were counted for radioactivity (see section IX).

The procedure for measurement of the uptake of radioactive TdR into whole cells was as follows. Samples (1.0 ml) were filtered through a cellulose acetate membrane filter (Oxoid, pore size 0.45 μm), and then rinsed with 5 to 10 ml of the medium in which cells had been suspended. The filters were dried and counted for radioactivity (see section IX).

For measurement of incorporation of radioactively-labelled BUdR into whole cells and TCA-insoluble material, procedures almost identical to the two above were adopted. The cells were grown or initiated to sporulate in media containing 5-bromo 2'-deoxy $[1',2'-^{3}H]$ uridine (1mCiml^{-1}, 30 Cimmol^{-1}, Amersham) at a final concentration of 0.5 μCiml^{-1}, with unlabelled BUdR in excess. Where required, samples were precipitated with 10% (w/v)TCA + BUdR (100 μgml^{-1}).

B. Protein synthesis

The procedure was identical to that used for the measurement of DNA synthesis, except that labelled L-methionine (4 Cimmol^{-1}) was added at 0.5 μCiml^{-1}, and unlabelled L-methionine at 20 μgml^{-1}. Samples were precipitated with 10% (w/v) TCA plus L-methionine (100 μgml^{-1})

C. RNA synthesis

The procedure was identical to that used for the measurement of DNA synthesis, except that $[5-^{3}H]$ uridine (27 Cimmol^{-1},

Amersham) was added at 0.5 $\mu Ciml^{-1}$, and unlabelled uridine (Sigma) at 25 μgml^{-1}. Samples were precipitated with 10% (w/v) TCA plus uridine (100 μgml^{-1}).

D. Preparation of inhibitors of DNA, RNA and protein synthesis

HPUra (6-(p-hydroxyphenylazo)uracil), an inhibitor of DNA synthesis in B. subtilis, was stored as a powder at $4^{o}C$, but a stock 5 $mgml^{-1}$ solution (in 0.05% (w/v) NaOH) was stored frozen at $-20^{o}C$.

Rifampicin (Sigma), an inhibitor of RNA synthesis, was stored as a powder at $-20^{o}C$, but a 0.2% (w/v) solution in methanol was made immediately before use. Chloramphenicol (Sigma) a protein synthesis inhibitor, was used in this work to inhibit initiation of DNA replication, and was stored as a powder at $4^{o}C$. A 1%(w/v) solution in ethanol was made immediately before use.

E. Preparation of BUdR

BUdR was stored as a powder at $-20^{o}C$, and a 0.2% (w/v) solution in distilled water sterilised by filtration made immediately before use.

IX. Scintillation counting

All samples were counted for 10 min in either a Nuclear Enterprises Scintillation Spectrometer (N.E.8320) or a Packard Tri-

Carb 300C scintillation counter.

Filtered samples were counted in 5 or 10 ml of either Packard TM299 scintillant, or a toluene -based scintillant. The composition of the toluene scintillant was; toluene, 1 litre; 2,5-diphenyloxazole, 4g; 1,4-bis (4-methyl-5-phenyl-oxazole-2-yl) benzene, 0.1g.

Aqueous samples containing CsCl were counted in 5 or 10 ml TM299 scintillation fluid in the Packard counter. Before counting, one-tenth the volume of distilled water was added to reduce the CsCl concentration and prevent the salt from coming out of solution.

When samples containing both $[^{3}H]$ and $[^{14}C]$ radioisotopes were measured for radioactivity, a correction had to be applied to the results to compensate for the contribution of $[^{14}C]$- derived emissions in the energy spectrum for $[^{3}H]$-derived emissions. The Packard counter can be programmed to count $[^{3}H]$- and $[^{14}C]$- derived emissions from a single sample, but a proportion of the counts in the $[^{3}H]$ energy range are derived from the $[^{14}C]$ source, as the total energy range of $[^{3}H]$ lies within the $[^{14}C]$ range. The actual count min^{-1} values due to the presence of a $[^{3}H]$ source were derived as follows. Duplicate standards were made up in scintillation vials.

These contained, per 5 ml scintillant,

0.025 μCi [2-^{14}C] TdR

0.1 ml CsCl solution (1.72 gml^{-1})

0.5 ml distilled water

5.0 ml Packard TM299 scintillant

The standards reflected the composition of a typical sample to be counted, except that no [^{3}H] source was present. They were counted for radioactivity, and the total of [^{14}C] counts (A + B) was obtained by adding the cpm values in both the [^{3}H] (A) and [^{14}C] (B) energy ranges. The ratio of the two values, A/B, the sample channels ratio (SCR), was calculated by the counter. The mean SCR of the standards was then used as a correction factor for the test samples.

The actual contribution of [^{3}H] and [^{14}C] sources were calculated as follows:-

1) [^{3}H] cpm = A - (mean SCR X B)

2) [^{14}C] cpm = B + (A - [^{3}H] cpm)

X. Chemicals

All radioactive material was supplied by Amersham International (formerly the Radiochemical Centre), Amersham, England. Casein hydrolysate, nutrient agar and purified agar were obtained

from Oxoid Ltd., Wade Rd., Basingstoke, Hants, England. Penassay broth was supplied by Difco Laboratories, Detroit, Michigan, USA. HPUra was a gift from Dr. B. A. Langley (ICI Ltd.), and Urografin was a gift from Dr. M. S. Sargent (National Institute for Medical Research, Mill Hill, London, England). Diethyl ether was obtained from Macfarlane-Smith Ltd., Wheatfield Rd., Edinburgh, Scotland.

All other chemicals were obtained from BDH Chemicals Ltd., Poole, England, or from Sigma London Chemical Co. Ltd.

RESULTS

I. The isolation of BUdR-tolerant strains

Although a BUdR-tolerant strain (But-(32)) was available it was decided to isolate and examine further strains for the following reasons. Firstly, to ensure that inhibition of sporulation but not growth by BUdR, was not merely a characteristic of strain But(32). Secondly, as strain But (32) occasionally reverted to BUdR-sensitivity (Coote, 1977), isolation of a more stable BUdR-tolerant (But) mutation might improve the reproducibility of the results. Thirdly, it was important to identify classes of But strains tolerant to different concentrations of BUdR, as these might represent different types of mutation giving rise to the But phenotype. In addition, two bromouracil (BU)-tolerant strains of B. subtilis were provided by other investigators (see Materials and Methods, section I), and these strains were used to assess whether or not tolerance to BUdR and BU are manifestations of the same mutation.

The selection procedure adopted to isolate further But strains was similar to that described for the isolation of BU-tolerant mutants of B. subtilis (Bishop and Sueoka, 1972). After treatment of the parent strain with the mutagen ethyl methanesulphonate (EMS) (Materials and Methods, section V), culture samples were plated on minimal agar plates including tryptophan (10 $\mu g ml^{-1}$), TdR (1 $\mu g ml^{-1}$) and BUdR (20 $\mu g ml^{-1}$). A small amount of the natural nucleoside had to be present, as even strain But(32) is unable to grow for more than a few generations

with BUdR alone. (see Introduction).

After incubation of the plates for 48h at 35°C, 100 colonies were picked at random from all selection plates, and reinoculated onto nutrient agar, followed by incubation at 35°C. All cells growing well after 24h incubation were again inoculated onto nutrient agar, grown overnight at 30°C, and streaked in turn on minimal agar supplemented with:-

a) tryptophan (10 $\mu g ml^{-1}$)

b) tryptophan (10 $\mu g ml^{-1}$) + TdR (10 $\mu g ml^{-1}$)

c) tryptophan (10 $\mu g ml^{-1}$) + TdR (1 $\mu g ml^{-1}$) + BUdR (20 $\mu g ml^{-1}$)

d) nutrient agar.

The plates were incubated at 35°C for 48h and *thy*$^+$ revertants discarded. Seven potential But derivatives, plus But(32) and the parent strain, were retested for growth on minimal agar supplemented with the additions in a), b) and c) above, plus two other TdR:BUdR concentration ratios, i.e. 1:10 $\mu g ml^{-1}$ and 1:30 $\mu g ml^{-1}$ respectively. After 48h incubation at 35°C, strains But(18), But(21) and But(24) were retained for future use as they had been independently isolated and appeared to grow as well as But(32) at the highest BUdR concentration, while still retaining the requirements for tryptophan and TdR.

Initial characterisation of BUdR-tolerant strains

Bishop and Sueoka (1972) isolated several classes of BU-tolerant mutants of *B. subtilis* (*thy*, *his*). In preliminary experiments, to test the possibility that there was also

more than one phenotype for BUdR-tolerance, a comparison was made of the growth rates, in minimal growth medium with various concentrations of BUdR, of strains But(32), But(18), But(21), But(24) and the parent strain.

In one experiment the range of tolerance of But(32) was compared to those of But(18) and But(24) (Table 3) and in another experiment its tolerance range was compared to those of But(21) and the parent strain(Table 4). In both experiments, relative to a control culture with TdR alone, the growth rate of But(32) was unaffected by BUdR at the two lowest concentrations, and only slightly affected at the highest concentration. Growth of the parent strain was inhibited at the lowest BUdR concentration (Table 4). Strain But(18) had a similar range of tolerance to that of But(32), but But(21) and But(24) were more sensitive to the analogue. The suggested order of tolerance obtained using these results, starting with the least-sensitive strain,is, But(32), But(18), But(21), But(24) and the parent strain. It appeared, therefore, that, as for BU-tolerance, classesof BUdR-tolerance might exist.

To test whether the most important factor determining the inhibitory effect of BUdR on growth was the absolute concentration of the analogue or the ratio of TdR to BUdR concentrations, the growth rates of two But strains, But(18) and But(24), differing in

TABLE 3. The growth rates of strains But(32), But(18) and But(24) in minimal medium (MM) (20ml) containing BUdR, at three different concentrations. (The symbol ∞ denotes cells which grew slightly, but did not reach an $E_{600\ nm}$ of 0.4, after which the $E_{600\ nm}$ decreased or remained at 0.4. These cells did not complete the normal exponential phase of growth.)

Strain	Doubling time (min)			
	TdR (8.5 $\mu g ml^{-1}$)	TdR (1 $\mu g ml^{-1}$) + BUdR (7.5 $\mu g ml^{-1}$)	TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$)	TdR (1 $\mu g ml^{-1}$) + BUdR (30 $\mu g ml^{-1}$)
But(32)	78	78	78	96
But(18)	84	84	84	96
But(24)	77	94	100	132 $\longrightarrow$ ∞

TABLE 4. The growth rates of strains But(32), But(21) and the parent strain in MM (20ml) containing BUdR at three different concentrations.

Strain	Doubling time (min)			
	TdR (8.5 μgml^{-1})	TdR (1 μgml^{-1}) + BUdR (7.5 μgml^{-1})	TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	TdR (1 μgml^{-1}) + BUdR (30 μgml^{-1})
But(32)	108	114	108	114
But(21)	77	99	114	$144 \longrightarrow \infty$
parent	78	195	198	$240 \longrightarrow \infty$

sensitivity to BUdR, were compared with growth of But(32) at the same ratio of TdR to BUdR used in the previous experiment (Table 4), but with increased absolute concentrations of both (Table 5). For strains But(18) and But(24) it appeared that the absolute concentration of BUdR was more important at the higher concentrations, as growth of these strains was inhibited at TdR and BUdR concentrations of 8.5 μgml^{-1} and 128 μgml^{-1}, respectively (1 : 15 ratio). However, at a ratio of 1 : 7.5 (TdR and BUdR concentrations of 8.5 μgml^{-1} and 64 μgml^{-1}, respectively) the growth rates of these strains were relatively unchanged with respect to control cells in TdR (8.5 μgml^{-1}) alone, suggesting that the ratio of TdR to BUdR concentrations is more important at lower BUdR concentrations. Strain But(32) was different in that the growth rate was relatively unaffected when the absolute concentrations were increased.

The parent strain can grow normally in the presence of low concentrations of BUdR (e.g. 3 μgml^{-1} BUdR + 1 μgml^{-1} TdR), but these concentrations had no dramatic effect on the levels of sporulation (Lauchlan, 1975). In fact, over a range of low concentrations of BUdR, with varying TdR to BUdR concentration ratios, there was no single concentration of BUdR which left growth of the parent strain unaffected and also inhibited sporulation to the extent observed with a BUdR-tolerant strain at higher BUdR concentrations (see Results section III). A TdR concentration of 8.5 μgml^{-1} was used for all control cultures

TABLE 5. The effect of maintaining the TdR : BUdR ratio, but altering the absolute concentrations of these compounds, on the growth rates in MM of strains But(32), But(18) and But(24). (see Table 4)

Strain	Doubling time (min)			
	TdR (8.5 $\mu g ml^{-1}$)	TdR (8.5 $\mu g ml^{-1}$) + BUdR (64 $\mu g ml^{-1}$) (ratio 1:7.5)	TdR (8.5 $\mu g ml^{-1}$) + BUdR (128 $\mu g ml^{-1}$) (ratio 1:15)	TdR (8.5 $\mu g ml^{-1}$) + BUdR (256 $\mu g ml^{-1}$) (ratio 1:30)
But(32)	54	66	72	78
But(18)	54	60	110	150 $\longrightarrow \infty$
But(24)	54	60	270	300 $\longrightarrow \infty$

since both the sensitive parent and the But strain could grow in medium with TdR at 1 μgml^{-1}, but when BUdR at 7.5 μgml^{-1} was also included (giving a total TdR + BUdR concentration of 8.5 μgml^{-1}), only growth of the parent strain was strongly inhibited (Table 8).

II. The basis of BUdR-tolerance

A. The production of isogenic BUdR-tolerant strains

Before a more thorough assessment was made of the degree of tolerance of the four BUdR-tolerant and two BU-tolerant strains available, the mutations were transferred into a thy A, thy B genetic background to render them isogenic. This procedure essentially ensured removal of any other undetected mutations which might have been present in the original isolates after mutagenesis, and which might have affected subsequent results. Any differences found between strains could then only be attributed to the But mutations.

A strain harbouring only the thy A, thy B genetic background was constructed by transformation of the parent thy A, thy B, trp C strain to trp^+. The new parent strain (thy A, thy B) was isolated after transformation of B. subtilis (thy A, thy B, trp C) using strain BD112, B. subtilis 168 (cysA14) as a DNA donor. Selection of trp^+ transformants was on minimal plates supplemented with only TdR (10 μgml^{-1}) . Out of 64 trp^+

colonies picked, only one had the desired phenotype, i.e. *trp*$^+$, *cys*$^+$, *thy*. The new strain, *B. subtilis* (*thy A*, *thy B*) was then used as a recipient in a transformation using But(32) as a DNA donor, and BUdR-tolerant derivatives selected on minimal growth medium containing TdR (1 $\mu g ml^{-1}$) plus BUdR (20 $\mu g ml^{-1}$). No tryptophan was present, in order to select against the donor strain. Transformation method 1, in which samples of donor cells excrete DNA during stationary phase, was used in both cases (see Materials and Methods, section VI B).

Direct transfer of the mutation *but-32* into the parent strain using this method was not possible as selection for BUdR-tolerant cells on plates supplemented with tryptophan, TdR and BUdR would have resulted in growth of strain But(32). Using this method both the donor and recipient cells must harbour mutations in two different loci so that selection can be made against growth of both strains. In this case selection against growth of the donor and untransformed recipient cells was possible by inclusion of only TdR and BUdR in minimal medium, since the new recipient strain, *B. subtilis* (*thy A*, *thy B*) was sensitive to BUdR (see Table 8), and But(32) has a requirement for tryptophan. Twenty-two potential TdR-requiring, But derivatives, plus the new parent strain and But(32), were inoculated on minimal medium supplemented with:-

a) Tryptophan (10 $\mu g ml^{-1}$)

b) TdR (10 $\mu g ml^{-1}$)

c) TdR (1 μgml^{-1}) + tryptophan (10 μgml^{-1}) + BUdR (7.5 μgml^{-1})

d) TdR (1 μgml^{-1}) + tryptophan (10 μgml^{-1}) + BUdR (15 μgml^{-1})

e) TdR (1 μgml^{-1}) + tryptophan (10 μgml^{-1}) + BUdR (30 μgml^{-1})

The first medium allowed growth of thy$^+$ revertants, and tryptophan was included in all media (except b) to allow growth of But(32). Two of the trp$^+$ transformants grew as well as But(32) at a TdR to BUdR ratio of 1 : 30 μgml^{-1}. One of these transformants, B. subtilis (thy A, thy B, but-32) was used for the remainder of the experiments, and will be referred to as but-32.

In a similar, but separate set of transformations, the mutations in strains But(18), But(21), and But(24), along with the BU-tolerance mutations from strains VUB112 and But-23.1 were transferred into the thy A, thy B genetic background. Transformants which grew as well as the new but-32 strain on minimal medium supplemented with TdR and BUdR at 1 and 20 μgml^{-1} respectively were retained for further analysis of the But phenotype.

To check that the isogenic derivative of But(32), but-32, had a similar degree of tolerance as strain But(32), a comparison was made of their growth rates and sporulation capacities in growth and resuspension media supplemented with TdR and BUdR at ratios of 1: 7.5 μgml^{-1}, 1: 15 μgml^{-1} and 1: 30 μgml^{-1} (Table 6). Tryptophan (20 μgml^{-1}) was included in all cases.

TABLE 6. Comparison of the growth rates and sporulation capacities of the isogenic strain (thy A, thy B, but-32) and the original strain (thy A, thy B, trp C, but-32).

Both strains were grown at 35°C in CH medium (20 ml), with the additions shown. During exponential phase the cells were transferred to SM (20 ml), with the additions shown (see Materials and Methods, section IV). The level of sporulation was determined at t_{20}. Figures in parentheses refer to the percentage sporulation in the presence of BUdR compared to control cultures with TdR alone.

Strain	Additions to medium							
	TdR (8.5 $\mu g ml^{-1}$)		TdR(1 $\mu g ml^{-1}$) + BUdR (7.5 $\mu g ml^{-1}$)		TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$)		TdR (1 $\mu g ml^{-1}$) + BUdR (30 $\mu g ml^{-1}$)	
	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$)	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$)	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$)	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$)
isogenic but-32 (thy)	39	56	48	3.6(10)	48	1.43(3)	78	1.03(2)
original But(32) (trp, thy)	39	21	48	2(6)	54	0.64(3)	78	0.5(2)

The growth rates of both strains were almost identical over the range of BUdR concentrations tested and in both strains the rate of growth was halved at the highest BUdR concentration (30 μgml^{-1}), compared with the control culture in TdR (8.5 μgml^{-1}) alone. In addition, the level of sporulation in both strains, relative to the control cultures, was inhibited to a similar extent at all BUdR concentrations tested.

B. Characterisation of isogenic BUdR-tolerant strains

To confirm that these strains also exhibited the same degree of tolerance to BUdR as their non-isogenic parent strains (see section I), and to determine whether sporulation capacities in the presence of BUdR could be categorised in the same way, a comparison was made of the growth rates and levels of sporulation between isogenic strains but-32, but-18, but-21, but-24, but-112 and but-23.1 in a single medium exhaustion system. (see Materials and Methods, section IV). The results of four separate experiments are given in Table 7. Experiments 1 and 2 were performed with all six strains at two different concentrations of BUdR, plus one control culture with TdR alone, whereas experiment 3 was carried out using strains but-18, but-21, but-24 and but-32. Experiment 4 was a comparison of strains but-112 and but-23.1 with but-32. Three different concentrations of BUdR were used in experiments 3 and 4, plus one control culture with TdR alone.

A feature of the results was the variation observed in the sporulation levels for each strain, even in control cultures,

TABLE 7. Growth rates and sporulation capacities of isogenic strains but-32, but-18, but-21, but-24, but-112 and but-23.1 at various BUdR concentrations in MM (20 ml).

(in experiments 1 and 2 the effect BUdR at the highest concentration, 30 μgml^{-1}, was not tested.)

Isogenic strain	Expt. No.	Additions to MM							
		TdR (8.5 μgml^{-1})		TdR (1 $ugml^{-1}$) + BUdR (7.5 μgml^{-1})		TdR (1 $ugml^{-1}$) + BUdR (15 μgml^{-1})		TdR (1 $ugml^{-1}$) + BUdR (30 μgml^{-1})	
		Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}
but-32	1	87	2.96	108	2.82	105	3.28	-	-
	2	84	9.00	120	5.26	150	4.80	-	-
	3	87	14	108	1.27	138	2.54	240	0.68
	4	102	12	108	0.06	123	0.06	204	0.05
but-18	1	72	7.48	93	5.16	117	4.70	-	-
	2	72	2.54	99	3.00	108	0.74	-	-
	3	87	13	108	2.24	141	1.86	180 → ∞	0.36
but-21	1	87	6.50	132	1.98	138	1.76	-	-
	2	66	4.46	144	3.52	318	2.44	-	-
	3	72	22	108	1.53	360	2.33	360 → ∞	1.26

TABLE 7. (contd.)

Isogenic strain	Expt. No.	Additions to MM							
		TdR (8.5 $\mu g ml^{-1}$)		TdR (1 $\mu g ml^{-1}$) + BUdR (7.5 $\mu g ml^{-1}$)		TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$)		TdR (1 $\mu g ml^{-1}$) + BUdR (30 $\mu g ml^{-1}$)	
		Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}
	1	66	4.78	78	3.22	108	2.56	-	-
but-24	2	75	6.10	96	5.36	174	2.02	-	-
	3	92	18	116	1.01	150	0.78	2.40 → ∞	0.45
but-112	1	156	0.44	183	0.52	201	0.32	-	-
	2	96	3.42	162	2.08	201	1.00	-	-
	4	84	3.00	108	0.02	204	0.01	240 → ∞	0.01
but-23.1	1	78	0.32	114	0.18	192	0.26	-	-
	2	75	3.14	141	0.88	366	0.26	-	-
	4	84	10	138	0.49	198	0.40	240 → ∞	0.20

but in general the trend was that spore incidence decreased with increasing BUdR concentration (The possibility that this progressive effect was due to an increasing effect on the viability of the cells was not tested in these experiments). However, experiments 1 and 2 show very poor sporulation in TdR alone, and the relative inhibitory effect of BUdR on sporulation appeared to diminish. Normally a high level of sporulation in control cultures is desirable, to render the results valid. Thus, the degree of variation in the sporulation levels in these experiments made it difficult to clearly state whether sporulation of some strains was more sensitive than others to the inhibitory action of BUdR.

In general the growth rates of these strains decreased with increasing BUdR concentration. In addition, the results indicated that there were differences between strains with regard to the BUdR concentrations at which the growth rate was markedly affected. For example, at a ratio of 1 : 15 μgml^{-1} of TdR : BUdR, strains but-21, but-112 and but-23.1 had relatively high doubling times compared to cells in TdR alone, whereas the times for strains but-32, but-18 and but-24 at this TdR :BUdR ratio, although higher than the control values, were relatively unaffected compared to those of the three more sensitive strains. The same division of strains was obtained when the doubling times at a TdR : BUdR ratio of 1 : 7.5 μgml^{-1} were compared. It may be possible to further categorise the "most-tolerant" class into one

containing strains but-18 and but-24, and another with but-32, as this latter strain had the only measurable doubling time during exponential growth at a BUdR concentration of 30 $\mu g ml^{-1}$.

It is also apparent that the relative inhibitory effect of BUdR on growth in MM of the isogenic strains but-32, but-18, but-21 and but-24 was stronger in these experiments compared to the preliminary experiments (Tables 3 and 4), where the original strains were used.

The results of these experiments (Table 7) might therefore suggest that at least three classes of tolerance to BUdR exist, containing but-32 in class I, but-18 and but-24 in class II, and but-21, but-112 and but-23.1 in class III. The first four strains mentioned are in an order of tolerance identical to that obtained in the earlier experiments using the non-isogenic mutant strains from which they were derived (see section I).

A somewhat similar order of tolerance was obtained from the results of another experiment (Table 8) in which the same strains, plus the BUdR-sensitive parent strain, were grown in CH medium with the same three concentrations of BUdR as in the previous experiments. Again strain but-32 appeared least sensitive to the effect of BUdR on growth, although in this case but-18 also grew exponentially at the highest BUdR concentration, although at half the rate of cells in TdR alone (Note that the

TABLE 8. The growth rates of isogenic BUdR - tolerant strains but-32, but-18, but-21, but-24, but-112 and but-23.1 and the parent strain, at various concentrations of BUdR in CH medium.

Strain	Doubling time (min)			
	TdR (8.5 μgml^{-1})	TdR (1 μgml^{-1}) + BUdR (7.5 μgml^{-1})	TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	TdR (1 μgml^{-1}) + BUdR (30 μgml^{-1})
but-32	57	57	63	72
but-18	60	81	102	120
but-21	60	78	120 → ∞	180 → ∞
but-24	60	78	87	120 → ∞
but-112	69	75	120 → ∞	180 → ∞
but-23.1	57	63	84	120 → ∞
parent	57	90	150 → ∞	200 → ∞

BUdR-sensitive strain had a measurable doubling time at the lowest BUdR concentration). The classes and order of BUdR-tolerant strains suggested by the results of this experiment are: class I, but-32; class II, but-18; class III, but-23.1 and but-24; class IV, but-112 and but-21, and class V, the parent strain. This order agrees with that suggested by the results in Table 7, except that but-23.1 appeared less sensitive to the effect of BUdR on growth rate in CH medium than in MM.

C. The effect of BUdR on growth of B. subtilis

The isogenic strain but-32 was chosen for further work as as it exhibited the greatest tolerance to BUdR. BUdR at 15 μgml^{-1} was used, as this concentration, in the presence ofTdR (1 μgml^{-1}), strongly inhibited growth of the parent strain, but had little effect on that of but-32 (Table 8). The doubling times obtained in Table 8 were relatively high, even in control cultures, and Fig. 8 shows the result of a more typical experiment where this comparison was again made. In this experiment BUdR at 15 μgml^{-1} had only a slight effect on the doubling time of but-32, but growth of the parent strain was inhibited at this concentration of BUdR, and the E_{600nm}, after reaching approximately 0.3-0.4, did not increase further.

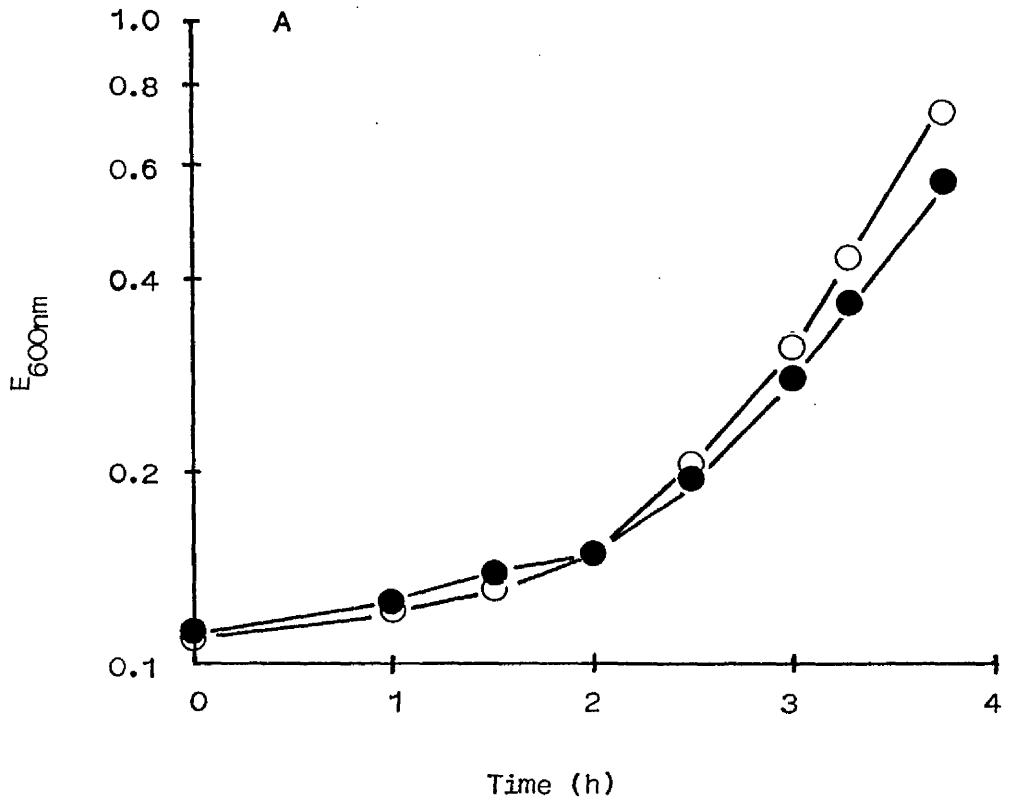
The inhibitory effect of BUdR on growth of the parent strain could have been explained by the effect of a limiting

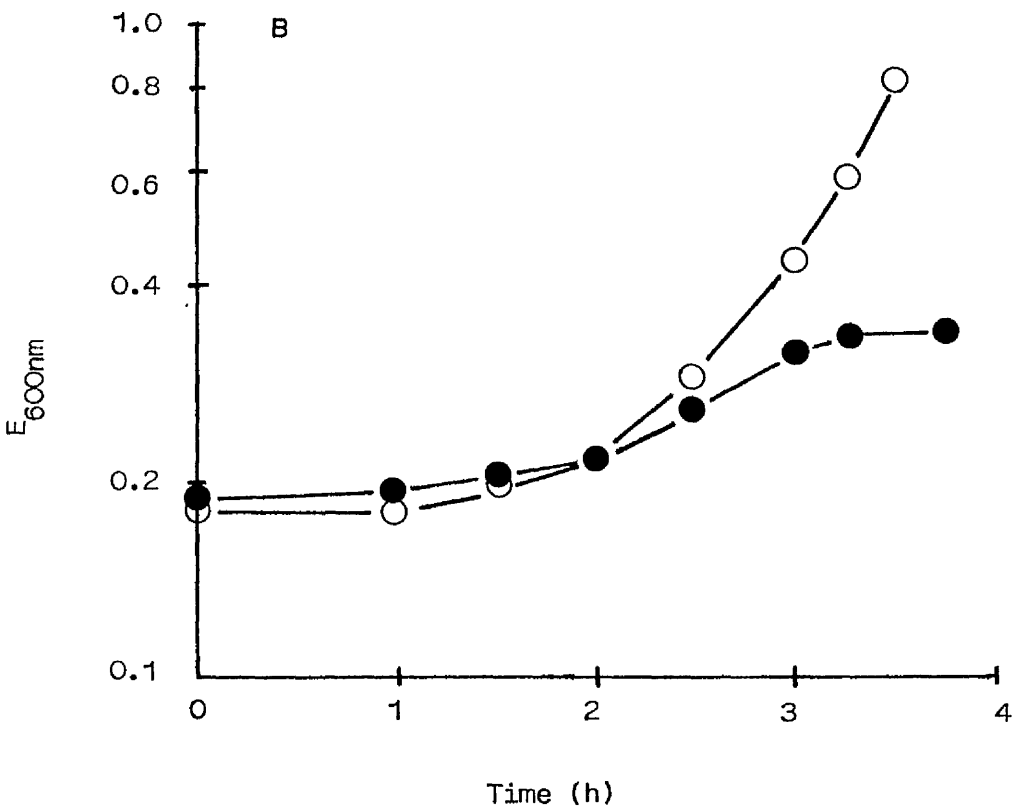
Figure 8. A comparison of the growth rates in CH medium of (A) strain but-32 and (B) the parent strain in the presence of BUdR.

Both strains were grown in CH medium (20 ml) containing either TdR (8.5 μgml^{-1}) (○) or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●). The doubling times (min) for this experiment are given below.

Additions to CH medium	Doubling time (min)	
	but-32	parent
TdR (8.5 μgml^{-1})	39	39
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	48	120 → ∞

Fig. 8


A
1.0
0.8
0.6
0.4
0.2
0.1
E_{600nm}
0
1
2
3
4
Time (h)


B
1.0
0.8
0.6
0.4
0.2
0.1
E_{600nm}
0
1
2
3
4
Time (h)

concentration of TdR in the medium. Therefore, the rate of growth of the parent strain over a range of TdR concentrations was measured (Fig.9). This experiment showed that growth was limited slightly above an E_{600nm} of 0.4 when TdR at 1.0 μgml^{-1} was supplied to the cells, indicating that the greater inhibition of growth in the presence of BUdR was probably due to the analogue itself. At a lower TdR concentration (0.1 μgml^{-1}), and even without TdR, some exponential growth occurred before a dramatic drop in the E_{600nm}, presumably due to cell death, took place. The limited growth observed for the culture without added TdR was presumably due to residual TdR in the intracellular nucleoside pools after growth of the overnight culture with TdR.

D. The effect of BUdR on DNA synthesis during growth

Laird and Bodmer (1967) reported preferential incorporation of TdR over BUdR into the DNA of a thymine-requiring mutant of B. subtilis 168 during growth in the presence of TdR and BUdR. Tolerance to BUdR by strain but-32 may have arisen as a result of an increase in the preference for TdR over BUdR. This was examined by a comparison of the rates of uptake of [2-^{14}C]TdR into acid-insoluble material during growth of both the parent strain and but-32 in the presence of BUdR (Fig. 10). The rates of incorporation in cultures containing only TdR at 8.5 μgml^{-1} or 1 μgml^{-1} were also measured and, in addition, the rate of incorporation of [^{3}H]-BUdR into acid-insoluble

Figure 9. The effect of altering the TdR concentration on growth in CH medium of the parent strain

The cells were grown in CH medium (20 ml) containing TdR at the concentrations shown below. The calculated doubling time (min) are given for those cultures not exhibiting a drop in E_{600nm} after a period of exponential growth (see text).

▽ , TdR (8.5 $\mu g ml^{-1}$) (36)

△ , TdR (1 $\mu g ml^{-1}$) (39)

▲ , TdR (0.5 $\mu g ml^{-1}$) (45)

○ , TdR (0.1 $\mu g ml^{-1}$)

● , No TdR

Fig.9

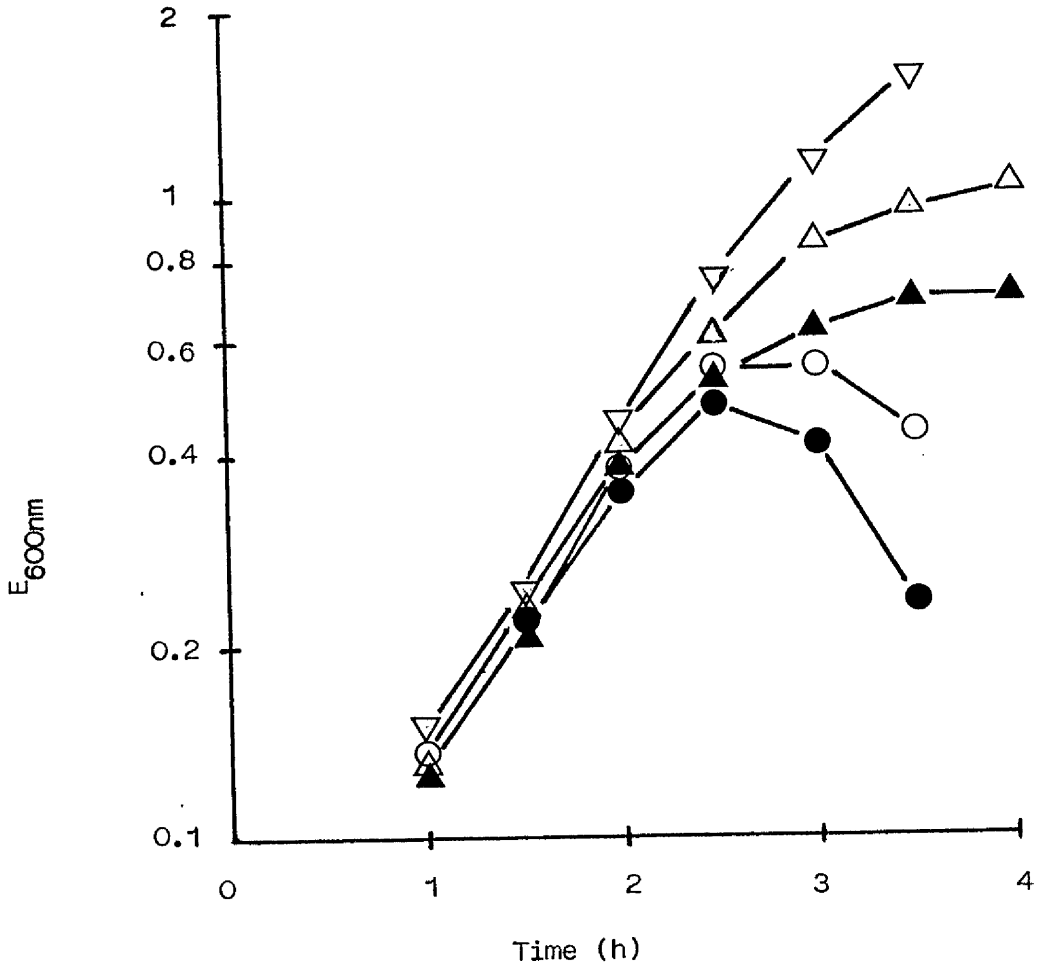


Figure 10. Incorporation of $[^{14}C]$ -TdR and $[^{3}H]$ - BUdR into acid-insoluble material during growth of A) strain but-32 and B) the parent strain.

Each strain was inoculated separately into four flasks containing CH medium (20 ml) plus the following additions:

1) TdR (8.5 $\mu g ml^{-1}$) (●)

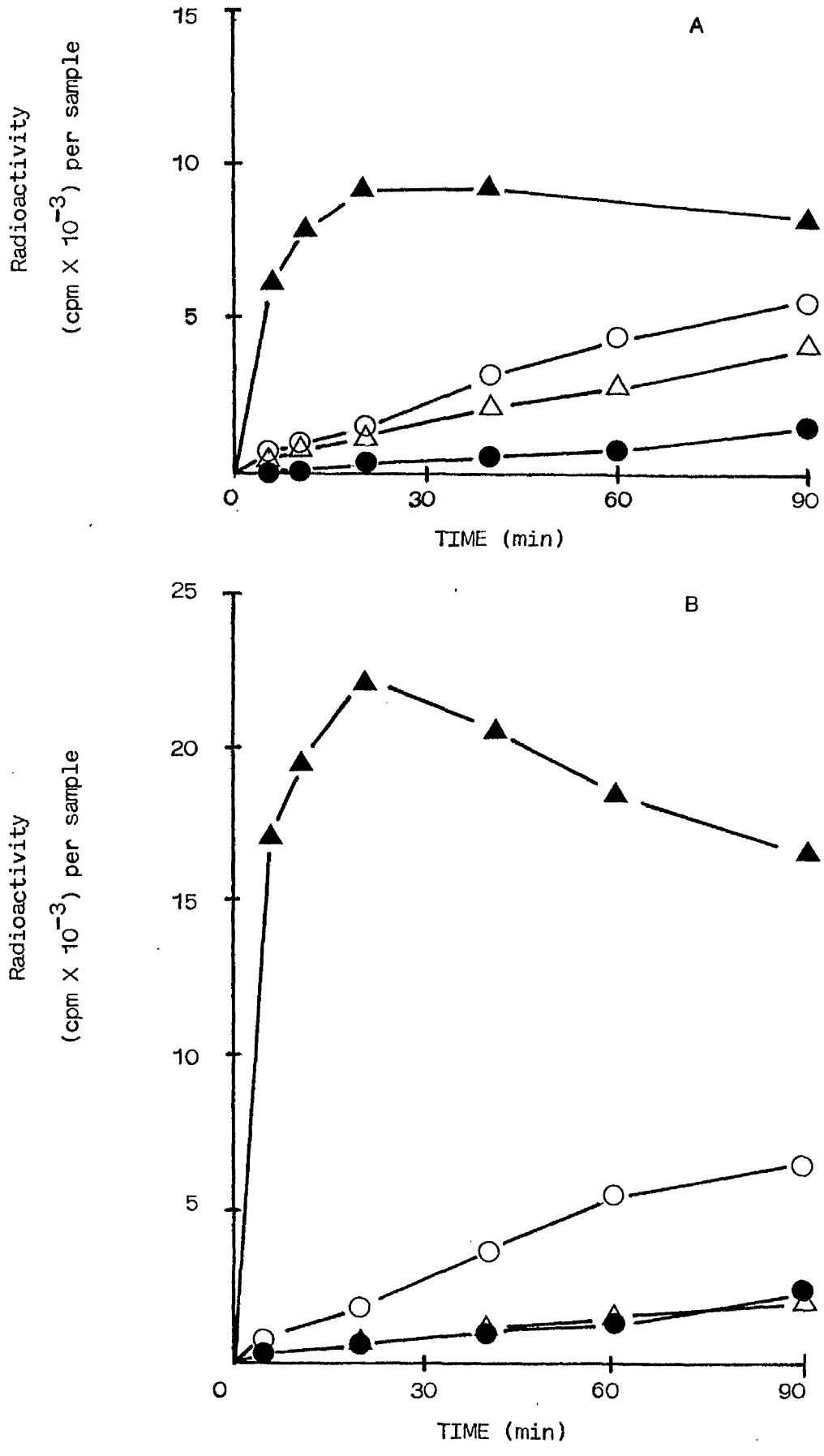
2) TdR (1 $\mu g ml^{-1}$) + BUdR (7.5 $\mu g ml^{-1}$) (△)

3) TdR (1 $\mu g ml^{-1}$) + BUdR (7.5 $\mu g ml^{-1}$) (▲)

4) TdR (1 $\mu g ml^{-1}$) (○)

After growing the cells to an E_{600nm} of approximately 0.6, $[^{14}C]$ - TdR (0.025 $\mu Ci ml^{-1}$, final concentration) was added to flasks 1,2 and 4, and $[^{3}H]$ - BUdR (0.5 $\mu Ci ml^{-1}$ final concentration) to flask 3. Incorporation of radioactivity into acid-insoluble material was measured at intervals after the addition of the label (see Materials and Methods, section VIII).

Fig. 10


A
B
Radioactivity
(cpm X 10^{-3}) per sample
15
10
5
25
20
0
30
60
90
TIME (min)

material in parallel cultures of both strains was measured. BUdR was added at 7.5 μgml^{-1} to allow some growth of the parent strain, and to bring the TdR + BUdR concentration to 8.5 μgml^{-1}, the concentration of TdR in the control culture.

$[^{3}H]$ - BUdR was incorporated rapidly into both strains, although the rate of incorporation rapidly declined. The parent strain incorporated more $[^{3}H]$ - BUdR than but-32. In both strains $[^{14}C]$ - TdR was incorporated at a greater rate at the lower TdR concentration due to decreased competition for incorporation of radioactive TdR by unlabelled TdR. When BUdR partially replaced TdR in the medium, the rate of incorporation of $[^{14}C]$ - TdR in the parent strain was almost identical to the rate found for the higher TdR concentration. However, in the But strain the rate of incorporation of $[^{14}C]$ - TdR in the presence of BUdR was closer to the rate found at the low TdR concentration than that found for the higher TdR concentration.

These results could perhaps be explained in two ways. If BUdR in some way slowed down the rate of DNA synthesis in the parent strain, then the tolerant strain might compensate for this by prematurely initiating rounds of DNA replication to offset a possible decrease in the overall rate of DNA synthesis induced by BUdR. This would have the effect of an apparent increase in the rate of $[^{14}C]$ - TdR incorporation into acid-insoluble material. An alternative possibility is that BUdR

normally competes with TdR for uptake into the cell or into the DNA and tolerance may arise as a result of total or partial exclusion of BUdR from the cell or the DNA. In this case there would again be a greater rate of $[^{14}C]$ - TdR incorporation as the excess BUdR would not compete as effectively with the radio-label for incorporation into acid-insoluble material. The greater degree of incorporation of $[^{3}H]$ - BUdR into acid-insoluble material of the parent strain compared to but-32 may be regarded as evidence for the latter hypothesis. An examination of the effect of BUdR on uptake of $[^{14}C]$ - TdR into whole cells is given below.

The possibility that the basis for the toxic effect of BUdR on the parent strain was via incorporation of the analogue into RNA was tested by monitoring the rate of incorporation of $[^{3}H]$ - BUdR into acid-insoluble material during growth in the presence of excess uridine, and by pre-treating cell samples with KOH to degrade RNA before addition of TCA (Fig. 11). In this experiment BUdR was included at 7.5 $\mu g ml^{-1}$ to allow some growth of the parent strain. Excess uridine in the growth medium had little effect on the rates of incorporation of $[^{3}H]$ - BUdR into acid-insoluble material in both strains, suggesting that BUdR toxicity was not related to incorporation into RNA. However, treatment of the cells with KOH before acid precipitation had a more marked effect on the uptake measured in the parent strain. Degradation of RNA in samples taken from the parent strain during

Figure 11. Incorporation of $[^3H]$ - BUdR into acid-insoluble material during growth of (A) but-32 and (B) the parent strain, in the presence of BUdR.

Each strain was grown in two flasks containing CH medium (20 ml) + TdR (1 μgml^{-1})+ BUdR (7.5 μgml^{-1}). Uridine (50 μgml^{-1}) was present in one culture. At an E_{600nm} of approximately 0.6, $[^3H]$ - BUdR (0.2 $\mu Ciml^{-1}$, final concentration) was added to all cultures. At intervals after addition of $[^3H]$ - BUdl duplicate samples (0.2 ml) were removed from each culture, one for immediate precipitation with TCA + BUdR (100 μgml^{-1}), the other for pre-treatment with 1 ml 1N KOH for 2h at 37°C before TCA precipitation.

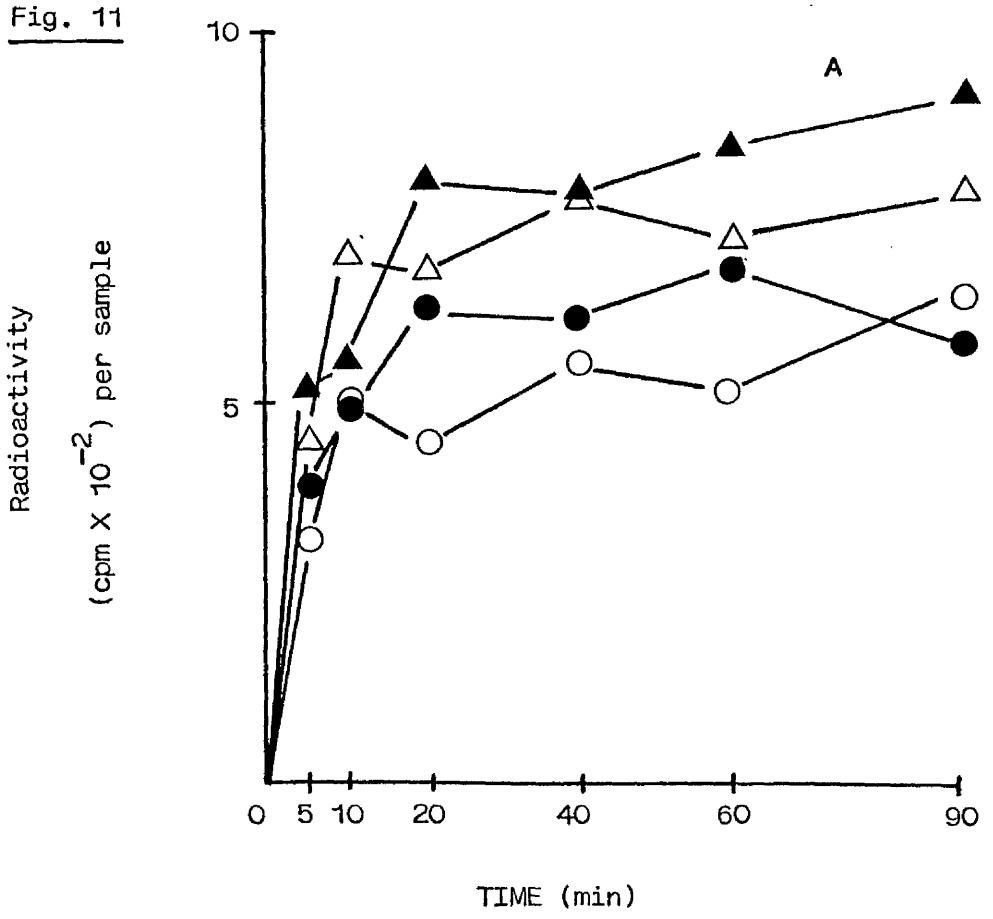
● uridine present in medium

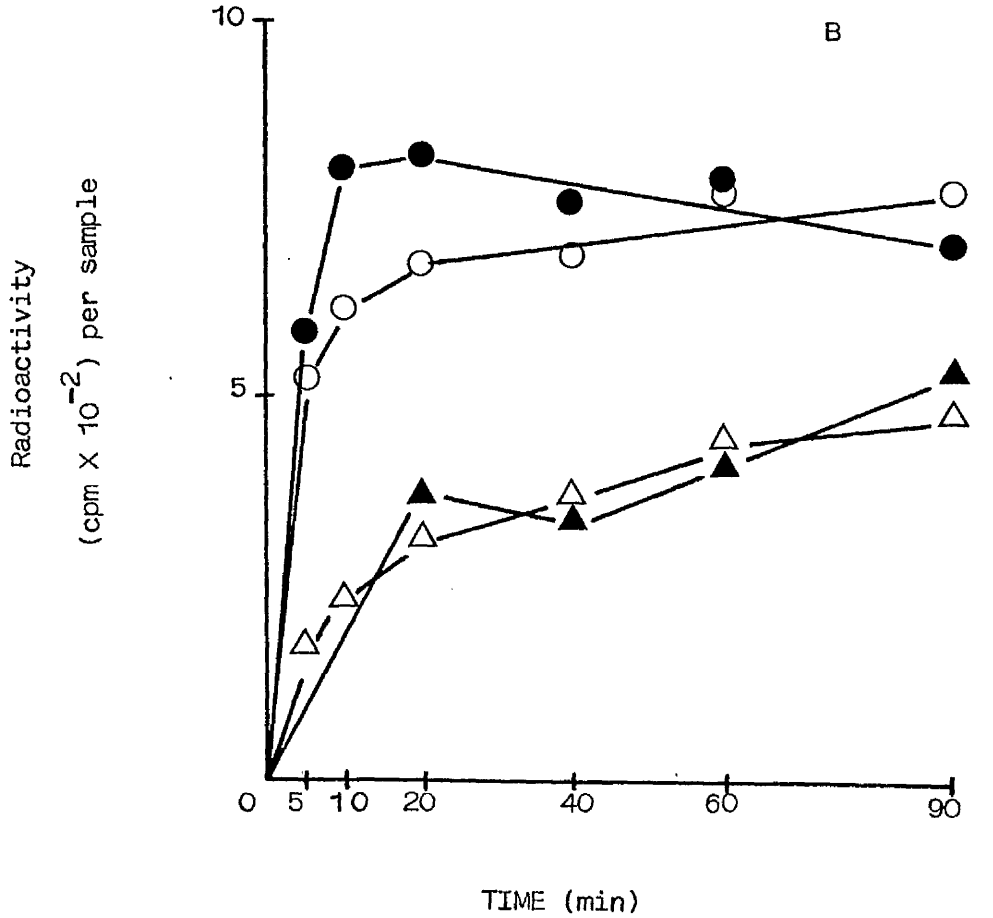
▲ uridine present in medium, samples pretreated with KOH

○ No uridine present in medium

△ No uridine present in medium, samples pretreatec with KOH

Fig. 11


A
10
5
Radioactivity
(cpm X 10^{-2}) per sample
0 5 10 20 40 60 90
TIME (min)


B
10
5
Radioactivity
(cpm X 10^{-2}) per sample
0 5 10 20 40 60 90
TIME (min)

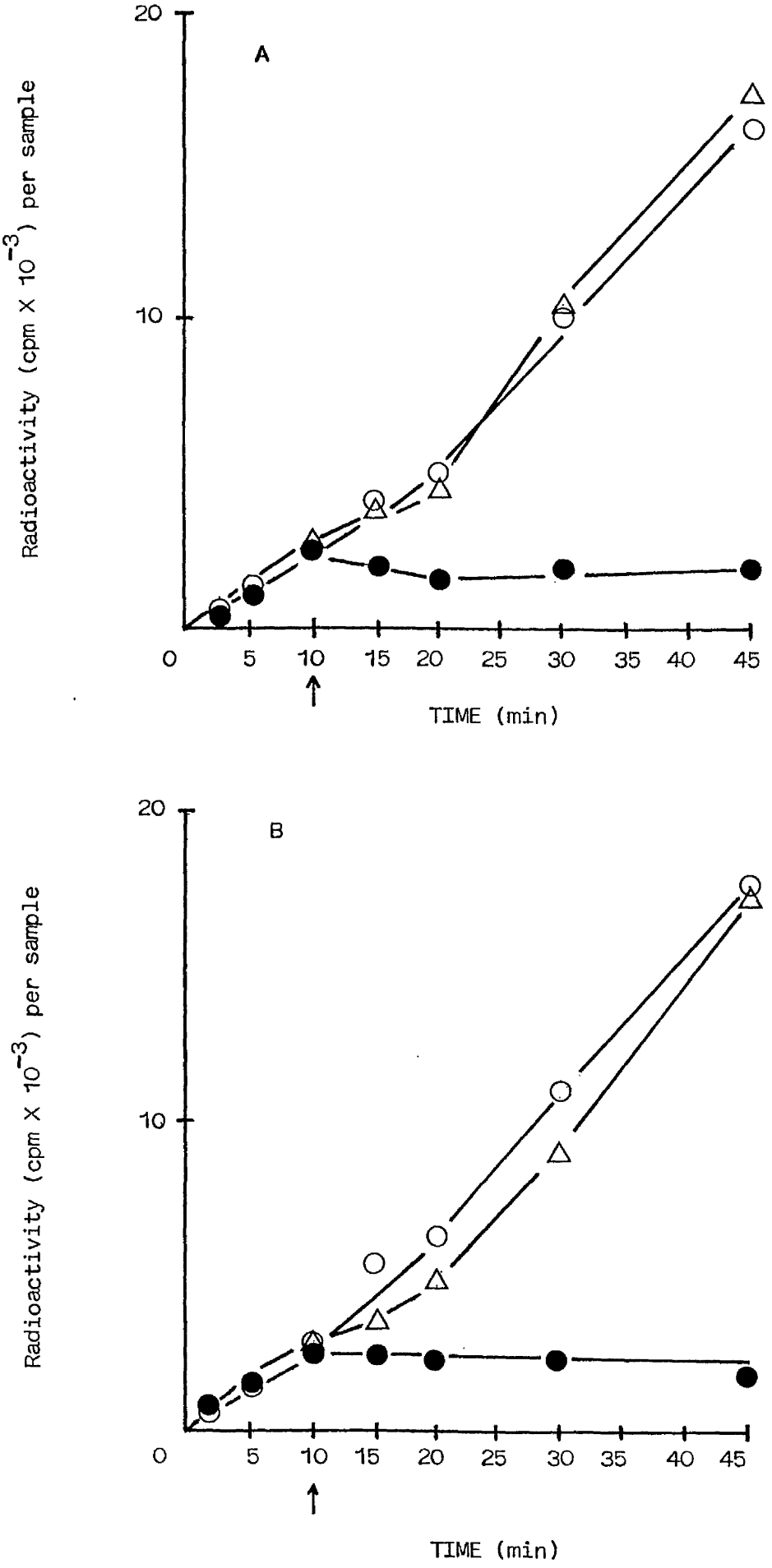
growth in the presence of BUdR resulted in a decreased rate of incorporation of [^{3}H]- BUdR, whereas this treatment did not decrease the sample counts for the But strain. In fact, they apparently increased. This result therefore suggested that the parent strain incorporated more BUdR into RNA than the tolerant strain. However, the results of another experiment (Fig. 12) indicated that BUdR had no effect on the rate of RNA synthesis, measured as the rate of incorporation of [^{3}H]- uridine into acid-insoluble material, during growth of both strains. On the other hand, rifampicin, a known inhibitor of RNA synthesis, completely inhibited further incorporation of [^{3}H]- uridine during growth of both strains. This provided further evidence that the effect of BUdR on growth of the parent strain was not due to incorporation of the analogue into RNA. It was concluded, therefore, that the result for KOH-treated cells (Fig. 11) may have been an artefact caused by the addition of KOH.

In some eukaryotic cells the compound deoxycytidine has been shown to overcome the toxic effect of BUdR (Meuth and Green, 1974). These workers suggested that BUdR-triphosphate, formed from BUdR in the cell, inhibits the enzyme ribonucleotide reductase, thus starving the cells of deoxycytidine residues (see Introduction). However, inhibition of growth of the parent strain by BUdR was unaffected by addition of an equal concentration of deoxycytidine, and surprisingly, growth was inhibited to an even greater extent when deoxycytidine was added in excess

Figure 12. The effect of BUdR on RNA synthesis during growth of (A) but-32 and (B) the parent strain.

Each strain was grown in CH medium (50 ml) + TdR (8.5 $\mu g ml^{-1}$) to an E_{600nm} of approximately 0.5. At this point three samples (each 10 ml) were removed from each culture, filtered and resuspended in pre-warmed CH medium (10 ml) containing i) TdR (8.5 $\mu g ml^{-1}$) (○), ii) TdR (8.5 $\mu g ml^{-1}$) (●) and iii) TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) (△). 0.2 ml of a $[^3H]$ - uridine solution (25 $\mu Ci ml^{-1}$ + 1.25 mg unlabelled uridine ml^{-1} in distilled water) was added to each culture (final concentration of 0.5 $\mu Ci ml^{-1}$). At intervals after addition of radio label samples (0.2 ml) were removed for measurement of incorporation of radioactivity into acid-insoluble material. (see Materials and Methods, section VIII (C)) In addition, 10 min after the addition of label, rifampicin (20 $\mu g ml^{-1}$, final concentration) was added to culture (ii). (↑).

Fig. 12


A
B
20
10
0
5
10
15
20
25
30
35
40
45
Radioactivity (cpm X 10^{-3}) per sample
TIME (min)

(Fig. 13). In the same experiment, the very slight inhibitory effect of BUdR on the growth rate of but-32 was also unaltered by addition of deoxycytidine at 15 $\mu g ml^{-1}$. However, as with the parent strain, increasing the concentration from 15 $\mu g ml^{-1}$ to 50 $\mu g ml^{-1}$ appeared to decrease the growth rate of but-32 in the presence of BUdR.

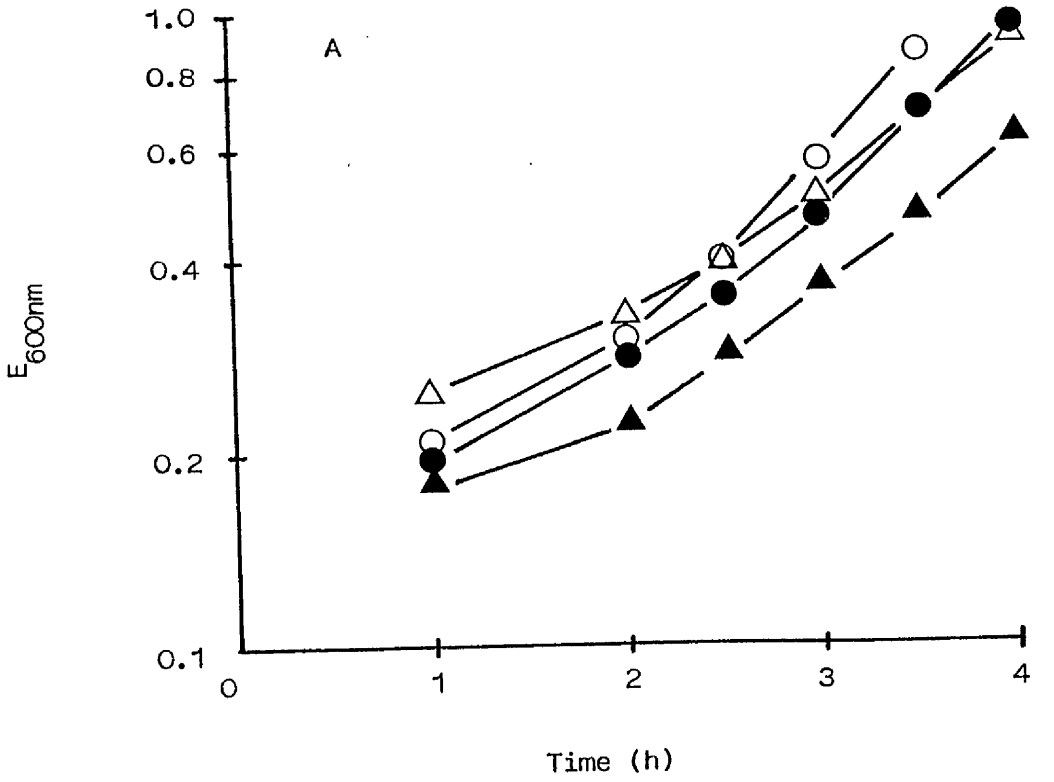
To further investigate the basis of the tolerance phenotype a comparison was made between the rates of $[^3H]$ - BUdR uptake into whole cells and acid-insoluble material of but-32 (Fig. 14) and the parent strain (Fig. 15) grown in the presence of BUdR. Unlike two previous experiments (Fig.'s 10 and 11), where the parent strain was grown with BUdR at a concentration of 7.5 $\mu g ml^{-1}$ to allow some growth, the cells in this experiment were grown with TdR alone until mid-exponential phase, when they were transferred to a BUdR-containing medium. In strain but-32 the label was rapidly taken up by the cells, but after approximately 10 min the curve plateaued (Fig. 14A), whereas the rate of incorporation into acid-insoluble material followed a more linear pattern (Fig. 14B). The pattern of uptake into whole cells was presumably due to turnover of the analogue in the nucleoside pools. It can also be seen that addition of rifampicin to the cultures had a much more marked effect on incorporation of $[^3H]$- BUdR into acid-insoluble material than into whole cells. If rifampicin only inhibits RNA synthesis then this result would suggest that

Figure 13. The effect of deoxycytidine (dCyd) on BUdR inhibition of growth.

Strain but-32 (A) and the parent strain (B) were grown in parallel cultures in CH medium (20 ml) containing TdR (8.5 μgml^{-1}) (○), TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●), TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + dCyd (15 μgml^{-1}) (△) and TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + dCyd (50 μgml^{-1}) (▲). The calculated doubling times are given below.

Additions to CH medium	Doubling time (min)	
	but-32	parent
TdR (8.5 μgml^{-1})	54	48
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	60	120 → ∞
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + dCyd (15 μgml^{-1})	60	130 → ∞
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + dCyd (50 μgml^{-1})	75	180 → ∞

Fig. 13


A
1.0
0.8
0.6
0.4
0.2
0.1
E_{600nm}
0
1
2
3
4
Time (h)

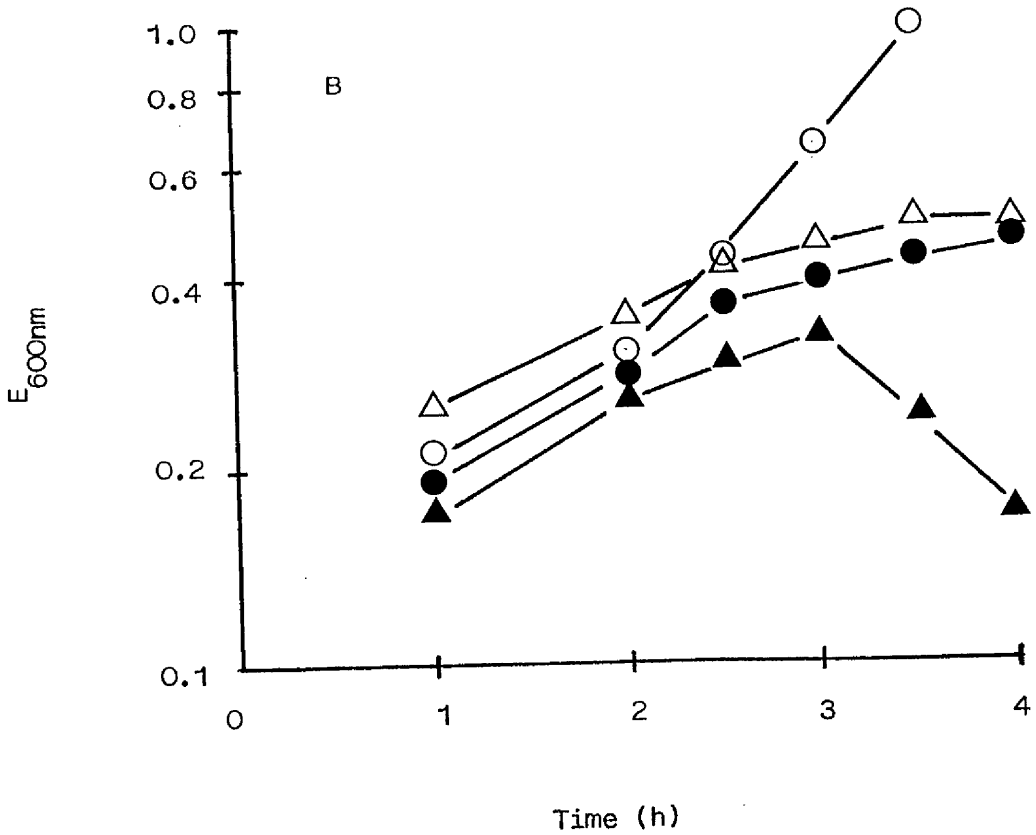

B
1.0
0.8
0.6
0.4
0.2
0.1
E_{600nm}
0
1
2
3
4
Time (h)

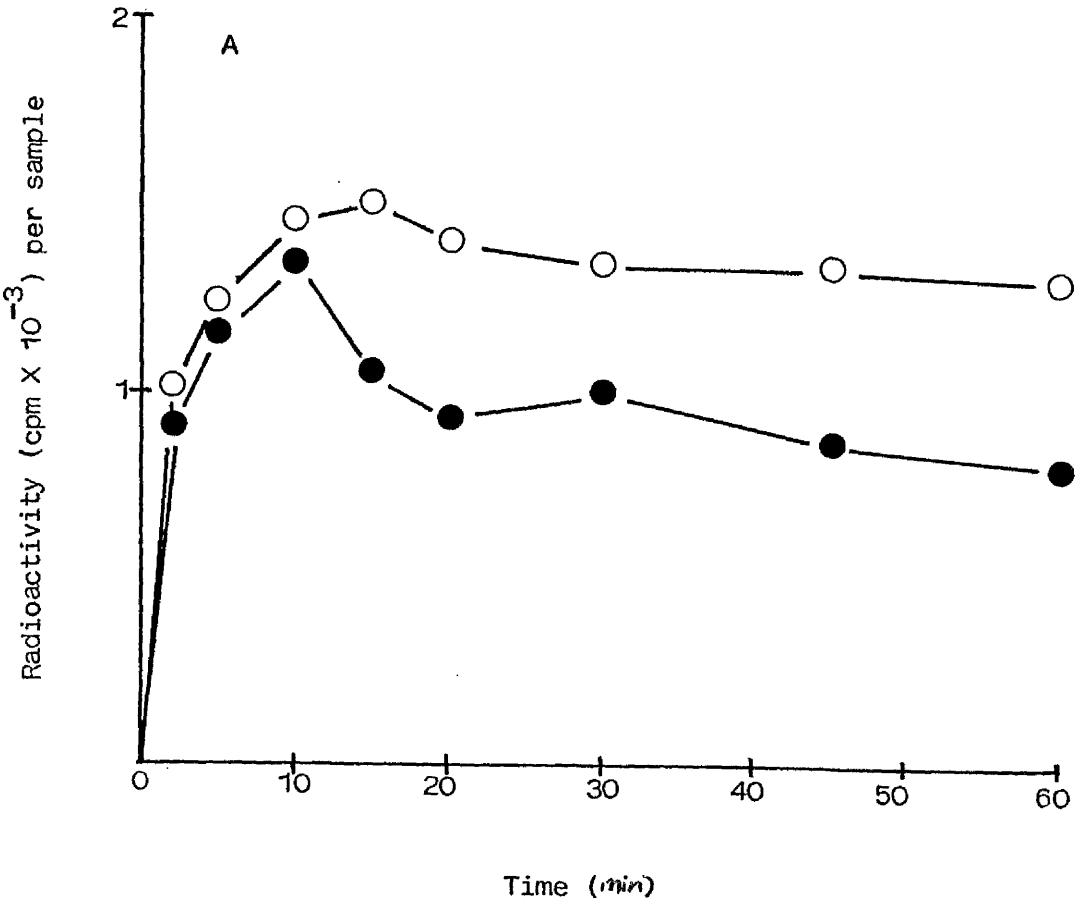
Figure 14. Incorporation of $[^3H]$ - BUdR into whole cells and acid-insoluble material of strain but-32 during growth in the presence of BUdR.

Strain but-32 was grown in CH medium (50 ml) + TdR (8.5 μgml^{-1}) to an E_{600nm} of approximately 0.5. Two samples, each 20 ml, were removed from the culture, filtered, washed and resuspended in pre-warmed CH medium (20 ml) plus TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). $[^3H]$ - BUdR (0.5 $\mu Ciml^{-1}$, final concentration) was added to both cultures, and rifampicin (20 μgml^{-1}, final concentration) to one culture. Two samples (each 0.2 ml) were removed at intervals from each culture, for measurement of incorporation of radioactivity into (A) whole cells and (B) acid-insoluble material (see Materials and Methods, section VIII).

○ no rifampicin added

● rifampicin present

Fig. 14


A
2
1
0
10
20
30
40
50
60
Radioactivity (cpm X 10^{-3}) per sample
Time (min)

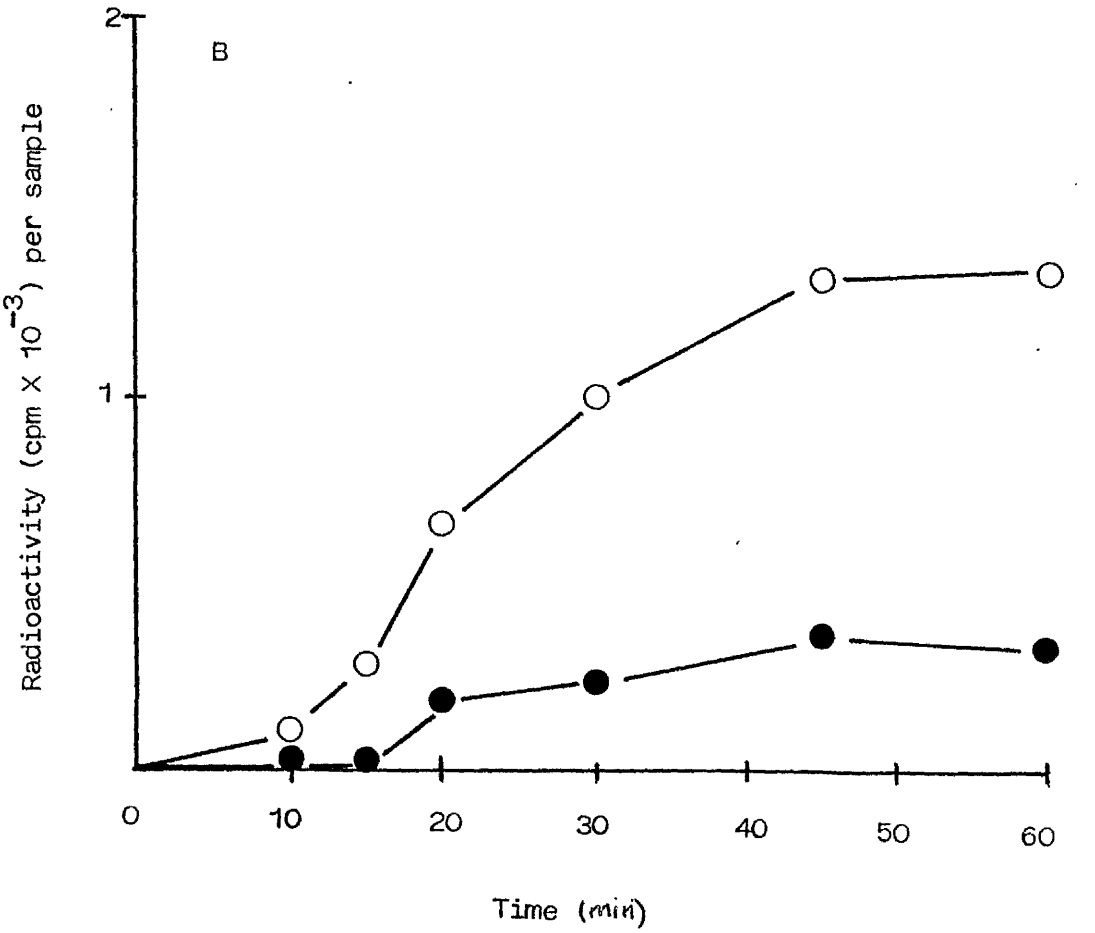

B
2
1
0
10
20
30
40
50
60
Radioactivity (cpm X 10^{-3}) per sample
Time (min)

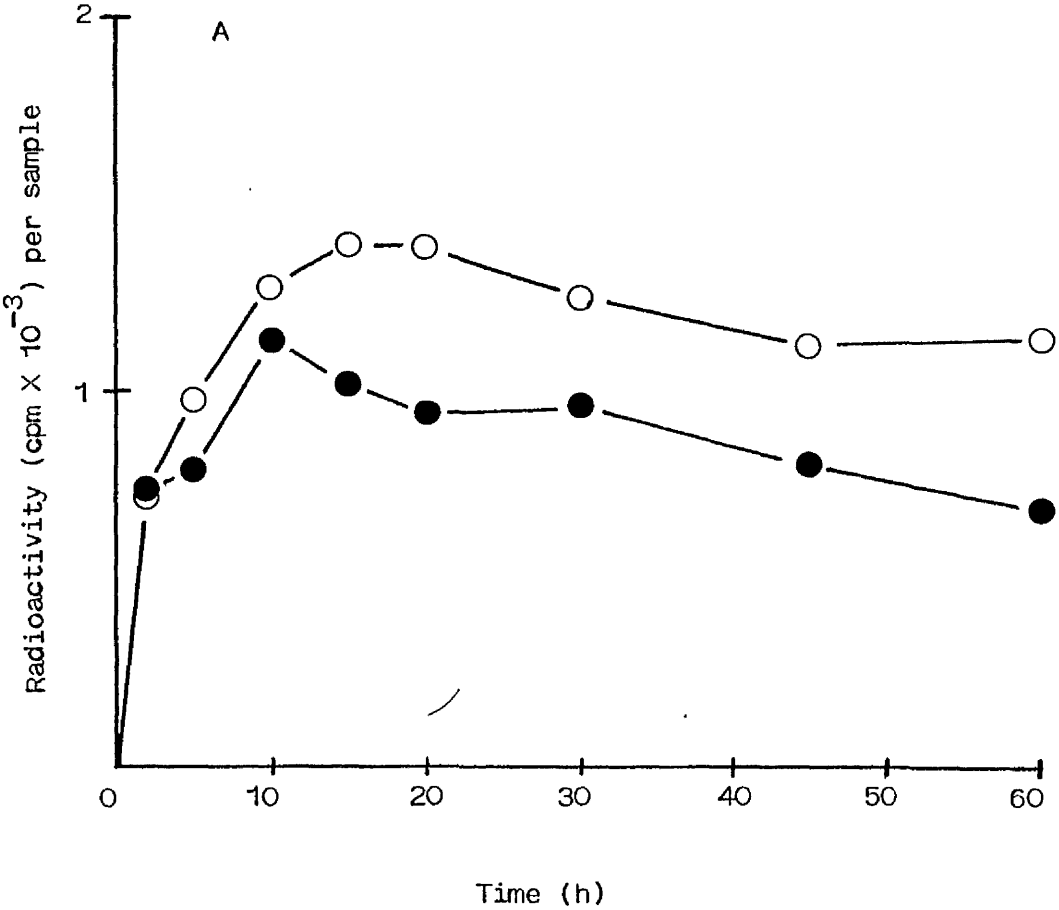
Figure 15 Incorporation of $[^3H]$ - BUdR into whole cells and acid-insoluble material of the parent strain during growth in the presence of BUdR.

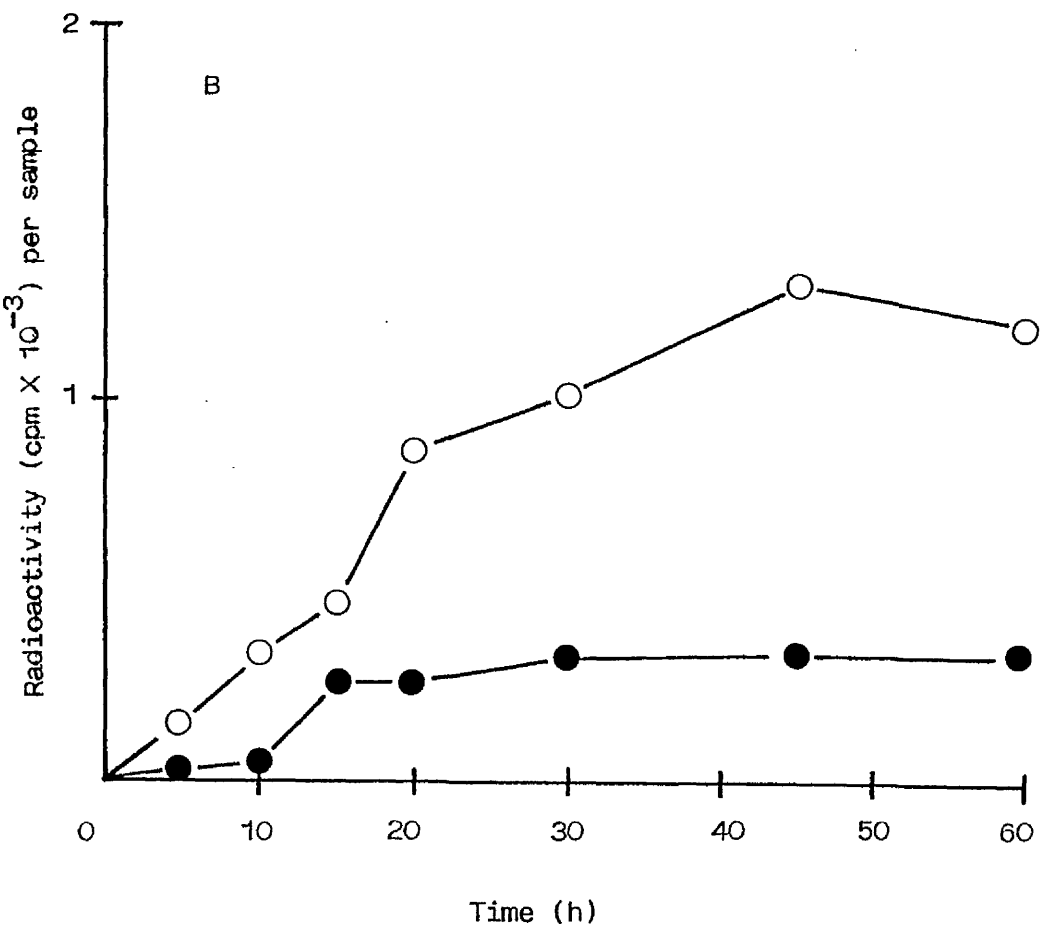
The parent strain was grown in parallel with strain but-32 (see Fig. 14) in CH medium (50 ml) +TdR (8.5 μgml^{-1}) to an E_{600nm} of approximately 0.5. Two samples, each 20 ml, were removed from the cultures, filtered, washed and resuspended in pre-warmed CH medium (20 ml) plus TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) $[^3H]$- BUdR (0.5 $\mu Ciml^{-1}$, final concentration) was added to both cultures, and rifampicin (20 μgml^{-1}, final concentration) added to one culture. Two samples (each 0.2 ml) were removed at intervals from each culture, for measurement of incorporation of radioactivity into (A) whole cells and (B) acid-insoluble material. (see Materials and Methods, section VIII).

○ no rifampicin added

● rifampicin present

Fig. 15


A
2
1
Radioactivity (cpm X 10^{-3}) per sample
0
10
20
30
40
50
60
Time (h)


B
2
1
Radioactivity (cpm X 10^{-3}) per sample
0
10
20
30
40
50
60
Time (h)

most of the BUdR taken up by the cells was incorporated in RNA. However, as rifampicin also inhibits DNA synthesis in Bacillus brevis by interference with DNA chain elongation (Bhattacharya and Sarkar, 1981) this hypothesis cannot be made.

In a parallel experiment, the patterns of uptake of [^{3}H]-BUdR into whole cells and acid-insoluble material of the parent strain (Fig. 15) were almost identical to those of but-32 (Fig. 14). This would suggest that as much BUdR is taken into the cells and incorporated into acid-insoluble material of the tolerant strain as in the parent, which is inconsistent with the results of two previous experiments (Fig.'s 10 and 11), in which more BUdR was incorporated into acid-insoluble material of the parent than the But strain.

Therefore, using a slightly different approach, a comparison was made between the rates of [^{14}C] - TdR uptake into whole cells and into acid-insoluble material of both strains during growth and sporulation. During both growth (Fig. 16) and sporulation (Fig.17) of but-32, the patterns of [^{14}C] - TdR uptake into whole cells and acid-insoluble material were similar, suggesting that BUdR was partially excluded from the cell in the tolerant strain during both growth and sporulation in the presence of the analogue. This was made clearer in a separate experiment where the same comparison was made using the parent strain (Fig.'s 18 and 19). Again the pattern of uptake was similar for

Figure 16. Incorporation of $[^{14}C]$ - TdR into whole cells and acid-insoluble material during growth of but-32.

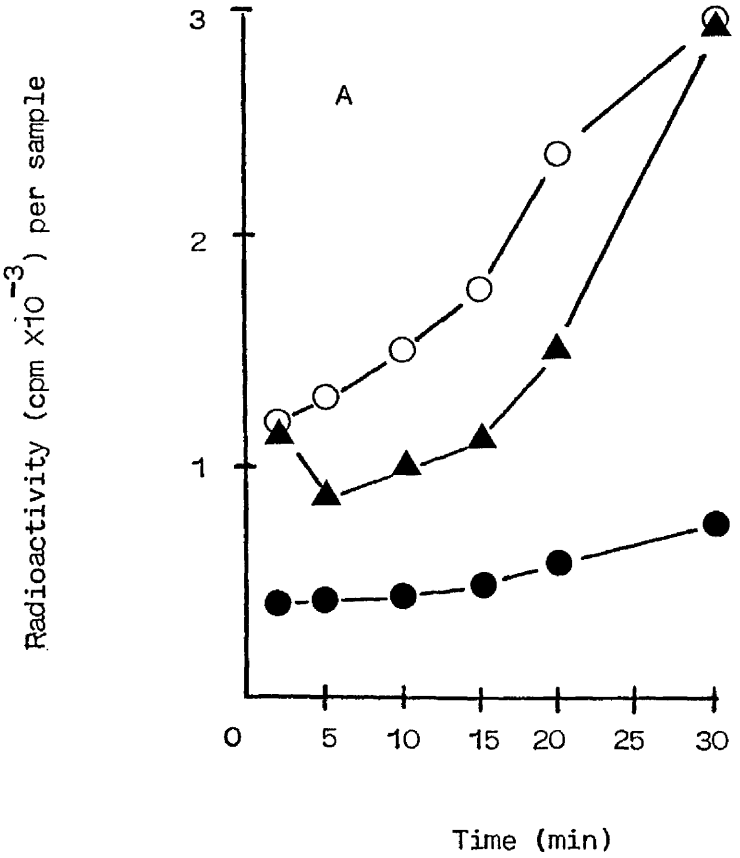
Three cultures of strain but-32 were grown to an E_{600nm} of approximately 0.5 in CH medium (20 ml) supplemented with TdR (1 μgml^{-1}), TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), or TdR (8.5 μgml^{-1}). $[^{14}C]$ - TdR was then added to each culture at 0.025 $\mu Ciml^{-1}$ final concentration. At intervals after addition of radioactive TdR two 0.2 ml samples were removed from each culture, one (A) used to measure uptake of radioactivity into whole cells and the other (B) into acid-insoluble material (see Materials and Methods, section VIII).

○ TdR (1 μgml^{-1})

▲ TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})

● TdR (8.5 μgml^{-1})

Fig. 16


A
Radioactivity (cpm X10^{-3}) per sample
3
2
1
0
5
10
15
20
25
30
Time (min)

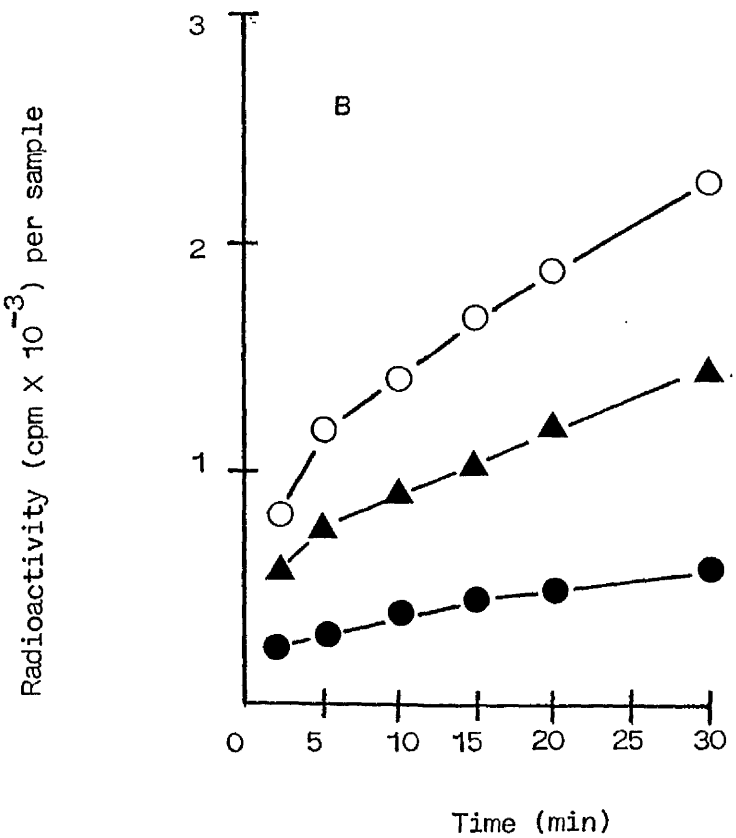

B
Radioactivity (cpm X 10^{-3}) per sample
3
2
1
0
5
10
15
20
25
30
Time (min)

Figure 17. Incorporation of $\left[^{14}C\right]$ - TdR into whole cells and acid-insoluble material during sporulation of but-32.

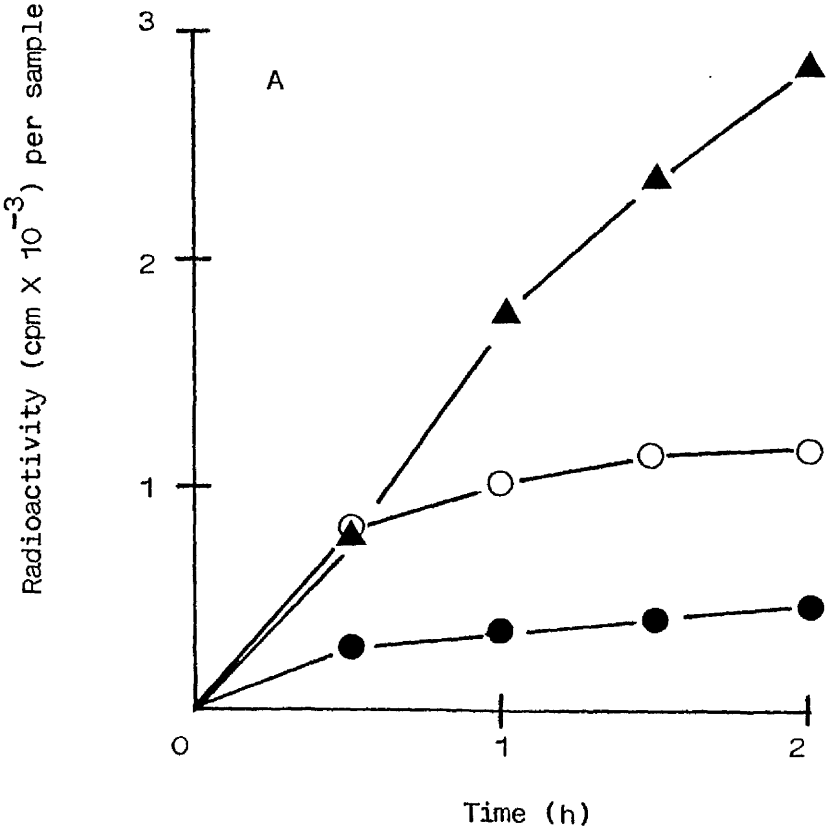
Three 20 ml cultures of but-32 in CH were grown in parallel with the three cultures described in the previous figure (Fig. 16) to an E_{600nm} of approximately 0.7. The cells were harvested by centrifugation and suspended in equal volumes (20 ml) of SM with identical additions. $\left[^{14}C\right]$ - TdR (0.025 $\mu Ciml^{-1}$) was added immediately after resuspension, and at intervals two 0.2 ml samples were removed from each culture for measurement of uptake of radioactivity into (A) whole cells and (B) acid-insoluble material.

○ TdR (1 μgml^{-1})

▲ TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})

● TdR (8.5 μgml^{-1})

Fig. 17


A
Radioactivity (cpm X 10^{-3}) per sample
3
2
1
0
1
2
Time (h)

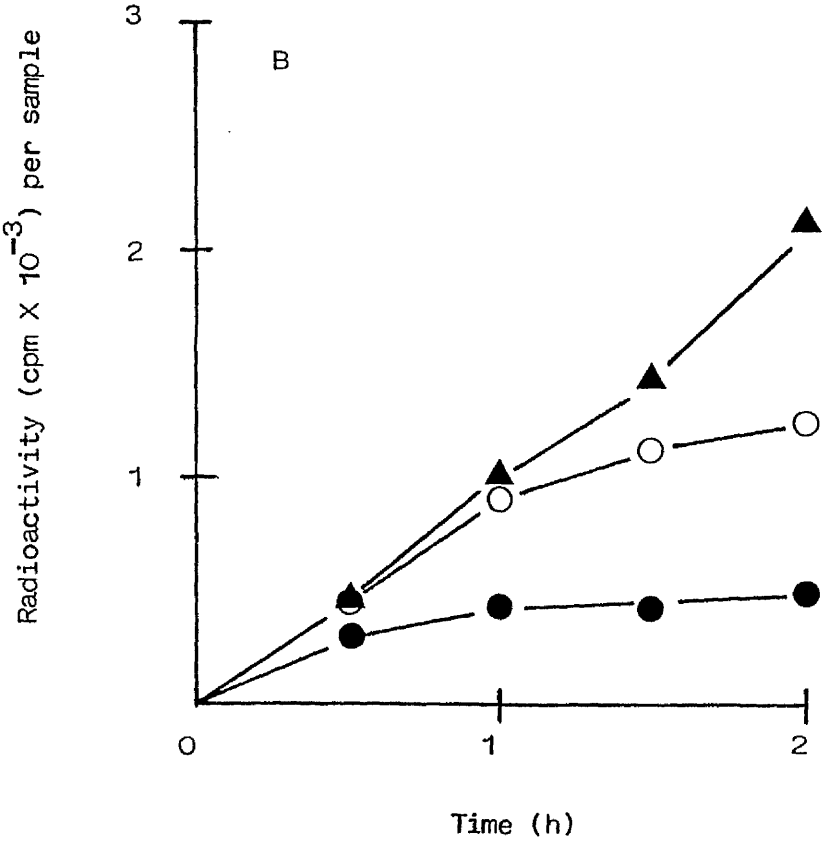
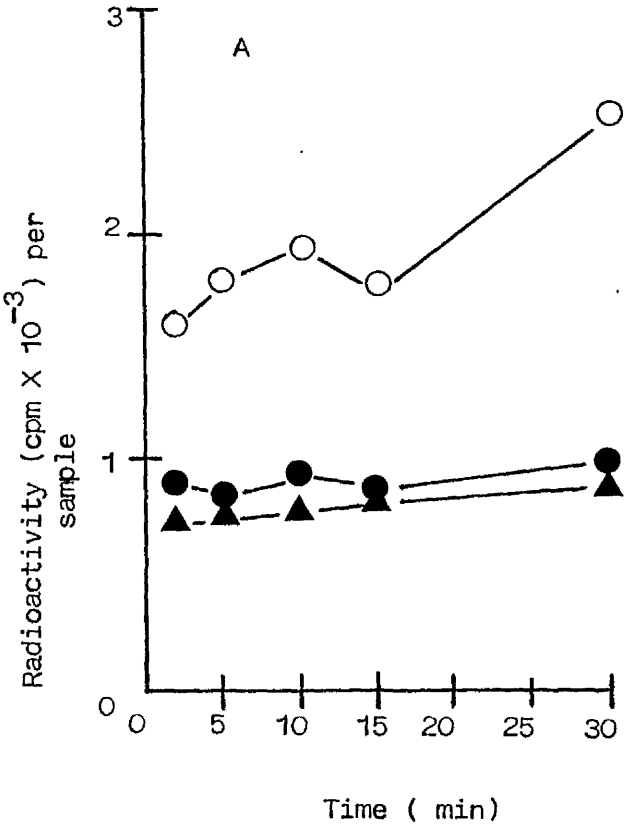

B
Radioactivity (cpm X 10^{-3}) per sample
3
2
1
0
1
2
Time (h)

Figure 18. Incorporation of $[^{14}C]$ - TdR into whole cells and acid-insoluble material during growth of the parent strain.

The parent strain was grown in parallel cultures in an identical manner to strain but-32 (Fig. 16), except that the concentration of BUdR in the medium was 7.5 μgml^{-1} instead of 15 μgml^{-1} to allow some growth. Incorporation of $[^{14}C]$ - TdR into (A) whole cells and (B) acid-insoluble material was measured as in the previous experiment, except that incorporation into acid-insoluble material was monitored over a longer period of time than for whole cells.

Fig. 18


A
Radioactivity (cpm X 10^{-3}) per sample
3
2
1
0
0
5
10
15
20
25
30
Time (min)

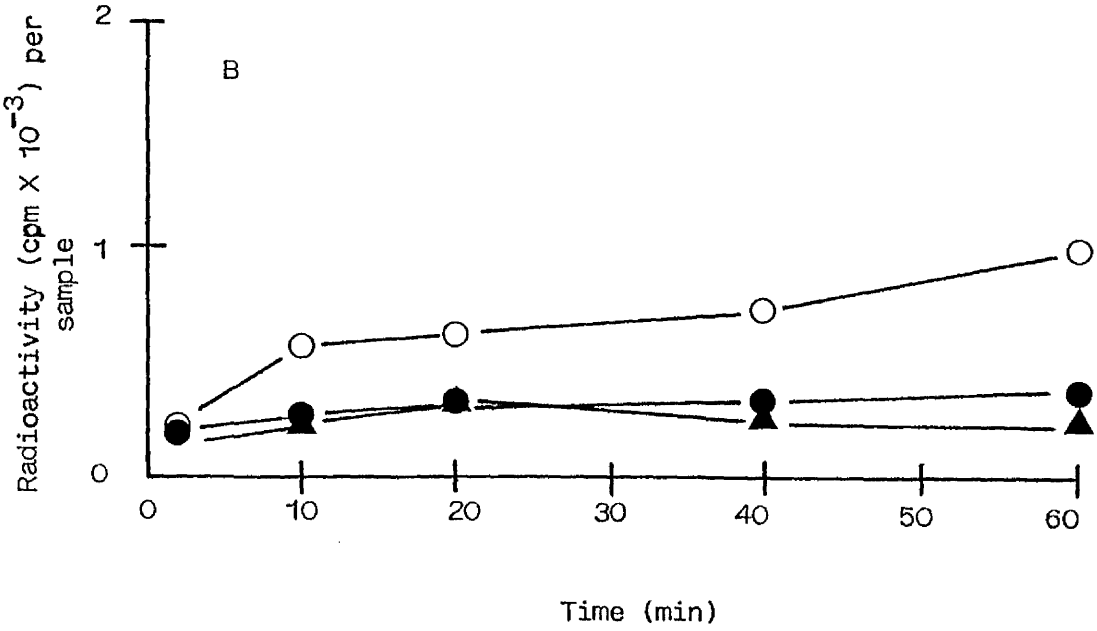
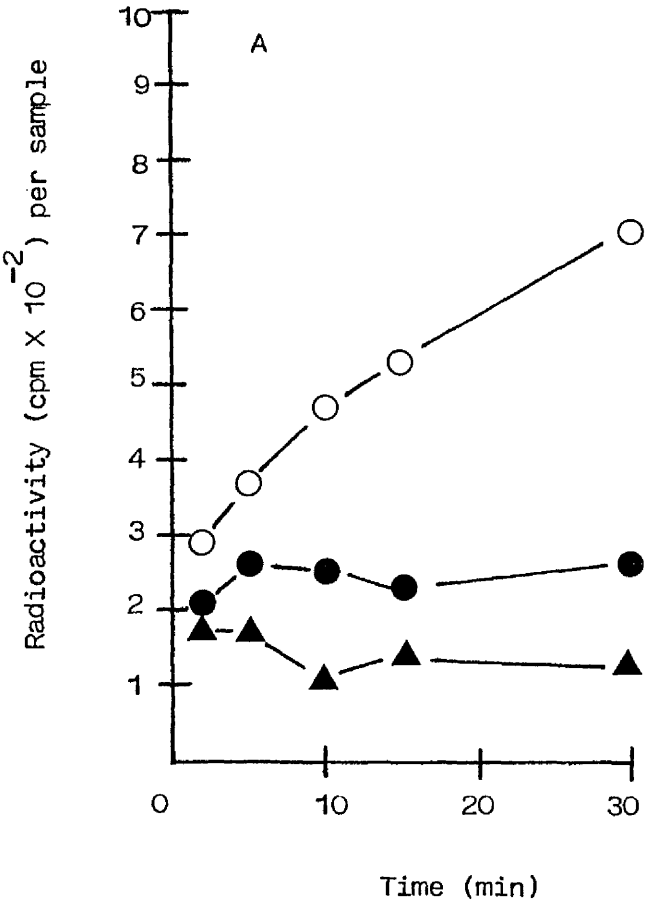
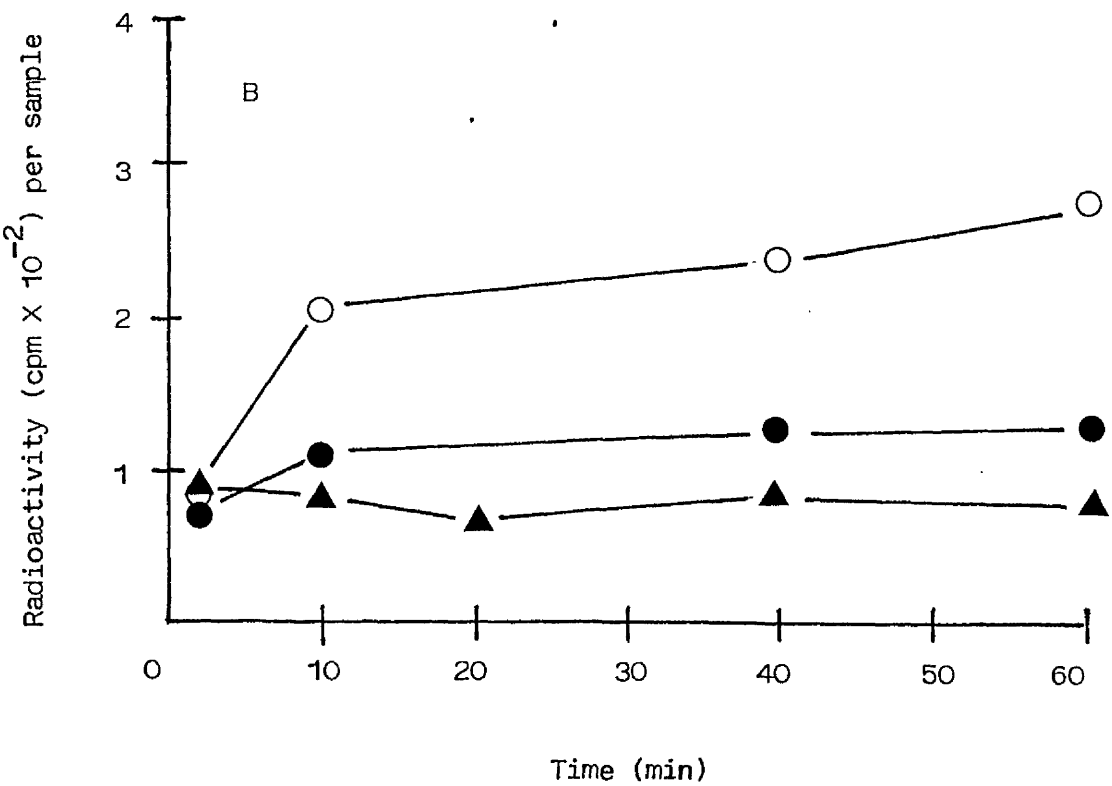

B
Radioactivity (cpm X 10^{-3}) per sample
2
1
0
0
10
20
30
40
50
60
Time (min)

Figure 19. Incorporation of $[^{14}C]$ - TdR into whole cells and acid-insoluble material during sporulation of the parent strain.

The parent strain was grown and initiated to sporulate in three cultures in an identical manner to strain but-32 (Fig. 17), except that the concentration of BUdR in the medium was 7.5 $\mu g ml^{-1}$ instead of 15 $\mu g ml^{-1}$. Incorporation of $[^{14}C]$-TdR into (A) whole cells and (B) acid-insoluble material was measured as in the previous experiment, except that incorporation into acid-insoluble material was monitored over a longer period of time than for whole cells.

Fig. 19


A
Radioactivity (cpm X 10^{-2}) per sample
10
9
8
7
6
5
4
3
2
1
0
10
20
30
Time (min)


B
Radioactivity (cpm X 10^{-2}) per sample
4
3
2
1
0
10
20
30
40
50
60
Time (min)

whole cells and acid-insoluble material, although it was different from that obtained with but-32. The difference was similar to that obtained previously (Fig. 10). The uptake in the presence of BUdR into both whole cells and acid-insoluble material followed more closely that of TdR (8.5 $\mu g ml^{-1}$) for the parent strain and TdR (1 $\mu g ml^{-1}$) for the But strain. It therefore seemed that the cells of but-32, both during growth and sporulation, discriminated against BUdR at the point of uptake into the cell rather than during DNA synthesis.

During sporulation of but-32 the rate of incorporation of radiolabel into acid-insoluble material in the presence of BUdR appeared constant over the sampling period, whereas the rate in either TdR concentration began to decrease after approximately 1½ to 2h (Fig. 17). During normal sporulation DNA replication must be terminated before the process can continue (Dunn et al, 1978), but this result suggested that BUdR might prolong DNA synthesis during sporulation of this strain, even though it may be partially excluded from the cell. It is not clear from the result of the same comparison using the parent strain (Fig. 19) if BUdR prolongs DNA synthesis during sporulation in this case as the levels of incorporation of $\left[^{14}C\right]$-TdR in the presence of BUdR were rather low during exposure of these cells to BUdR during both growth and sporulation. The effect of BUdR on viability in this case may have reduced the level of incorporation of $\left[^{14}C\right]$- TdR below the TdR (8.5 $\mu g ml^{-1}$) level.

To overcome this problem the parent strain was grown in CH medium containing only TdR (8.5 μgml^{-1}) before resuspension in SM with TdR (1 μgml^{-1}) plus BUdR (15 μgml^{-1}). The rate of incorporation of $\left[^{14}C\right]$ - TdR into acid-insoluble material in the presence of BUdR during sporulation was compared to the rates at two different TdR concentrations (Fig. 20 B). The same comparison was made for strain but-32 during sporulation (Fig. 20 A).

The patterns of incorporation were similar but not identical to those obtained previously (Fig.'s 17 and 19). In this case the rate of incorporation of $\left[^{14}C\right]$ - TdR into acid-insoluble material in the parent strain during sporulation in the presence of BUdR was almost half the rate achieved by but-32, but compared with the incorporation in TdR (1 μgml^{-1}), the relative degree of incorporation of $\left[^{14}C\right]$ - TdR in the presence of BUdR was greater than that obtained during growth (Fig. 10).

Therefore, the main conclusion from this work was that the tolerance to BUdR exhibited by strain but-32 probably derived from its ability to preferentially exclude BUdR from the cells when given a mixture of TdR and BUdR.

E. CsCl density gradient analysis of incorporation of BUdR into DNA

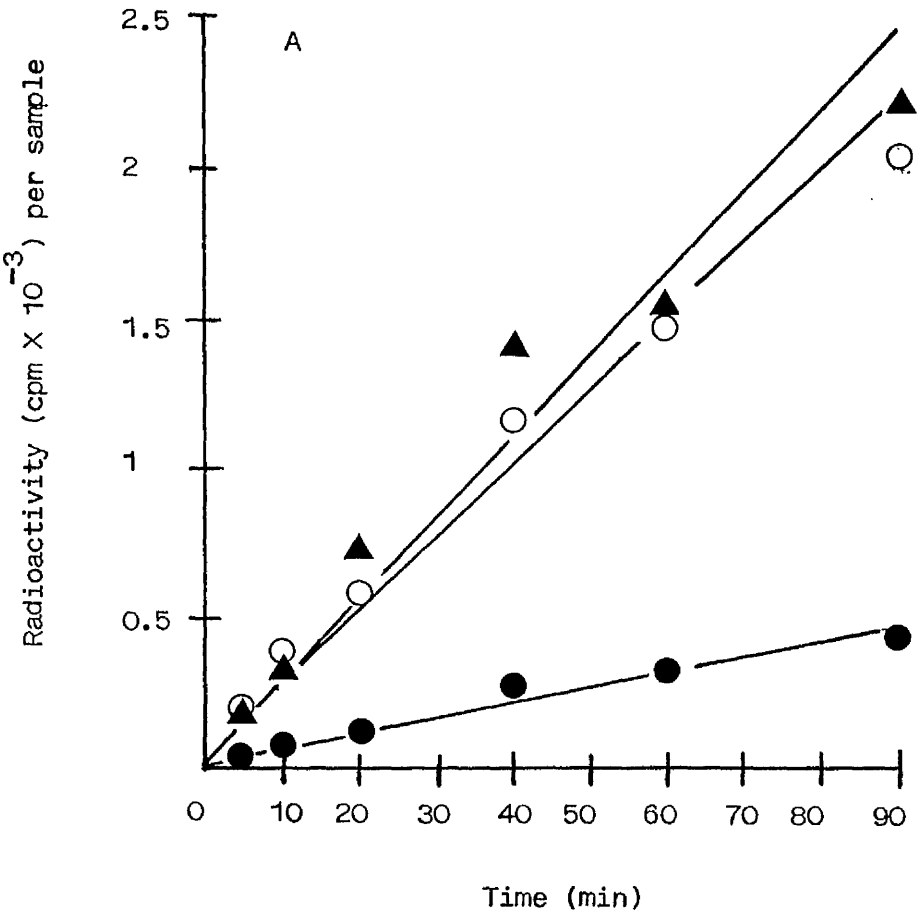
The results of the previous section suggested that BUdR was partially excluded from whole cells and DNA of the BUdR-

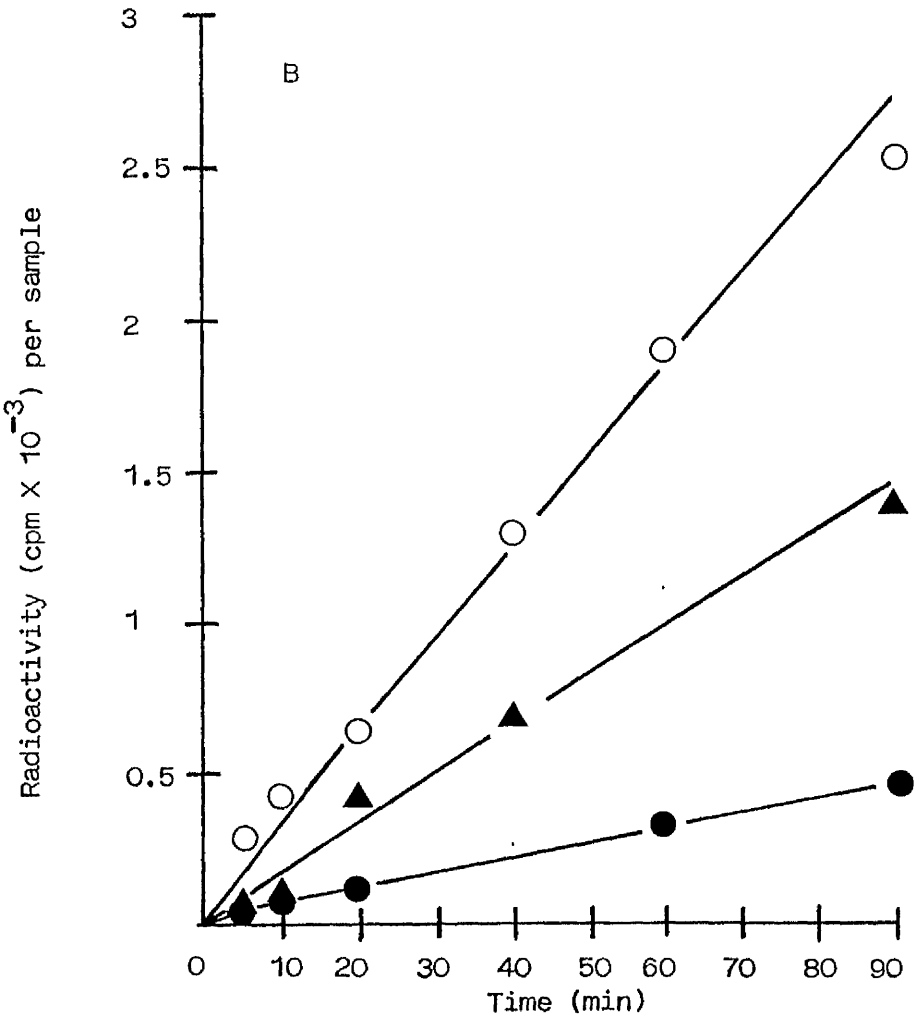
Figure 20. Incorporation of $[^{14}C]$- TdR into acid-insoluble material during sporulation of strain but-32 (A) and the parent strain (B).

Both strains were grown in parallel in CH medium (80 ml with TdR (8.5 $\mu g ml^{-1}$). At an E_{600nm} of 0.7 the cultures were divided into four 20 ml volumes, the cells harvested by centrifugation, and resuspended in SM (20 ml) containing $[^{14}C]$ - TdR (0.025 $\mu Ci ml^{-1}$, final concentration) and either TdR (8.5 $\mu g ml^{-1}$) (●), TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) (▲) or TdR (1 $\mu g ml^{-1}$) (○). The remaining 20 ml was used for measurement of incorporation of $[^{3}H]$ - BUdR into acid-insoluble material (see Fig. 25, results section III).

Incorporation of radioactivity into acid-insoluble material was measured at intervals after resuspension (as described in Materials and Methods, section VIII).

Fig. 20


A
Radioactivity (cpm X 10^{-3}) per sample
2.5
2
1.5
1
0.5
0
10
20
30
40
50
60
70
80
90
Time (min)


B
Radioactivity (cpm X 10^{-3}) per sample
3
2.5
2
1.5
1
0.5
0
10
20
30
40
50
60
70
80
90
Time (min)

tolerant strain, whereas the sensitive parent strain apparently did not exclude BUdR to as great an extent. To compare the degree of incorporation of the analogue into the DNA of both strains, measurements were made of the buoyant density in CsCl density gradients of DNA prepared from cells of both strains grown in the presence of BUdR (Fig. 21). In this experiment the BUdR concentration used was 15 $\mu g ml^{-1}$, which was inhibitory to growth of the parent strain. Therefore, both strains were grown to mid-exponential phase in TdR alone before transferring the cells to CH medium containing TdR plus BUdR, together with $[^{14}C]$- TdR. At intervals after transfer to BUdR medium, samples were removed for preparation of DNA. The buoyant density profiles of these samples were compared with those obtained using DNA prepared from the two strains transferred to growth medium containing only added TdR.

The results show that at each time interval after exposure to BUdR, but-32 DNA not only had more $[^{14}C]$- TdR incorporated than DNA prepared from the parent strain, but it was also at a lower buoyant density. The degree of BUdR substitution can be estimated from the difference in buoyant density between poly d(A-T) and poly d(A-BU), which is 200 $mg ml^{-1}$ (Wake and Baldwin, 1962). B. subtilis 168 DNA contains 28% TdR residues (Schildkraut et al, 1962), compared with 50% in poly d(A-T), and so complete substitution of BUdR for TdR should give a density increase of 112 $mg ml^{-1}$. The increase in buoyant density

Figure 21. The buoyant density in a CsCl gradient of DNA prepared from the parent strain and but-32 during growth in the presence of BUdR

Both strains were grown at 35°C in CH medium (100 ml) plus TdR (8.5 $\mu g ml^{-1}$) to an E_{600nm} of approximately 0.6. Two 30 ml volumes of each culture were removed, and the cells harvested by centrifugation. The pellets were resuspended in 30 ml pre-warmed CH medium plus the following additions :-

1) TdR (8.5 $\mu g ml^{-1}$) + [^{14}C] - TdR(0.025 $\mu Ci ml^{-1}$, final concentration),
2) TdR(1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + [^{14}C] - TdR (0.025 $\mu Ci ml^{-1}$, final concentration).

Incubation was continued at 35°C. 10ml samples were removed from each culture at intervals after resuspension and lysates prepared as in Materials and Methods, section VII (B), except that the DNA was not precipitated with ethanol. A sample of each lysate was centrifuged to equilibrium in a CsCl gradient, along with control DNA samples prepared from but-32 cells grown in the presence of [methyl-^{3}H] - TdR in either TdR or TdR + BUdR medium (see Materials and Methods, section VII).

(A) but-32 lysate prepared from TdR medium 45 min after addition of [^{14}C] - TdR.

(C), (E), and (G). but-32 lysates prepared from BUdR medium at 15, 30 and 45 min respectively after addition of [^{14}C] - TdR.

(B) parent strain lysate prepared from TdR medium 45 min after addition of $[^{14}C]$ - TdR.

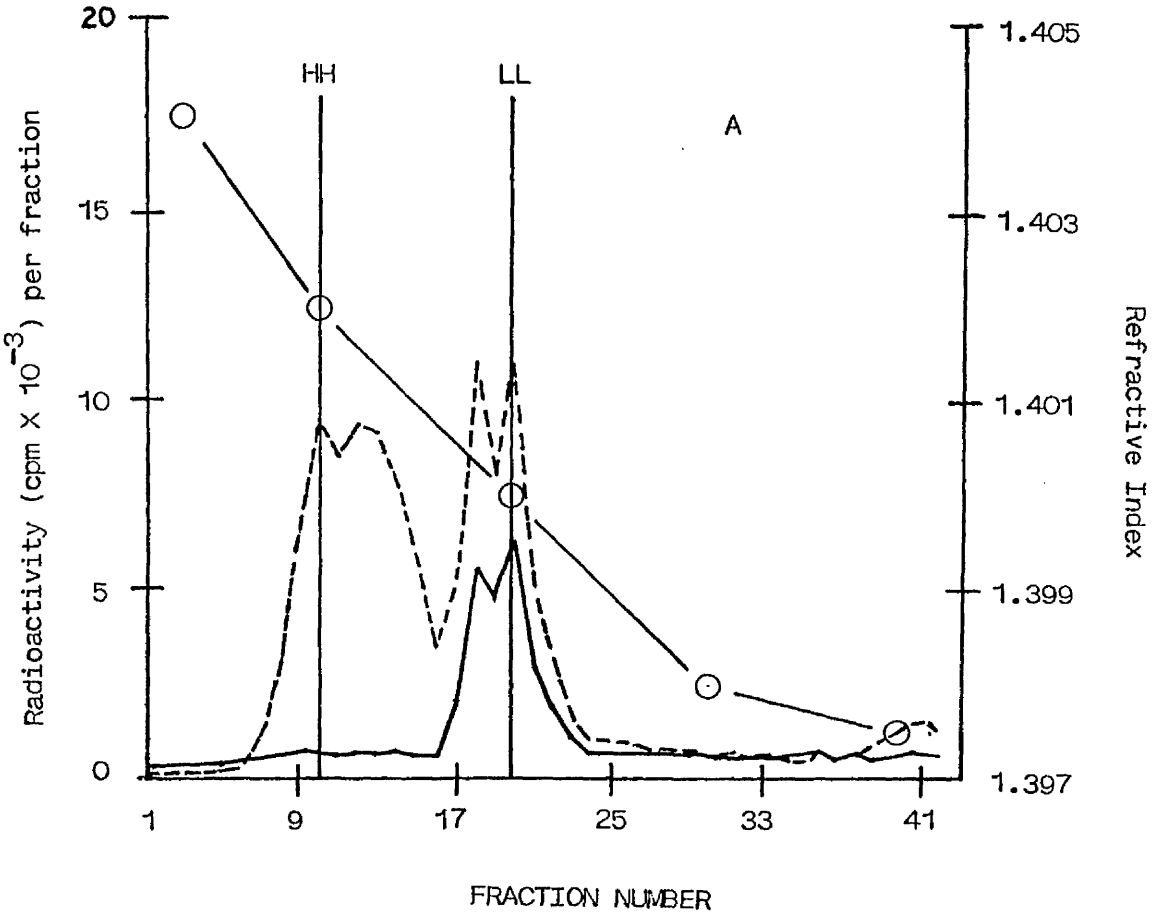
(D), (F), and (H). parent strain lysates prepared from BUdR medium 15, 30, and 45 min after addition of $[^{14}C]$ - TdR.

———— $[^{14}C]$ - TdR labelled test DNA

--------- $[^{3}H]$ - TdR labelled reference DNA (heavy-heavy (HH) and light-light (LL) density)

○ Refractive index of selected CsCl fractions

Fig. 21


20
15
10
5
0
HH
LL
A
1.405
1.403
1.401
1.399
1.397
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
1
9
17
25
33
41
FRACTION NUMBER

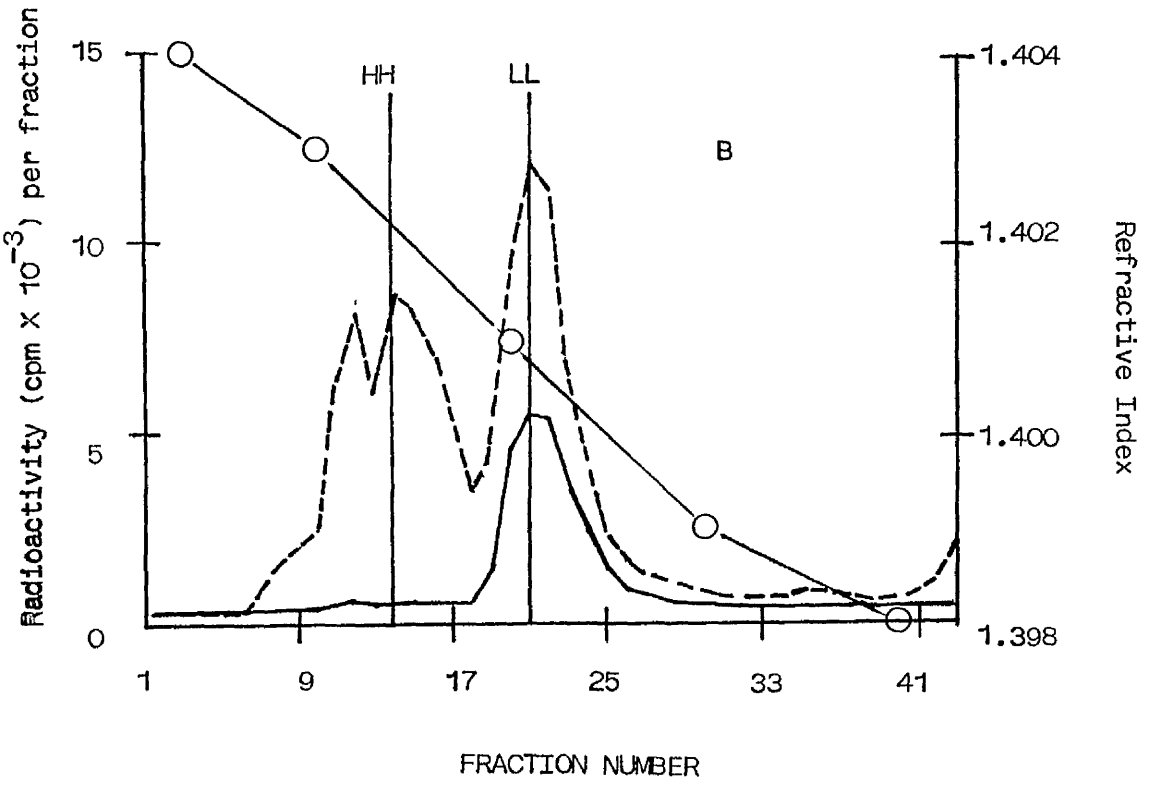
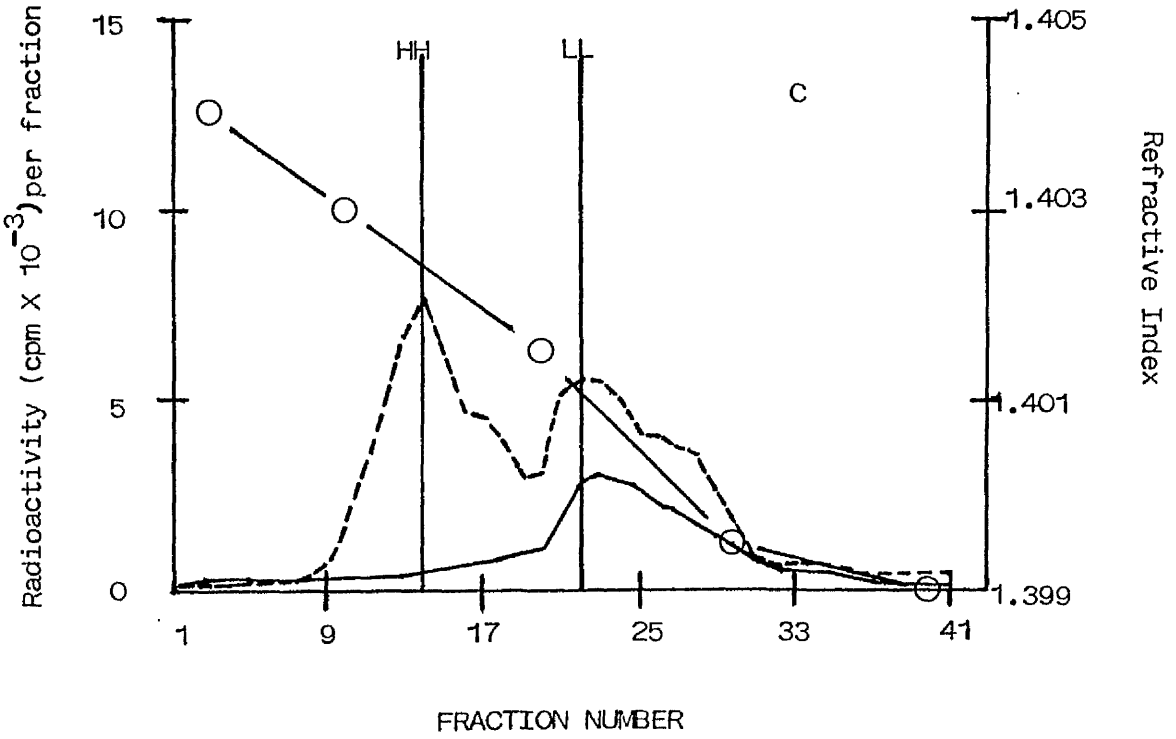

15
10
5
0
HH
LL
B
1.404
1.402
1.400
1.398
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
1
9
17
25
33
41
FRACTION NUMBER

Fig. 21 (cont.)


Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
HH
LL
C
15
10
5
0
1.405
1.403
1.401
1.399
1
9
17
25
33
41
FRACTION NUMBER

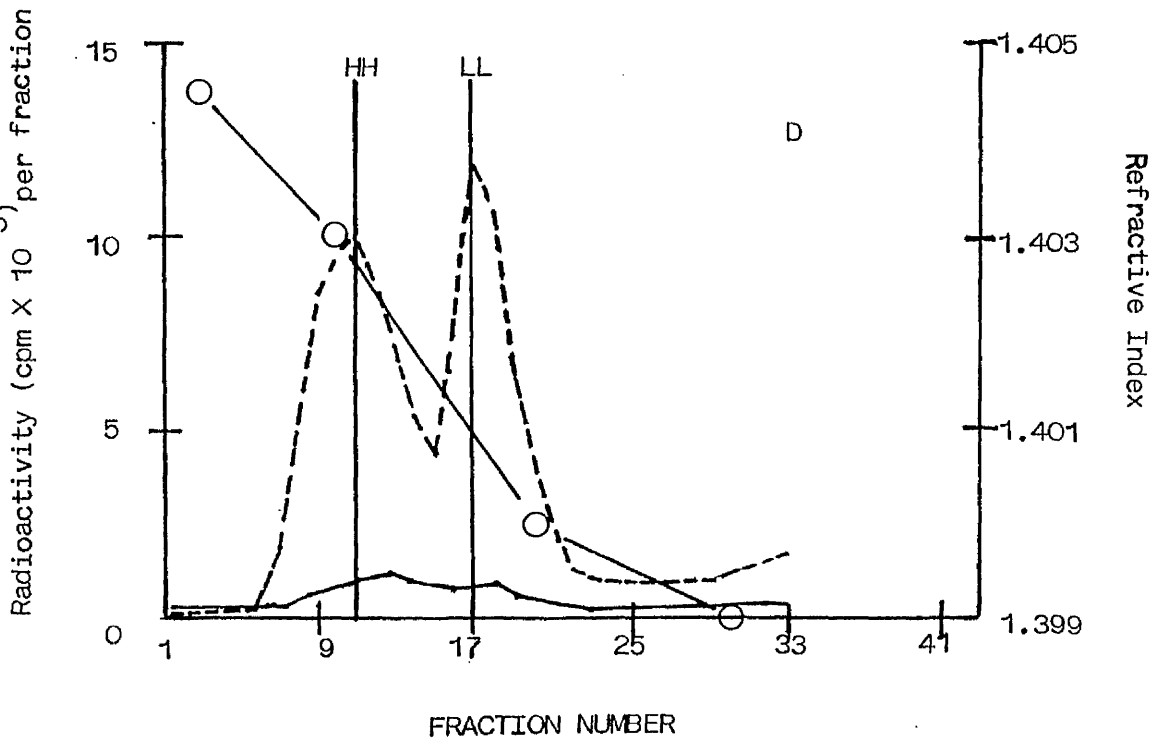
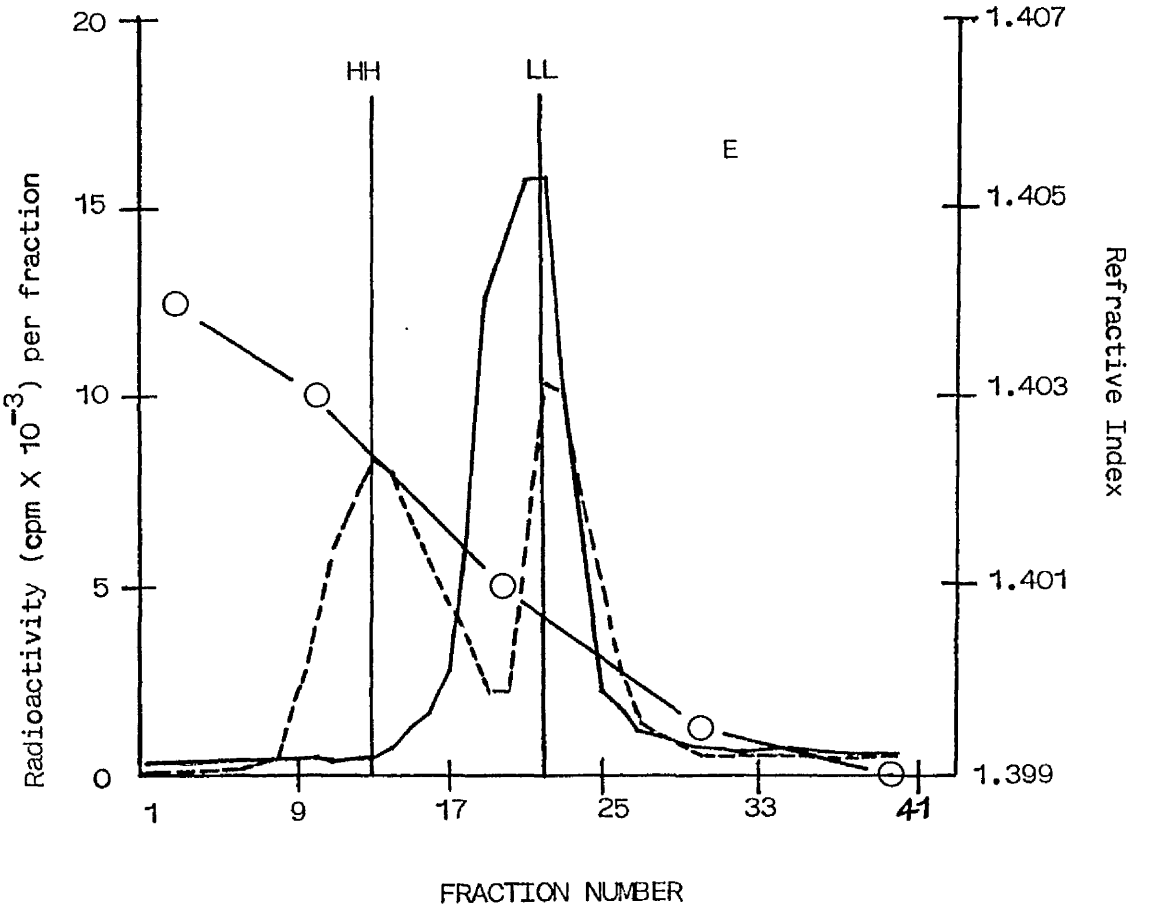

Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
HH
LL
D
15
10
5
0
1.405
1.403
1.401
1.399
1
9
17
25
33
41
FRACTION NUMBER

Fig. 21 (cont.)


HH
LL
E
20
15
10
5
0
1.407
1.405
1.403
1.401
1.399
1
9
17
25
33
41
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
FRACTION NUMBER

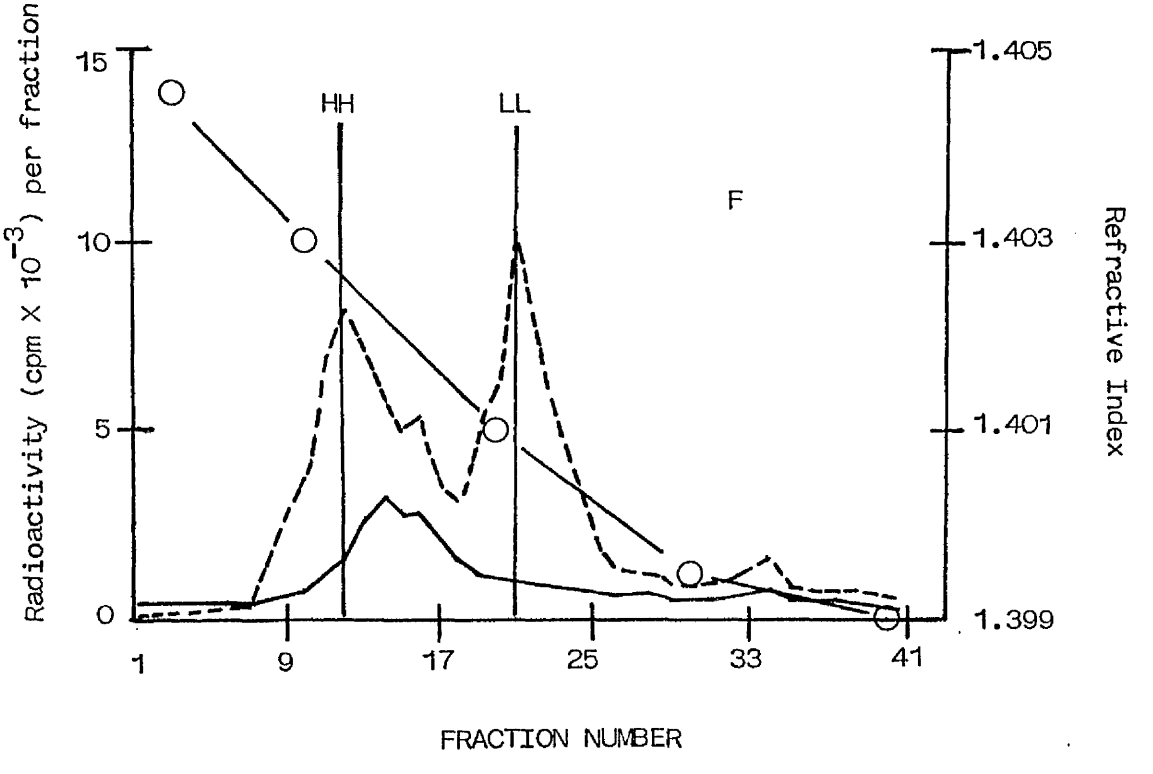
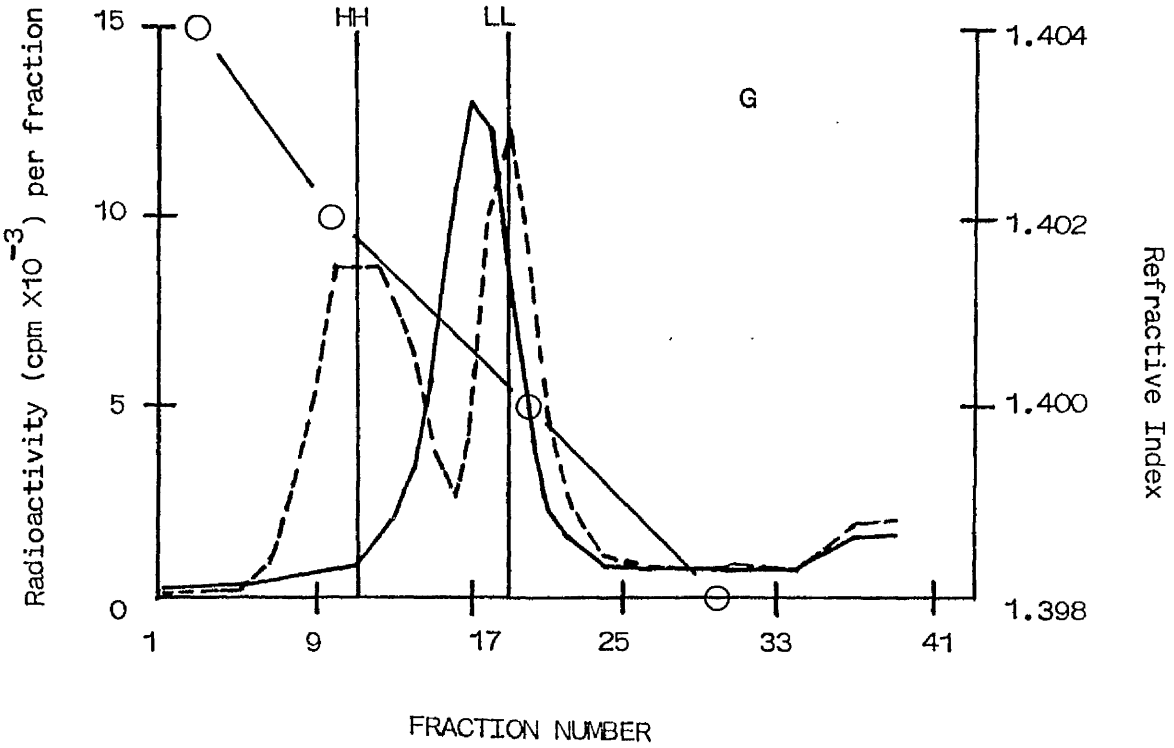
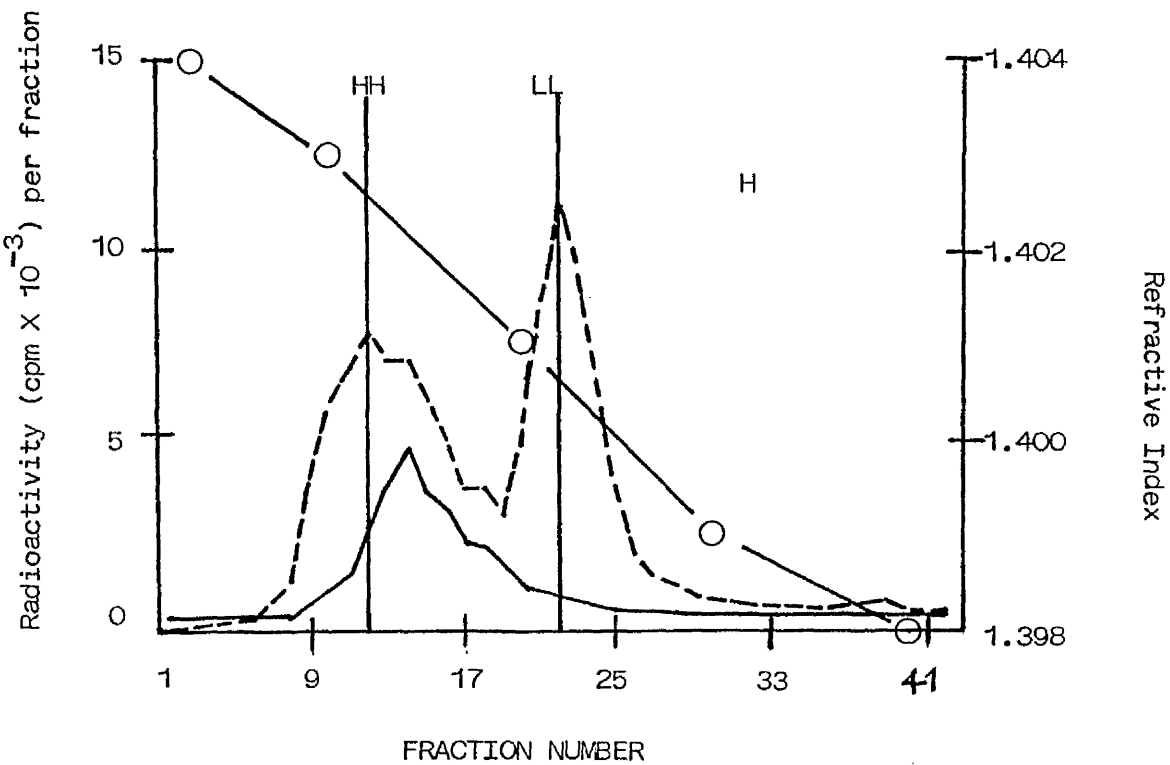

HH
LL
F
15
10
5
0
1.405
1.403
1.401
1.399
1
9
17
25
33
41
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
FRACTION NUMBER

Fig. 21 (cont.)


Radioactivity (cpm X10^{-3}) per fraction
15
10
5
0
HH
LL
G
1.404
1.402
1.400
1.398
Refractive Index
1
9
17
25
33
41
FRACTION NUMBER


Radioactivity (cpm X 10^{-3}) per fraction
15
10
5
0
HH
LL
H
1.404
1.402
1.400
1.398
Refractive Index
1
9
17
25
33
41
FRACTION NUMBER

of DNA prepared from the parent strain and but-32 in this experiment (Fig. 21) represents values of 15% and 6% substitution of BUdR for TdR residues respectively after 45 min in the presence of BUdR. These results therefore substantiated the earlier indication that tolerance to BUdR resulted in less incorporation of the analogue into DNA.

F. Marker-frequency analysis of DNA replication

During growth of B. subtilis DNA replication proceeds from a fixed origin in a bidirectional manner, and during exponential growth in a rich medium one or more rounds of chromosome replication may be initiated before termination of existing rounds of replication (see Introduction). This dichotomous replication results in a multiforked chromosome (Oishi et al, 1964). Therefore, under these conditions there must be on average a greater number of copies of genes, or markers, closer to the origin than the terminus of the bacterial chromosome. Gene or marker frequencies in DNA prepared from growing cells can be analysed by genetic transformation and used to estimate the average number of replication positions per chromosome (Sueoka and Yoshikawa, 1965).

The original parent strain, B. subtilis (thy A, thy B, trp C) has a number of replication origins during growth (Ephrati-Elizur and Borenstein, 1971), and bromouracil is thought to induce multi-forked replication (Nagley and Wake, 1969).

Therefore, it was decided to investigate the effect of BUdR on the extent of initiation of new rounds of DNA replication.

DNA was prepared from growing cells of the parent strain and but-32, and used to transform a recipient strain, Mu8u5u16 (pur A16, leu-8, met B5). The pur A16 marker is located near the origin of replication, and represents a requirement for adenine. The met B5 marker is located near the terminus. The ratio of these markers correlates with the average number of replication positions per chromosome (Sueoka and Yoshikawa, 1965). In these experiments DNA was prepared from cells during growth in the presence of BUdR, and the ratio of pur^+ to met^+ transformants compared to the ratio obtained for DNA from cells grown in TdR alone.

In the first experiment (Table 9) the effect of slowing down the rate of DNA replication on the marker ratios was also examined by using the drug 6-(p-hydroxyphenylazo) uracil (HPUra), a specific inhibitor of DNA synthesis in B. subtilis, at a concentration not inhibitory to growth (see results section III). Samples were removed from the cultures of both strains at two points during growth, early and late exponential phase. The transformations were performed at least three times per sample, and the mean values are presented. These values were also standardised with respect to the ratios obtained for spore DNA. Spores of B. subtilis contain completed chromosomes (Oishi et al,

TABLE 9. Marker-frequency analysis of DNA replication during growth of but-32 and the parent strain.

Both strains were grown in CH medium (100 ml) supplemented with A) TdR(8.5 μgml^{-1}), B) TdR(1 μgml^{-1})+BUdR(15 μgml^{-1}) or C) TdR(8.5 μgml^{-1})+HPUra(0.5 μgml^{-1}). Samples (10 ml) were removed for extraction of DNA at $E_{600\ nm}$ readings of 0.4 and 0.8 (Early (E) and late (L) exponential phase). The results of 3 separate transformations, with the mean values, are presented, along with standardised means, which are the mean values divided by the *pur*$^+$/*met*$^+$ ratio obtained for spore DNA (see text).

Strain	Medium	Doubling time (min)	Sampling point (E or L)	*pur*$^+$/*met*$^+$ transformants			Mean ratios	Standardised mean ratios
				Exp 1	Exp 2	Exp 3		
but-32	A	39	E	2.9	5.4	9.4	5.9	4.3
			L	6.6	4.6	10.1	7.1	5.1
	B	48	E	3.8	1.6	1.9	2.4	1.7
			L	6.0	3.8	6.9	5.6	4.1
	C	39	E	4.8	4.4	7.9	5.7	4.1
			L	11.1	2.3	8.0	7.1	5.1
parent	A	33	E	5.9	3.7	11.9	7.2	5.5
			L	11.1	3.0	11.1	8.4	6.5
	B	90 → ∞	E	2.9	2.5	3.6	3.0	2.3
			L*	-	-	-	-	-
	C	30	E	5.3	2.9	4.9	4.3	3.3
			L	10.6	8.3	6.4	8.4	6.5

*The parent strain did not grow to the required E_{600nm} in BUdR medium, therefore no "late" sample was taken (-). The "early" sample was taken at a reading of 0.4. These cells grew slowly until this point, after which the E_{600nm} decreased.

1964; Sargent, 1980 b), and the *pur*$^+$/ *met*$^+$ marker ratio obtained with spore DNA from each strain was used as a correction factor to standardise the ratios obtained during growth to a value of 1.0 for spore DNA. DNA prepared from spores of the wild-type 168 (*trp C*) strain gave a *pur*$^+$/ *met*$^+$ ratio of 0.99 (average of three determinations) whereas DNA from spores of the parent strain and *but-32* formed in the presence of TdR alone gave ratios of 1.30 and 1.38 respectively (average of three determinations each). The discrepancy between the ratios of the wild-type and the TdR-requiring strains is probably due to a lack of isogenicity around the *met B* region in the latter, which lowers transformation efficiency at the *met B5* locus (Callister and Wake, 1974).

Although the marker ratios obtained varied from experiment to experiment, for a particular sampling time, the mean *pur*$^+$/ *met*$^+$ ratios for *but-32* grown in the presence of BUdR were not higher than those obtained in TdR (8.5 $\mu g ml^{-1}$) alone, suggesting that the number of replication positions in DNA of this strain is not increased by BUdR. In addition, although BUdR did not allow much growth of the parent strain the mean marker ratio of DNA prepared from cells sampled at an E_{600nm} of 0.4 was lower than that of the control at either sampling time. HPUra did not affect the growth rate of either strain when added at 0.5 $\mu g ml^{-1}$, and had little effect on the marker ratios compared to the control values.

It was apparent that *pur*$^+$/*met*$^+$ ratios of DNA prepared from cells sampled later during growth were consistently higher than those given by DNA from cells sampled at an earlier stage. This variation in *pur*$^+$/*met*$^+$ ratios was also seen in a somewhat different experiment, using the same strains (Table 10). Instead of sampling at two points during the exponential phase of growth, a comparison was made between the *pur*$^+$/ *met*$^+$ ratios of DNA prepared from cells removed from the cultures just prior to centrifugation during mid-exponential growth, and at the point of resuspension in sporulation medium, t_{zero}. The effect on the ratios of lowering the TdR concentration to 1 μgml^{-1} was also determined.

As in the previous experiment, there appeared to be little difference between the marker ratios of DNA prepared from cells in the presence or absence of BUdR. However, in this case there was no uniform increase in the marker ratios between the two sampling points, although they did increase slightly for strain *but-32* in all media except that containing HPUra.

The addition of a low concentration of TdR, 1 μgml^{-1}, although allowing an almost normal growth rate for both strains, almost doubled the values of the marker ratios obtained from cells grown with the higher TdR concentration (8.5 μgml^{-1}). A similar effect of a limiting thymine concentration has been reported previously (Ephrati-Elizur and Borenstein, 1971).

TABLE 10. Marker-frequency analysis of DNA replication in strain but-32, and the parent strain during growth, and at the point of resuspension in SM.

Both strains were grown in CH medium (20 ml) supplemented with (A) TdR(8.5 μgml^{-1}), (B) TdR(1 μgml^{-1})+BUdR(15 μgml^{-1}), (C) TdR(8.5 μgml^{-1})+HPUra(0.5 μgml^{-1}) or (D) TdR(1 μgml^{-1}). At an E_{600nm} of 0.7 samples(10 ml) were removed for extraction of DNA. The parent strain only grew slowly to a E_{600nm} of 0.41 in culture (B), but a sample was removed at this point and the remaining cells, together with the cells in the remaining 10 ml volumes of all the other cultures were harvested by centrifugation and suspended in SM(10 ml) containing the same additions. DNA was extracted from the resuspended cells at this point (t_{zero}). The mean values and standardised means (see legend to Table 9) for 3 or 4 separate transformations are presented.

Strain	Medium	Doubling time (min)	Sampling point	pur+/met+ transformants				Mean pur+/met+ ratios	Standardised mean ratios
				Exp.1	Exp.2	Exp.3	Exp.4		
but-32	A	36	Growth	3.3	3.2	3.4	4.8	3.7	2.7
			t_{zero}	-	3.3	4.9	7.0	5.0	3.6
	B	54	Growth	5.1	2.9	3.4	4.4	4.0	2.9
			t_{zero}	-	3.9	7.0	3.9	4.9	3.6
	C	42	Growth	4.0	5.0	2.5	4.9	4.1	3.0
			t_{zero}	-	4.7	3.3	4.0	4.0	2.9
	D	42	Growth	4.4	7.2	5.3	7.6	6.1	4.4
			t_{zero}	-	4.0	10.0	7.2	7.1	5.1

TABLE 10. (contd.)

Strain	Medium	Doubling time (min)	Sampling point	pur$^+$/met$^+$ transformants				Mean pur$^+$/met$^+$ ratios	Standardised mean ratios
				Exp.1	Exp.2	Exp.3	Exp.4		
parent	A	36	Growth	-	3.4	2.5	3.9	3.3	2.5
			t_{zero}	-	3.5	2.7	3.6	3.3	2.5
	B	114 → ∞	Growth	3.0	3.1	-	3.8	3.3	2.5
			*t_{zero}	2.2	-	2.2	2.4	2.3	1.8
	C	48	Growth	-	6.6	2.9	7.2	5.6	4.3
			t_{zero}	-	5.0	1.8	6.0	4.3	3.3
	D	48	Growth	-	5.9	4.2	5.9	5.3	4.1
			t_{zero}	-	10.6	2.7	4.6	6.0	4.6

(-) not determined;

* < 100 transformants per plate

It would seem from this result that BUdR, in the presence of TdR (1 $\mu g ml^{-1}$), was not creating conditions of limiting thymidine whereby the cells would compensate for a consequent decrease in the rate of DNA replication by increasing the number of replication forks. This would in turn produce a higher *pur*$^+$/ *met*$^+$ ratio from the DNA on transformation.

The selection plates used in the latter two experiments contained medium lacking TdR, and as the *met B* locus is linked to the *thy B* locus (Wilson *et al*, 1966) it was thought that the *pur*$^+$/ *met*$^+$ ratios obtained in these experiments may have been artificially increased by discrimination against *thy*$^-$ recombinants among the *met*$^+$ transformants. Therefore, the effect of including TdR in the selective media was examined (Table 11). The results of two separate transformations carried out in parallel suggested a clear difference in the marker ratios obtained with and without TdR in the selection medium. On average, the number of *met*$^+$ transformants, relative to *pur*$^+$ transformants, obtained when TdR was included, was approximately double the relative number of *met*$^+$ to *pur*$^+$ transformants obtained in the other set of transformations, when TdR was not included in the selection medium. It could be argued therefore, that the lack of TdR on selection plates explains why a value of 1.0 was not obtained for the *pur*$^+$/ *met*$^+$ ratios of DNA prepared from spores of *but-32* and the parent strain, both TdR-requiring strains. If the values for spore DNA from these two strains had been

TABLE 11. Marker frequency analysis of DNA replication during growth of but-32.

B. subtilis but-32 was grown in four separate 20 ml volumes of CH medium including (A) TdR (8.5 μgml^{-1}), (B) TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), (C) TdR (8.5 μgml^{-1}) + HPUra (0.5 μgml^{-1}) or (D) TdR (1 μgml^{-1}). During exponential growth duplicate samples (each 10 ml) were removed for extraction of DNA. These DNA preparations were used in two transformation experiments where pur$^+$ and met$^+$ transformants were selected separately, but in one set of transformations (b) TdR (20 μgml^{-1}) was added to all selective media whereas in the other it was not added (a).

Medium	Doubling time (min)	Sample	pur$^+$ transformants		met$^+$ transformants		pur$^+$/met$^+$ ratio	
			a	b	a	b	a	b
A	36	1	325	260	105	203	3.1	1.3
		2	355	321	122	254	2.9	1.3
B	42	1	504	284	140	191	3.6	1.5
		2*	-	-	-	-	-	-
C	45	1	434	436	194	254	2.2	1.7
		2	524	311	195	155	2.7	2.0
D	42	1	300	199	121	139	2.5	1.4
		2	294	161	135	140	2.2	1.2

* this sample was lost during preparation.

determined using selection plates with TdR, they would probably have decreased from approximately 1.3 to a value closer to 1.0.

However, regardless of the effect of TdR on the number of *met*$^+$ transformants detected, in both transformations the values for the marker ratios of DNA from cells exposed to BUdR, HPUra (0.5 μgml^{-1}) or a low TdR concentration were relatively close to the values for cells grown in TdR (8.5 μgml^{-1}). The result for BUdR- or HPUra-treated cells agreed with those obtained previously (Tables 9 and 10) but, surprisingly, similar ratios were obtained with TdR (1 μgml^{-1}), which did not agree with the higher ratios obtained previously (Table 10).

G. A comparison of the effects of BUdR and bromouracil on growth

Finally, the effects of bromouracil (BU) on the rate of growth and the sporulation capacity of the parent strain and *but-32* were compared to the effects of BUdR (Table 12). Whereas BUdR inhibited growth of the parent strain, BU had a much less dramatic effect, and these cells grew exponentially, although more slowly than cells in TdR alone, to a point where they could have been transferred to resuspension medium. Strain *but-32* grew as normal in the presence of either BU or BUdR at the concentrations used. However, like BUdR, BU inhibited sporulation of both strains without drammatically affecting the viability

TABLE 12. The effects of BUdR and BU on growth and sporulation of the parent strain and but-32.

Both strains were grown as follows. Three cultures were grown in CH medium (20 ml) + TdR (8.5 μgml^{-1}) for subsequent sporulation in SM (20 ml) containing (A) TdR (8.5 μgml^{-1}), (B) TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) or (C) TdR (1 μgml^{-1}) + BU (15 μgml^{-1}). Samples of these cultures were removed at t_3 for a viable cell count (see Materials and Methods, section IVC), and the level of sporulation determined at t_{20} (see Materials and Methods, section IVB). Figures in parentheses represent the percentage values for sporulation levels relative to the control cultures. Also, four other cultures (see overleaf) were grown in parallel, two containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), and two containing TdR (1 μgml^{-1}) + BU (15 μgml^{-1}). The cells in these cultures were not induced to sporulate.

GROWTH			SPORULATION				
Additions to CH medium	Doubling time (min)		Additions to SM	Spores ml^{-1} (X 10^{-6}) at t_{20}		Viable cells ml^{-1} (X 10^{-6}) at t_3	
	but-32	parent		but-32	parent	but-32	parent
TdR (8.5 μgml^{-1})	42	39	TdR (8.5 μgml^{-1})	40 (100)	38 (100)	424	206
TdR (8.5 μgml^{-1})	42	36	TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	1.44 (4)	0.001 (0.02)	198	270
TdR (8.5 μgml^{-1})	42	39	TdR (1 μgml^{-1}) + BU (15 μgml^{-1})	3.22 (8)	0.001 (0.02)	272	508

TABLE 12. (contd.)

GROWTH		
Additions to CH medium	Doubling time (min)	
	but-32	parent
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	42	120 → ∞
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	48	120 → ∞
TdR (1 μgml^{-1}) + BU (15 μgml^{-1})	42	60
TdR (1 μgml^{-1}) + BU (15 μgml^{-1})	42	60

of cells during sporulation.

A similar effect was obtained in another experiment, where the growth rate and sporulation capacity of the parent strain in CH medium containing BU or BUdR were examined (Table 13). The rate of growth was relatively unaffected by BU compared to the effect of BUdR, but sporulation was inhibited by both, although the level of sporulation in CH medium containing TdR (1 $ugml^{-1}$) was very low compared to typical values in resuspension medium, and as such the relative inhibitory action of BU and BUdR on sporulation was weaker. TdR at 1 μgml^{-1} itself does not inhibit sporulation when the control level of sporulation in TdR (8.5 μgml^{-1}) is reasonably high (see Table 14, results section III).

Laird and Bodmer (1967) noted that the preference for thymine over BU was greater than the preference for TdR over BUdR in a thymine-requiring mutant of B. subtilis 168, and this, coupled with the fact that TdR was present in the medium instead of thymine, may have relieved the inhibitory effect of BU on growth of the parent strain in these experiments.

III. The basis of inhibition of sporulation by BUdR

A. The effect of BUdR on sporulation

The inhibitory effect of BUdR on growth of the parent strain, and the relative insensitivity of the isogenic BUdR-tolerant strain but-32 to this inhibition, were examined in the

TABLE 13. The effects of BUdR and BU on growth rate and sporulation capacity of the parent strain in CH medium.

The cells were grown and allowed to sporulate in CH medium (20ml) containing the additions shown. Figures in parentheses refer to a separate experiment in which only the growth rate was measured.

Additions to CH medium	E_{600} doubling time (min)	Spores ml^{-1} ($X10^{-2}$) at t_{20}
TdR (1 $\mu g ml^{-1}$)	42 (33)	378
TdR (1 $\mu g ml^{-1}$) + BU (15 $\mu g ml^{-1}$)	48 (45)	168
TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$)	120→∞ (140→∞)	108

previous section. In addition, data was presented in Table 12 to indicate the inhibitory effect of BUdR (and BU) on sporulation of this strain. In a similar experiment (Table 14), it was found that sporulation of but-32 was also inhibited when the cells were grown and resuspended in the presence of BUdR. In addition, when these cells were grown in the presence of BUdR, but initiated to sporulate in TdR (8.5 μgml^{-1}) alone, a normal sporulation level was achieved. Inhibition of sporulation in BUdR medium was not due to a limiting TdR concentration, as the sporulation capacity of but-32 was normal in SM with TdR at 1 μgml^{-1} instead of 8.5 μgml^{-1}, the normal control level.

This analysis was extended to a comparison of the effect of BUdR on the sporulation capacity and viability of cells during sporulation of the parent strain and but-32, together with the effect on sporulation and cell viability of adding an inhibitor of DNA synthesis, HPUra, to the medium at a concentration which only slows down the rate of DNA synthesis in B. subtilis 168 (Anderson and Ganesan, 1976). Sporulation was initiated by the replacement method in all the experiments carried out for this section of work (see Materials and Methods, section IV).

BUdR reduced the final yield of spores in resuspension cultures of both strains compared to control cultures with TdR alone, without significantly affecting the viability of the cells during sporulation (Table 15). The inhibitory effect of

TABLE 14. The effect of BUdR on sporulation of but-32.

B. subtilis but-32 was grown in CH medium (20 ml) with TdR (8.5 μgml^{-1}) (three cultures), TdR (1 μgml^{-1}) (one culture) and TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (two cultures) to an E_{600nm} of 0.7. The cells grown in TdR (8.5 μgml^{-1}) cultures were harvested by centrifugation and resuspended in SM containing either TdR (8.5 μgml^{-1}), TdR (1 μgml^{-1}) or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). The cells grown in TdR (1 μgml^{-1}) were resuspended in SM + TdR (1 μgml^{-1}). The cells from one of the cultures containing BUdR were initiated to sporulate in SM + TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), whereas the cells from the other BUdR culture were resuspended in SM + TdR (8.5 μgml^{-1}). The level of sporulation in all cultures was determined at t_{20} (see Materials and Methods, section IV B). Figures in parentheses refer to the percentage sporulation, by comparison with a culture grown and sporulated in medium with TdR (8.5 μgml^{-1}).

Growth		Sporulation	
Additions	Doubling time (min)	Additions	Spores ml^{-1} ($X10^{-4}$) at t_{20}
TdR (8.5 μgml^{-1})	36	TdR (8.5 μgml^{-1})	532 (100)
TdR (8.5 μgml^{-1})	42	TdR (1 μgml^{-1})	590 (111)
TdR (8.5 μgml^{-1})	36	TdR (1 μgml^{-1})+ BUdR (15 μgml^{-1})	55 (10)
TdR (1 μgml^{-1})	42	TdR (1 μgml^{-1})	564 (106)
TdR (1 μgml^{-1})+ BUdR (15 μgml^{-1})	42	TdR (1 μgml^{-1})+ BUdR (15 μgml^{-1})	38 (7)
TdR (1 μgml^{-1})+ BUdR (15 μgml^{-1})	42	TdR (8.5 μgml^{-1})	630 (118)

TABLE 15. The effect of BUdR and a sub-inhibitory concentration of HPUra on the growth rate, viability during sporulation and sporulation capacity of but-32 and the parent strain.

Both strains were grown in CH medium (100 ml) containing the additions shown. Three parallel cultures (100 ml) containing only TdR(8.5 μgml^{-1}), were used to grow the cells of both strains before initiating sporulation in equal volumes of SM containing the additions shown, to determine the specific effect of these compounds on sporulation. The cultures used for measurement of the growth rates were also used for marker-frequency analysis of DNA replication during growth (see Table 9, results section II, part F). Figures in parentheses refer to the percentage sporulation relative to the control cultures.

Strain	Additions	Doubling time (min) in CH medium	Viable count during sporulation (c.f.u.$ml^{-1}X10^{-6}$)				Sporulation (spores $ml^{-1}X10^{-6}$ at t_{20})
			t_{zero}	t_2	t_4	t_6	
but-32	TdR(8.5 μgml^{-1})	39	186	440	550	500	32 (100)
	TdR(1 μgml^{-1})+BUdR(15 μgml^{-1})	48	166	640	464	468	3.56(11)
	TdR(8.5 μgml^{-1})+HPUra(0.5 μgml^{-1})	39	218	642	540	524	11 (35)
parent	TdR(8.5 μgml^{-1})	33	116	258	374	422	136 (100)
	TdR(1 μgml^{-1})+BUdR(15 μgml^{-1})	$120 \rightarrow \infty$	140	251	332	576	3.26 (2)
	TdR(8.5 μgml^{-1})+HPUra(0.5 μgml^{-1})	30	172	314	406	488	40 (29)

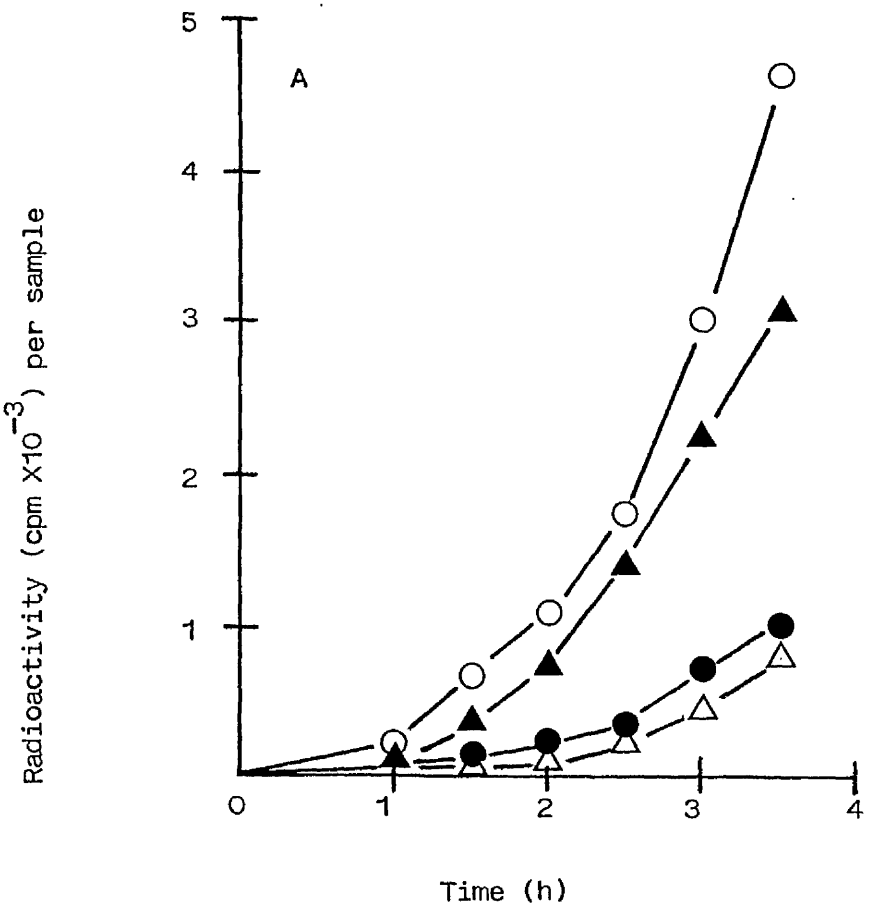
BUdR on sporulation of but-32 was relatively weak in this experiment, reducing the level of sporulation to only 10% of the control value. However, sporulation in the control culture in this experiment was also rather low. On the other hand, sporulation of the parent strain was reduced to 2% of the level in TdR alone. Addition of HPUra at 0.5 $\mu g ml^{-1}$ had no effect on the growth rate or viability during sporulation of both strains, but appeared to decrease the rate of incorporation of $[^{14}C]$- TdR into acid-insoluble material during growth and sporulation of both strains (Fig.'s 22 and 23). The inhibitory effect of HPUra at this concentration on DNA synthesis during sporulation of the parent strain was more marked than in strain but-32, although even in this strain HPUra inhibited the relative rate of DNA replication to a greater extent during sporulation than during growth. The effect of HPUra (at 0.5 $\mu g ml^{-1}$) on DNA synthesis was also associated with inhibition of sporulation, lowering the spore incidence to 35% of the control value in TdR alone for but-32, and to 29% of the control value for the parent strain. This result was unexpected, as only slight inhibition of sporulation was observed when the wild-type strain was initiated to sporulate at a similar HPUra concentration (Young and Jeffs, 1978). It was important, however, in indicating that sporulation was apparently inhibited by merely decreasing the rate of DNA replication during the process.

The few spores that do form in the presence of BUdR are

Figures 22 and 23. Incorporation of [^{14}C] - TdR into acid-insoluble material during (A) growth and (B) sporulation of but-32 (Fig. 22) and the parent strain (Fig. 23).

Both strains were grown in CH medium (20 ml) containing TdR (8.5 μgml^{-1}) (●), TdR (8.5 μgml^{-1}) + HPUra (0.5 μgml (△), TdR (1 μgml^{-1}) (0) or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (▲). [^{14}C] - TdR (0.025 $\mu Ciml^{-1}$, final concentration) was added to all cultures at the start of the experiment. As the parent strain grew only poorly in TdR + BUdR, one extra culture containing TdR (8.5 μgml^{-1}) was grown for subsequent initiation of sporulation in BUdR medium. After growth of all cultures, sporulation was initiated in SM (20 ml) with the same additions, plus [^{14}C] - TdR (0.025 $\mu Ciml^{-1}$, final concentration). The cells in the extra culture of the parent strain were resuspended in SM with TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). Incorporation of radioactivity intc acid-insoluble material was measured throughout both growth and sporulation.

Fig. 22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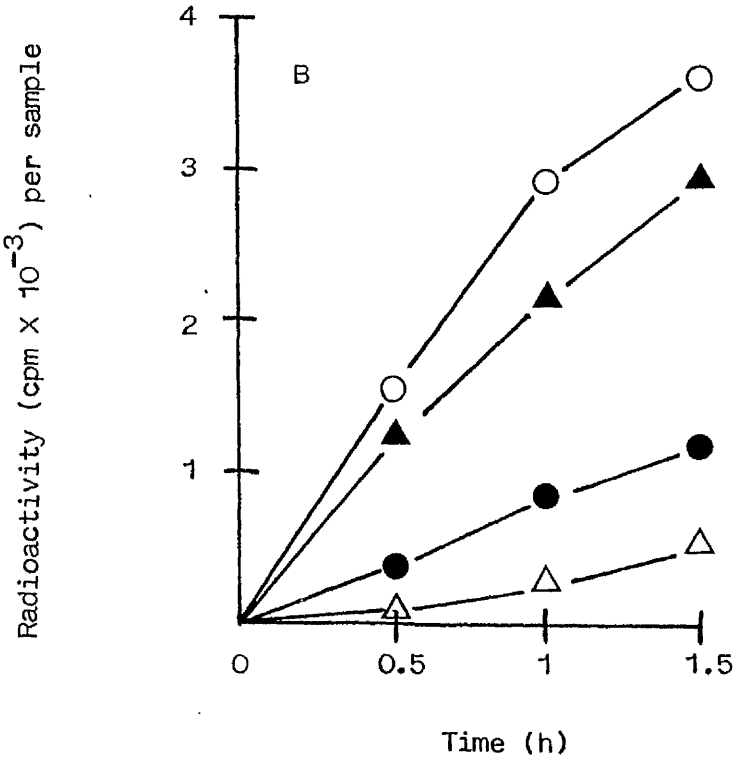
5
4
3
2
1
0
1
2
3
4
Time (h)


B
Radioactivity (cpm X 10^{-3}) per sample
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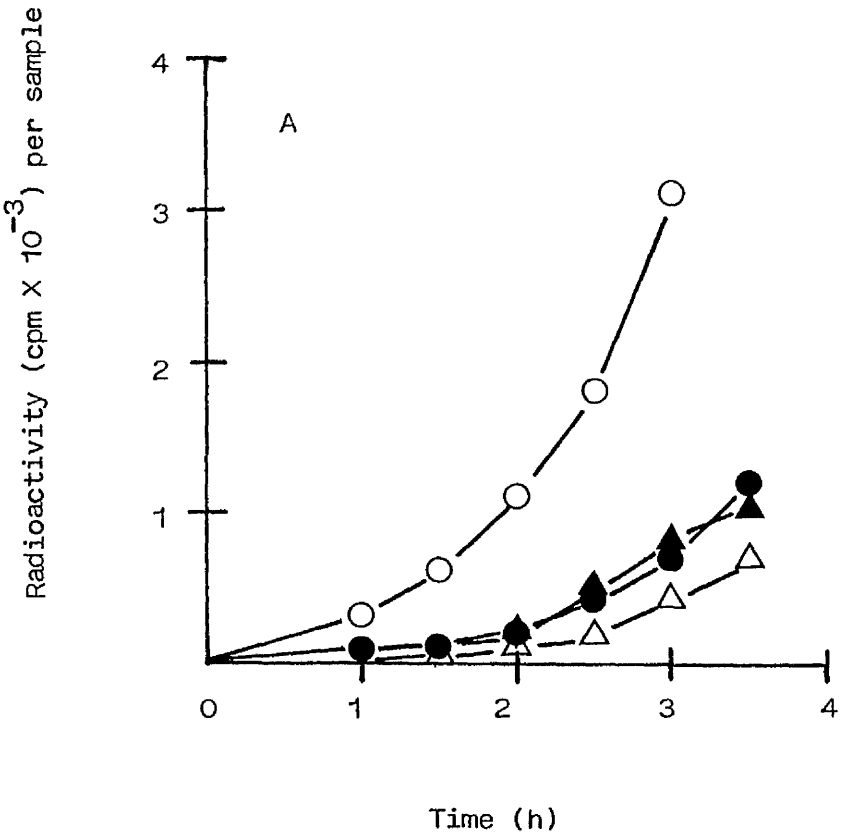
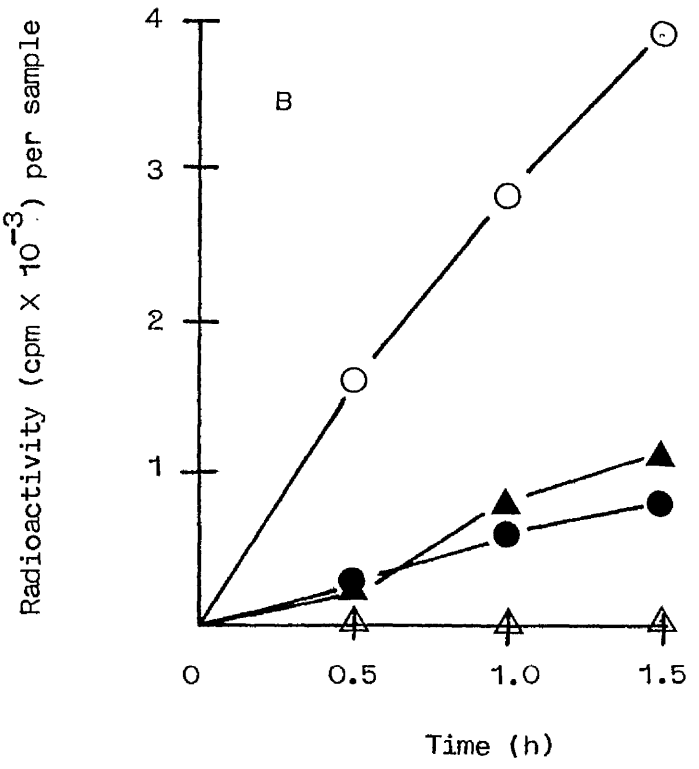
3
2
1
0
0.5
1
1.5
Time (h)

Fig. 23


A
Radioactivity (cpm X 10^{-3}) per sample
4
3
2
1
0
1
2
3
4
Time (h)


B
Radioactivity (cpm X 10^{-3}) per sample
4
3
2
1
0
0.5
1.0
1.5
Time (h)

not subsequently resistant to the effect of BUdR if they are germinated, grown and reinitiated to sporulate in the presence of BUdR (Table 16). The purified spores in this case could not be germinated in either CH medium or Penassay broth, probably due to carry over of SDS from the preparation, even after centrifugation through Urografin and repeated washing in sterile distilled water (see Materials and Methods, section VI, part 2(a)). However, it was possible to germinate and grow these spores on nutrient agar.

A problem encountered during the course of this work was the day-to-day variation in the sporulation levels of but-32 in TdR alone. This is demonstrated by reference to Tables 14, 15 and 16. Sporulation of the wild-type strain in SM was consistently higher than that obtained for but-32, but this strain could not be used to analyse inhibition of sporulation by BUdR, as a BUdR concentration as high as 1 $\mu g ml^{-1}$ was required to lower the spore incidence of this strain to 60% of the control level (Table 17).

The possibility that BUdR inhibited sporulation by interference with protein synthesis was tested by measurement of the rate of incorporation of $[^{3}H]$- methionine into acid-insoluble material during sporulation (Fig. 24). For the first hour after initiation of sporulation it appeared that BUdR had little

TABLE 16. The effect of BUdR on the sporulation capacity of spores formed in the presence of BUdR.

Strain but-32 was grpwn in duplicate in CH medium (300 ml) containing TdR(8.5 μgml^{-1}). The cells in one culture were initiated to sporulate in SM (300 ml) containing TdR(8.5 μgml^{-1}), and the other cells initiated in the same volume of SM with TdR(1 μgml^{-1})+BUdR(15 μgml^{-1}). At t_{20} the levels of sporulation were determined (first sporulation) and the spores purified (see Materials and Methods, section VI). A loopful of spores from both cultures was germinated by streaking on nutrient agar and incubation overnight at 30° C. Enough cells to give a starting E_{600nm} of approximately 0.1 were transferred by a sterile loop into CH medium (20 ml) with TdR(8.5 μgml^{-1}), and grown to an E_{600nm} of 0.7. The cultures were halved and the cells in each portion harvested by centrifugation. The cells from one half were resuspended in SM (10 ml) with TdR(8.5 μgml^{-1}), and the remaining cells resuspended in the same volume of SM with TdR(1 μgml^{-1})+BUdR(15 μgml^{-1}). At t_{20} the levels of sporulation were determined (second sporulation).

First sporulation		Second sporulation	
Additions to medium	Spores $ml^{-1}(X10^{-6})$ at t_{20}	Additions to medium	Spores $ml^{-1}(X10^{-6})$ at t_{20}
TdR(8.5 μgml^{-1})	72	TdR(8.5 μgml^{-1})	62
		TdR(1 μgml^{-1})+BUdR(15 μgml^{-1})	5.2
TdR(1 μgml^{-1})+ BUdR(15 μgml^{-1})	2.08	TdR(8.5 μgml^{-1})	74
		TdR(1 μgml^{-1})+BUdR(15 μgml^{-1})	3.4

TABLE 17. The effect of a high BUdR concentration on growth and sporulation of B.Subtilis 168 (trpC).

The wild-type strain was grown in CH medium (20 ml) with BUdR at two concentrations, 0.5 and 1.0 $mgml^{-1}$. Distilled water was included instead of BUdR in a control culture. The cells from each culture were initiated to sporulate in the same volume of SM with the same additions. The level of sporulation was determined at t_{20}.

Additions to medium	Doubling time (min) in CH medium	Spores $ml^{-1}(X10^{-6})$ at t_{20}
BUdR (0.5 $mgml^{-1}$)	51	119
BUdR (1.0 $mgml^{-1}$)	54	92
H_2O	57	156

Fig. 24. The effect of BUdR on the rate of incorporation of L- [2-^{3}H] -methionine into acid-insoluble material during but-32 sporulation.

Strain but-32 was grown in CH medium (100 ml) with TdR (8.5 $\mu g ml^{-1}$). Before resuspension of cells in sporulation medium the culture was divided into five portions, and the cells from each harvested by centrifugation. The pellets were resuspended in SM (20 ml) containing L- [^{3}H]- methionine (0.5 $\mu Ci ml^{-1}$) and L-methionine (20 $\mu g ml^{-1}$), plus the following additions.

● TdR (8.5 $\mu g ml^{-1}$)

○ TdR (1 $\mu g ml^{-1}$)

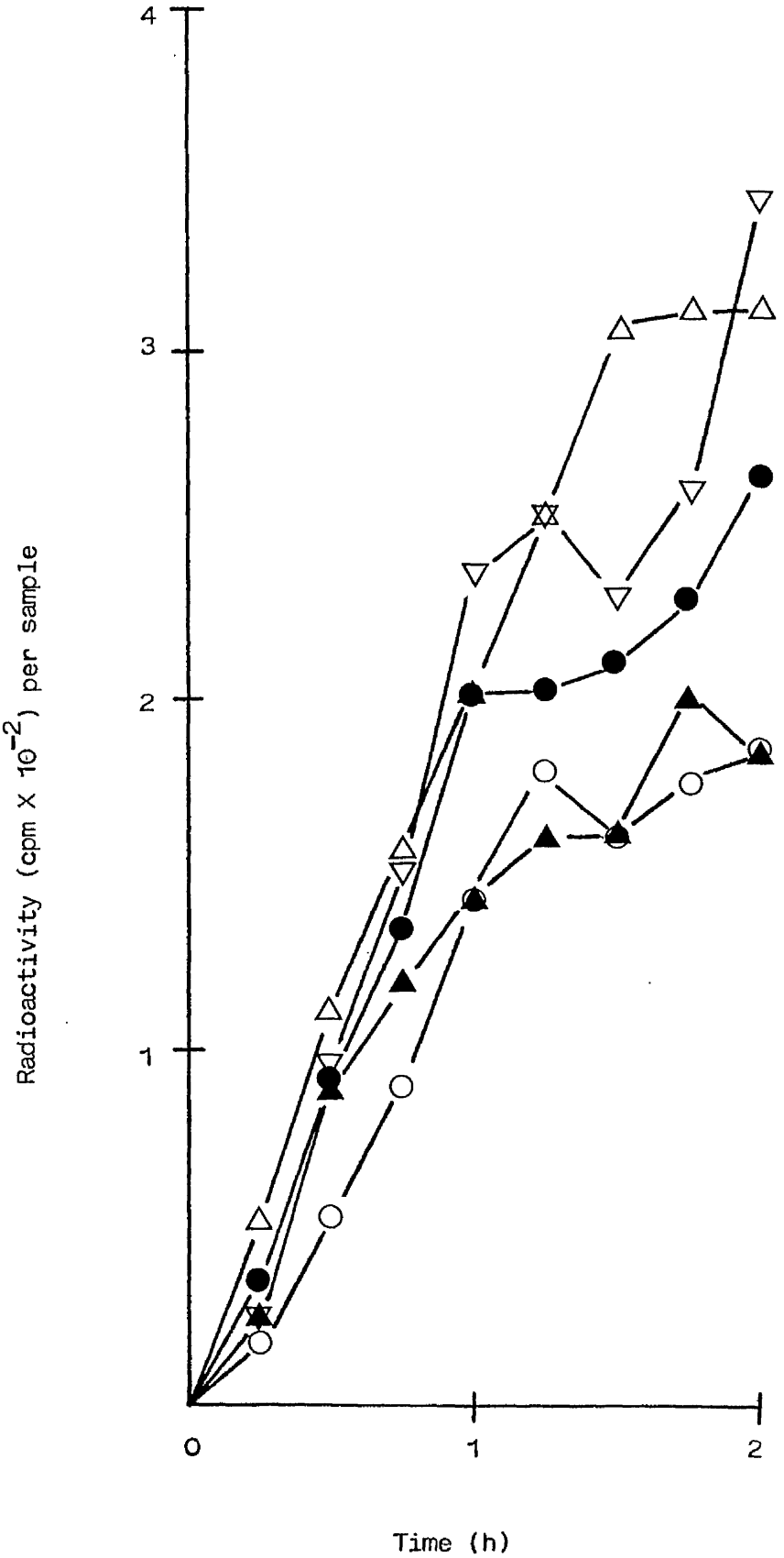
△ TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$)

▽ TdR (8.5 $\mu g ml^{-1}$) + HPUra (0.5 $\mu g ml^{-1}$)

▲ TdR (8.5 $\mu g ml^{-1}$) + HPUra (50 $\mu g ml^{-1}$)

At intervals after resuspension the cells were sampled and measured for incorporation of radioactivity into acid-insoluble material (see Materials and Methods, Section VIII).

Fig. 24



effect on the rate of gross protein synthesis by comparison with the control culture TdR containing (8.5 μgml^{-1}), although by 1½h after t_{zero} the rate levelled off, whereas in the control culture incorporation of radioactive methionine continued to rise, although not in a linear manner. Slowing down the rate of DNA synthesis using HPUra at 0.5 μgml^{-1} appeared to have little effect on the overall rate of incorporation. However, complete inhibition of DNA synthesis by addition of HPUra at 50 μgml^{-1} final concentration, although initially having no effect on the rate of protein synthesis, resulted in a decrease in the rate about 45 min after t_{zero}. Unexpectedly, the rate of protein synthesis in the presence of TdR at only 1 μgml^{-1} appeared to be lower than the rate in TdR (8.5 μgml^{-1}).

The non-linear pattern of incorporation observed approximately after 1h in SM could have been due to the rapid protein turnover which occurs soon after initiation of sporulation, and the experiment could have been improved by measuring incorporation of labelled methionine for 30 or 60 min intervals rather than continuously. The effect of chloramphenicol in an extra culture could also have been tested.

The presence of excess uridine in the sporulation medium did not alter the effect of BUdR on but-32 sporulation (Table 18), and degradation of RNA with KOH did not decrease the level of $[^3H]$-BUdR incorporated into acid-insoluble material during sporulation of but-32 and the parent strain (Fig. 25). This is in contrast

TABLE 18. The effect of uridine (Urd) and deoxycytidine (dCyd) on inhibition of sporulation by BUdR.

Strain but-32 was grown in CH medium (120 ml) supplemented with TdR (8.5 $\mu g ml^{-1}$). The cells were harvested from six equal portions of the culture during mid-exponential growth, and each portion resuspended in SM (20 ml) with the additions shown. The level of sporulation was determined at t_{20}, and figures in parentheses represent the percentage sporulation relative to the control culture.

Additions to SM	Spores $ml^{-1}(X10^{-6})$ at t_{20}
TdR (8.5 $\mu g ml^{-1}$)	15 (100)
TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$)	1.30 (9)
TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + dCyd (15 $\mu g ml^{-1}$)	1.62 (11)
TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + dCyd (50 $\mu g ml^{-1}$)	1.50 (10)
TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + Urd (15 $\mu g ml^{-1}$)	0.90 (6)
TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + Urd (50 $\mu g ml^{-1}$)	1.44 (10)

Figure 25. Incorporation of $[^3H]$- BUdR into acid-insoluble material during sporulation of but-32 and the parent strain.

Each strain was grown to mid-exponential phase in CH medium (80 ml) with TdR (8.5 $\mu g ml^{-1}$). 20 ml aliquots of each culture were centrifuged and the pelleted cells resuspended in SM with TdR (1 $\mu g ml^{-1}$), BUdR (15 $\mu g ml^{-1}$), and $[^3H]$- BUdR (0.5 $\mu Ci ml^{-1}$). (The remaining 60 ml of each culture was used for another experiment (Fig. 20). Duplicate samples were removed at intervals after resuspension, one treated with 1 ml 1N KOH for 2h at 37°C and the other untreated, before TCA precipitation and measurement of incorporated radioactivity, as described in Materials and Methods, section VIII.

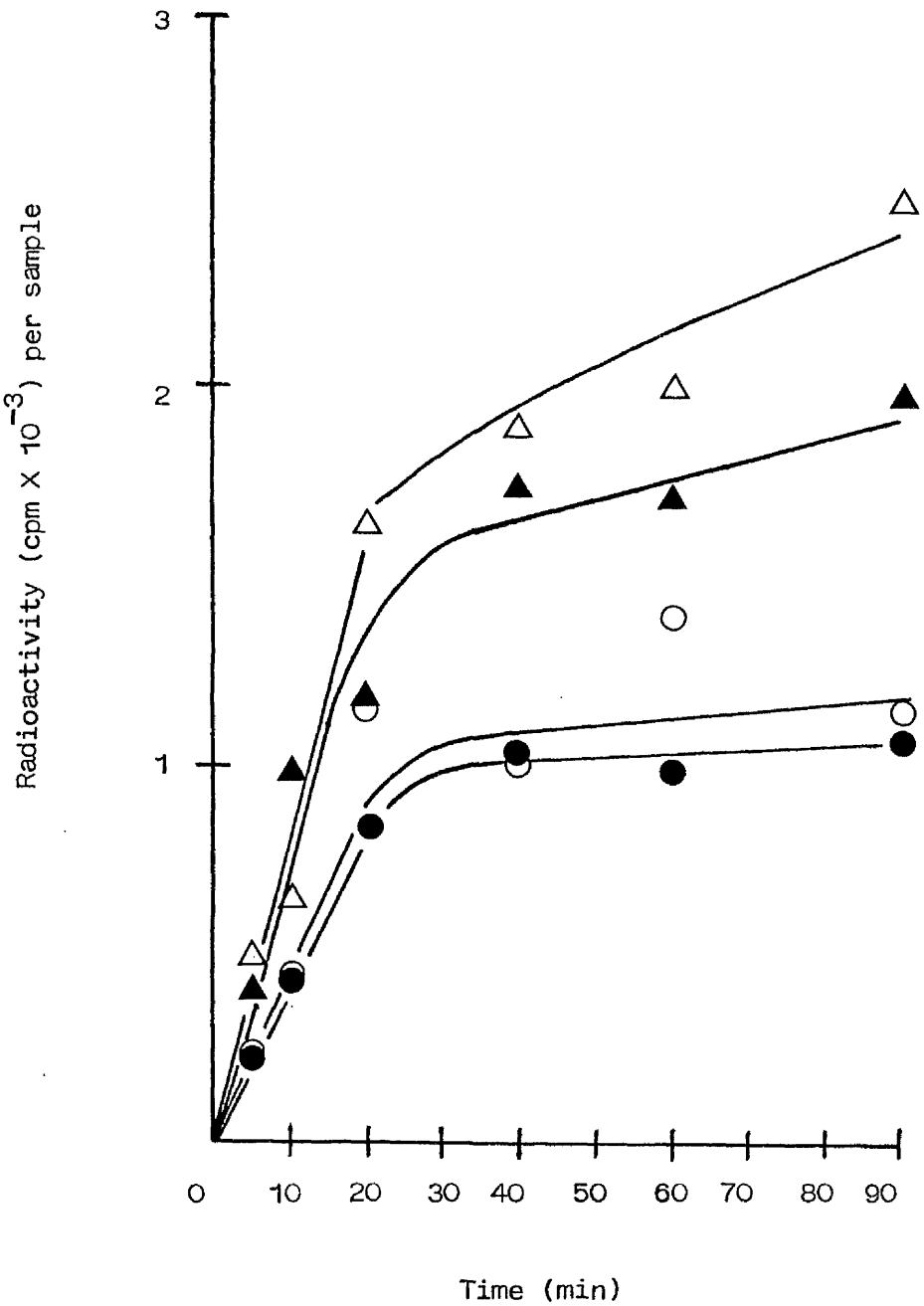
△ parent strain, samples pretreated with KOH

▲ parent strain, samples untreated

○ but-32, samples pretreated with KOH

● but-32, samples untreated

Fig. 25


3
2
1
0
10
20
30
40
50
60
70
80
90
Radioactivity (cpm X 10^{-3}) per sample
Time (min)

to the effect of KOH during growth in the presence of BUdR (Fig. 11B). However, as in growing cells, the parent strain still incorporated more [^{3}H]-BUdR than but-32. In addition, BUdR had no effect on the rate of gross RNA synthesis, measured as the rate of incorporation of [^{3}H]-uridine into acid-insoluble material, during sporulation of but-32 and the parent strain (Fig. 26). Therefore, it seemed unlikely that BUdR exerts its inhibitory action on sporulation via incorporation into RNA.

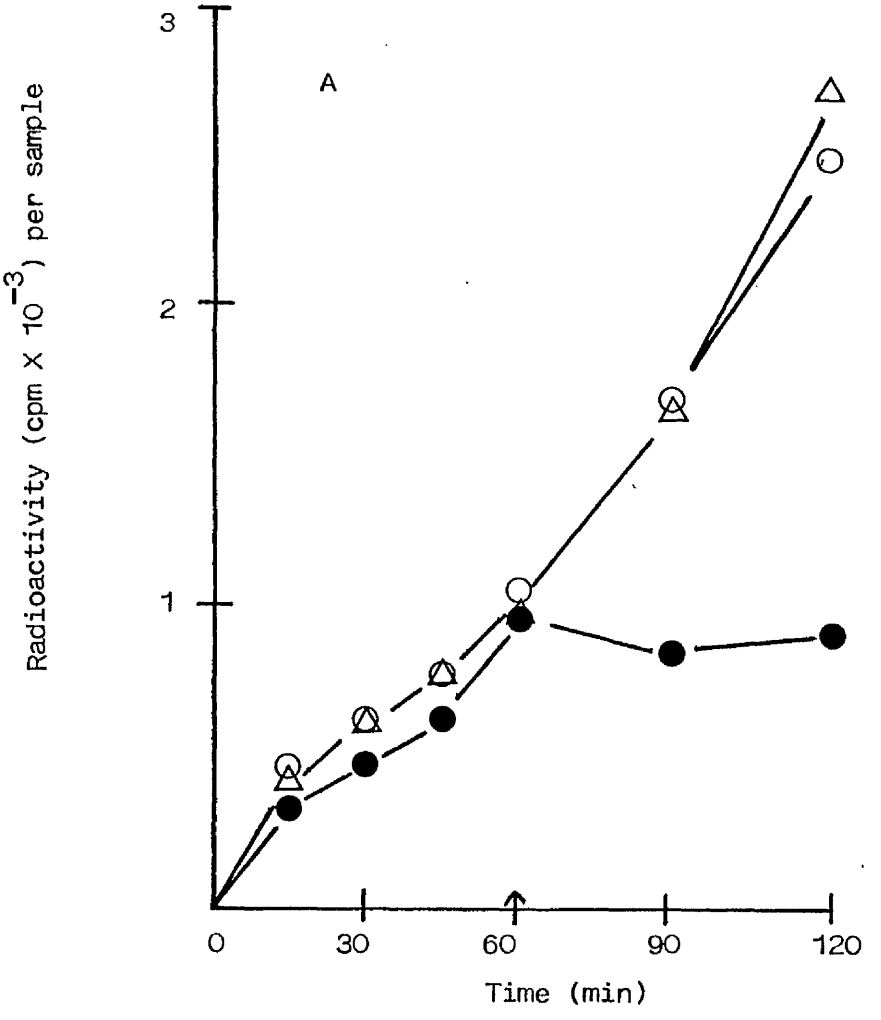
A compound which has been shown to overcome inhibition of differentiation by BUdR in some eukaryotic cells is deoxycytidine (Davidson and Kaufman, 1977; see Introduction), but it did not alter the effect of BUdR on but-32 sporulation (Table 18).

B. Escape from BUdR inhibition of sporulation

The next possibility investigated was that inhibition of sporulation by BUdR was associated with the known requirement for DNA replication during sporulation. The use of HPUra has shown that successful sporulation depends on the completion of chromosome replication during the onset of sporulation (Dunn et al, 1978). In these experiments, the wild-type strain was initiated to sporulate, and a fully-inhibitory concentration of HPUra added at intervals after initiation. When added at t_{zero}, HPUra almost completely inhibited sporulation, but the cells began to escape the inhibitory effect on sporulation some 35 min after

Figure 26. The effect of BUdR on RNA synthesis during sporulation of (A) but-32 and (B) the parent strain.

Each strain was grown in CH medium (60 ml) + TdR (8.5 $\mu g ml^{-1}$) to an E_{600nm} of approximately 0.7, and the cultures each divided into three 20 ml portions. Each portion was centrifuged at 2000 X g in a MSE "Super Minor" centrifuge for 15 min to pellet the cells. The pellets were resuspended in SM (20 ml) containing i) TdR (8.5 $\mu g ml^{-1}$) (⊖), ii) TdR (8.5 $\mu g ml^{-1}$) (●) and iii) TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) (△). 0.4 ml of a $[^3H]$- uridine solution (see Fig. 12) was added to each culture (0.5 $\mu Ci ml^{-1}$, final concentration). At intervals after addition of radiolabel samples (0.2 ml) were removed for measurement of incorporation of radioactivity into acid-insoluble material (see Materials and Methods, section VIII (C)). In addition, rifampicin (20 $\mu g ml^{-1}$, final concentration) was added to culture (ii) 60 min after the addition of $[^3H]$- uridine. (↑)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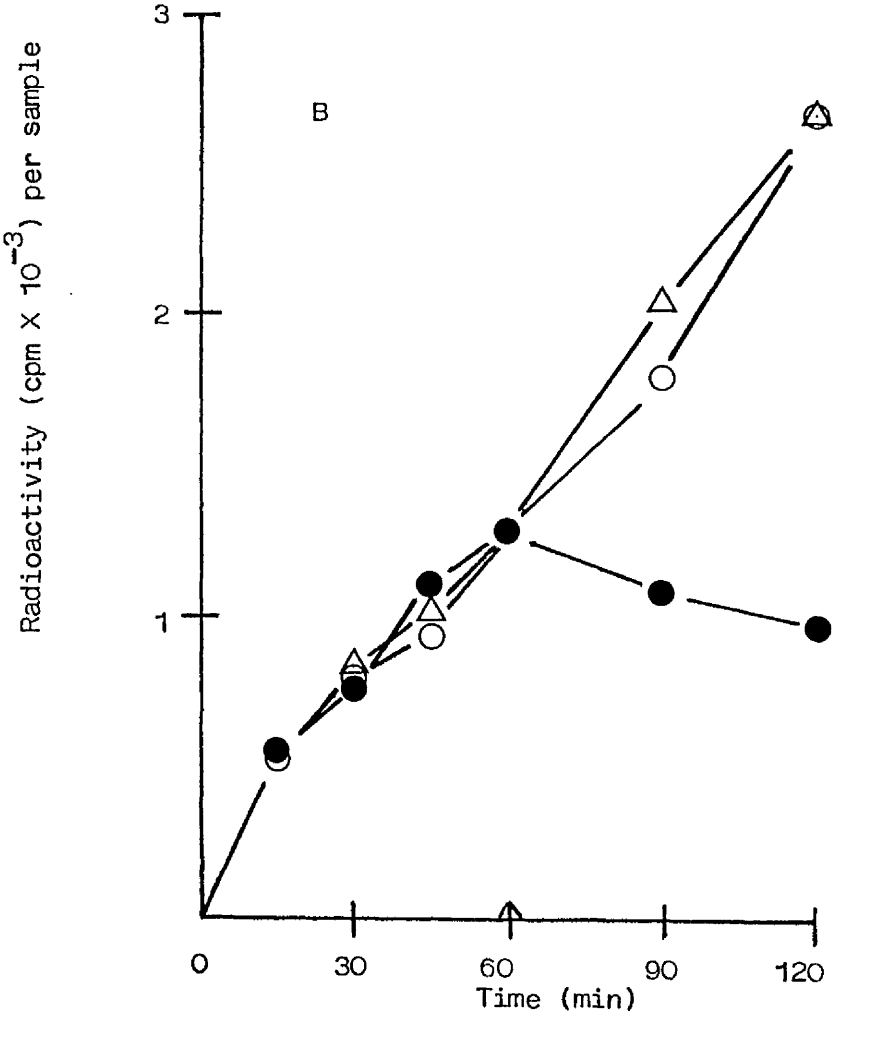
Radioactivity (cpm X 10^{-3}) per sample
3
2
1
0
30
60
90
120
Time (min)


B
Radioactivity (cpm X 10^{-3}) per sample
3
2
1
0
30
60
90
120
Time (min)

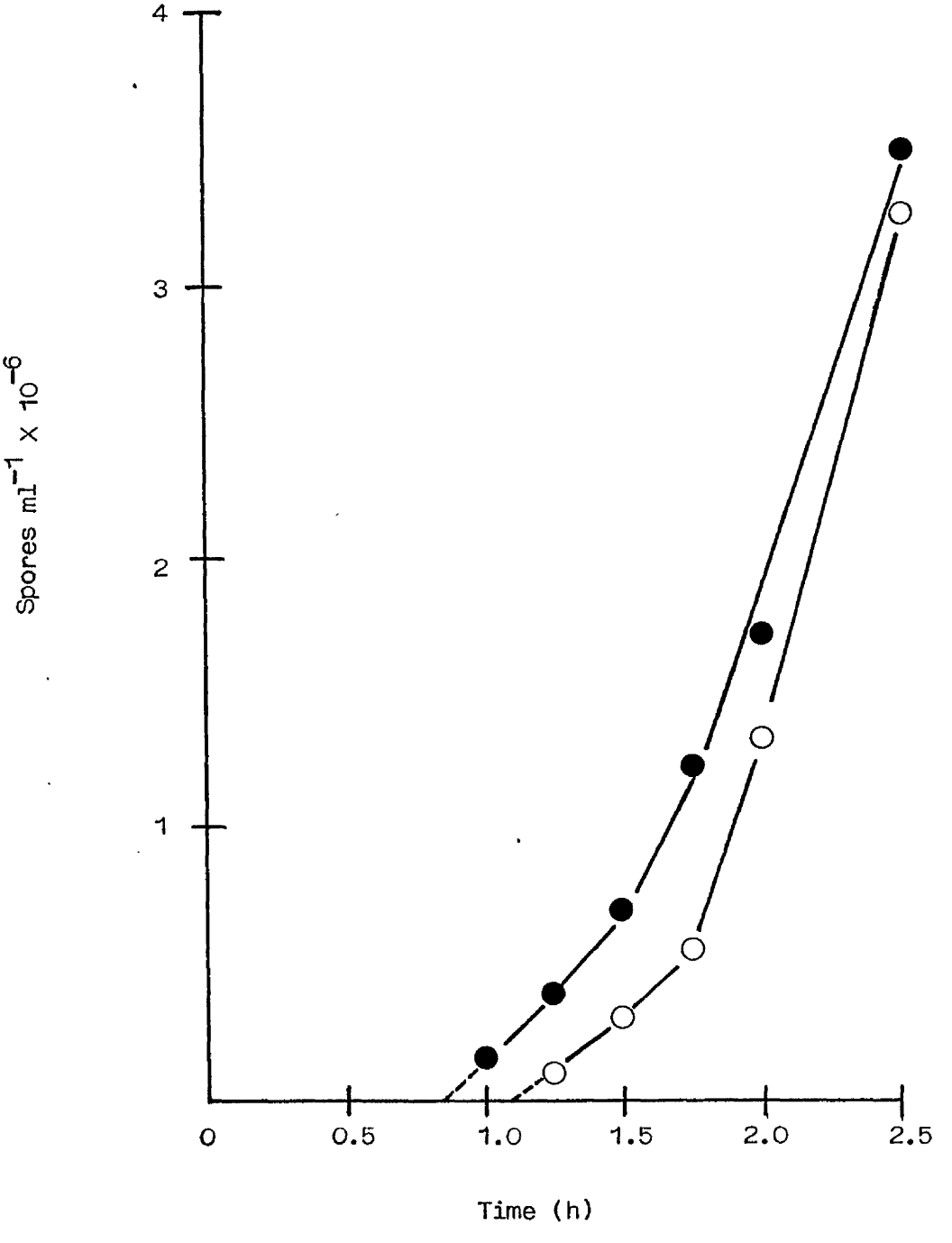
initiation, and this escape correlated with termination of DNA replication, as monitored by marker frequency analysis.

This approach therefore offered a means whereby the effect of BUdR on the rate of termination of replication during sporulation could be tested, and the escape of but-32 from inhibition of sporulation by both HPUra and BUdR was examined (Fig. 27). Unlike the wild-type, but-32 began to escape the inhibitory effect of HPUra at a later time, about 75 min after initiation. The pattern of escape from BUdR inhibition of sporulation was similar, and slightly preceded that from HPUra. Therefore, the effect of BUdR on sporulation appeared to be limited to the period of DNA replication. Escape from HPUra inhibition in the wild-type was essentially complete 2h after initiation of sporulation (Dunn et al, 1978), but a separate experiment showed that the spore incidence in but-32 was still rising 6h after initiation (Fig. 28). This experiment also showed that addition of a sub-inhibitory concentration of HPUra to sporulating cells of but-32 delayed the escape of sporulation from both HPUra and BUdR inhibition of sporulation. Thus again BUdR inhibition of sporulation appeared to be closely associated with DNA replication during sporulation. The concentration of HPUra used in this experiment, 1 $\mu g ml^{-1}$, reduced the spore incidence to about 2% of that in the control culture, but also decreased the rate of growth in the CH medium prior to resuspension (doubling time of 63 min compared to 42 min in TdR alone). Therefore, the inhibitory effect of this concentration on sporulation

Figure 27. Escape of strain but-32 from HPUra and BUdR inhibition of sporulation.

The cells were grown and resuspended in media (200 ml) containing TdR (8.5 μgml^{-1}). At intervals after resuspension two 10 ml portions were removed; to one portion was added HPUra (50 μgml^{-1}) (○), and the other cells were collected on a membrane filter, washed with SM and suspended in SM (10 ml) containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●). Incubation was continued until t_{20}, when the level of sporulation was determined in each culture (see Materials and Methods, section IV).

Fig. 27



any initiation of DNA replication after one rinse could have been partially inhibited.

The continued incorporation of $\left[^{14}C\right]$- TdR in the presence of BUdR suggested that BUdR was either delaying termination of replication or perhaps allowing reinitiation of DNA replication.

In another two experiments comparisons were made of the rates of $\left[^{14}C\right]$- TdR incorporation after the reverse transfer, i.e. from BUdR to TdR medium at various times during sporulation. The data indicated that the length of time the cells were exposed to the analogue correlated with a decrease in the overall rate of DNA replication (Fig.'s 35 and 36). Fig. 35 shows that whereas incorporation in BUdR medium was still rising at t_4, the rate in cells exposed to TdR alone from t_{zero} decreased to zero by t_3. The rate after transfer from BUdR to TdR medium at t_3 was similar to that of cells in TdR alone from t_{zero}. However, the rate after transfer at t_4 was less than that at t_3, and the rate after transfer at t_5 was in turn less than that at t_4. In addition, Fig. 36 shows that the rate after transfer of cells from BUdR to TdR medium at t_7 was less than that at t_3, which in this experiment was in turn less than that of cells transferred at t_{zero}. Also, in this experiment the incorporation of radioactivity was monitored over a longer period during sporulation and it can be seen that the level of incorporation by cells exposed to TdR from

Fig. 34

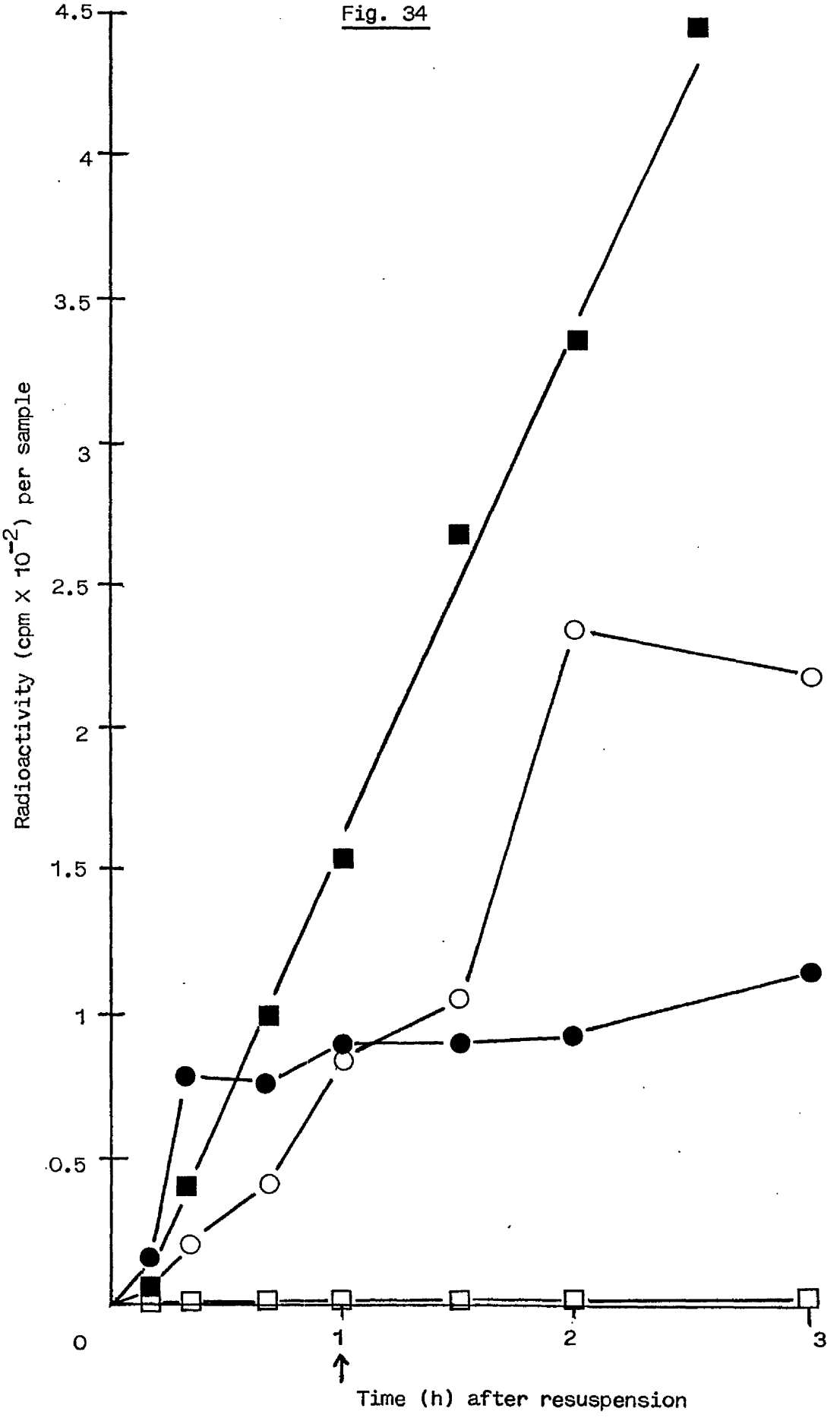

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Radioactivity (cpm X 10^{-2}) per sample
1
2
3
Time (h) after resuspension

Figure 34. DNA synthesis after resuspension at t_{zero}

Strain but-32 was grown and resuspended in 40 ml media containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). Immediately the cells from four 10 ml portions were collected on membrane filters, each washed with and resuspended in SM (10 ml). TdR (8.5 μgml^{-1}) was added to three cultures, TdR (1μgml^{-1}) + BUdR (15 μgml^{-1}) to the other. $\left[^{14}C\right]$- TdR (0.025 $\mu Ciml^{-1}$) was added to all cultures. HPUra (50 μgml^{-1}) was added to one culture containing TdR (□), chloramphenicol (100 μgml^{-1}) to another (●), and the third was left as a control (0). The BUdR culture was also left untreated (■). At intervals samples were removed for measurement of incorporation of $\left[^{14}C\right]$- TdR into acid-insoluble material.

1h after t_{zero} (↑) the cells in the culture containing chloramphenicol were collected on a membrane filter, washed with and suspended in SM (10 ml) with TdR (8.5 μgml^{-1}) + $\left[^{14}C\right]$- TdR (0.025 $\mu Ciml^{-1}$).

but the cells were lost in the process.

In a parallel experiment the rate of DNA synthesis from t_{zero} was measured (Fig. 34). The cells still incorporated [^{14}C]-TdR after 3h in the presence of BUdR. Therefore, at the time they had been transferred at t_3 from BUdR to TdR medium (Fig. 33) DNA replication was still in progress. However, in TdR alone, the level of incorporation did not increase after t_2, suggesting that DNA synthesis had finished by this time. Addition of HPUra (50 $\mu g ml^{-1}$) at t_{zero} completely inhibited DNA synthesis, but the rate in the presence of chloramphenicol markedly decreased after approximately 30 min from t_{zero}, suggesting that in TdR alone either some degree of reinitiation of DNA replication occurs during sporulation, or that some cells do not initiate DNA replication until a later time. One hr after initiation the chloramphenicol was rinsed out of the culture, to determine whether initiation of DNA replication could occur in SM after the time when these cells begin to escape the effect of HPUra (50 $\mu g ml^{-1}$) on sporulation (Fig. 27). Little evidence of reinitiation was observed when the chloramphenicol was removed, suggesting that most of the cells in TdR alone had terminated DNA replication by this time. The cpm values did start to increase by the end of the sampling period, however, and it may have been that the apparent lack of initiation was due to traces of chloramphenicol still persisting, as the cells were only rinsed once before transfer to TdR medium. In this way

Fig. 33

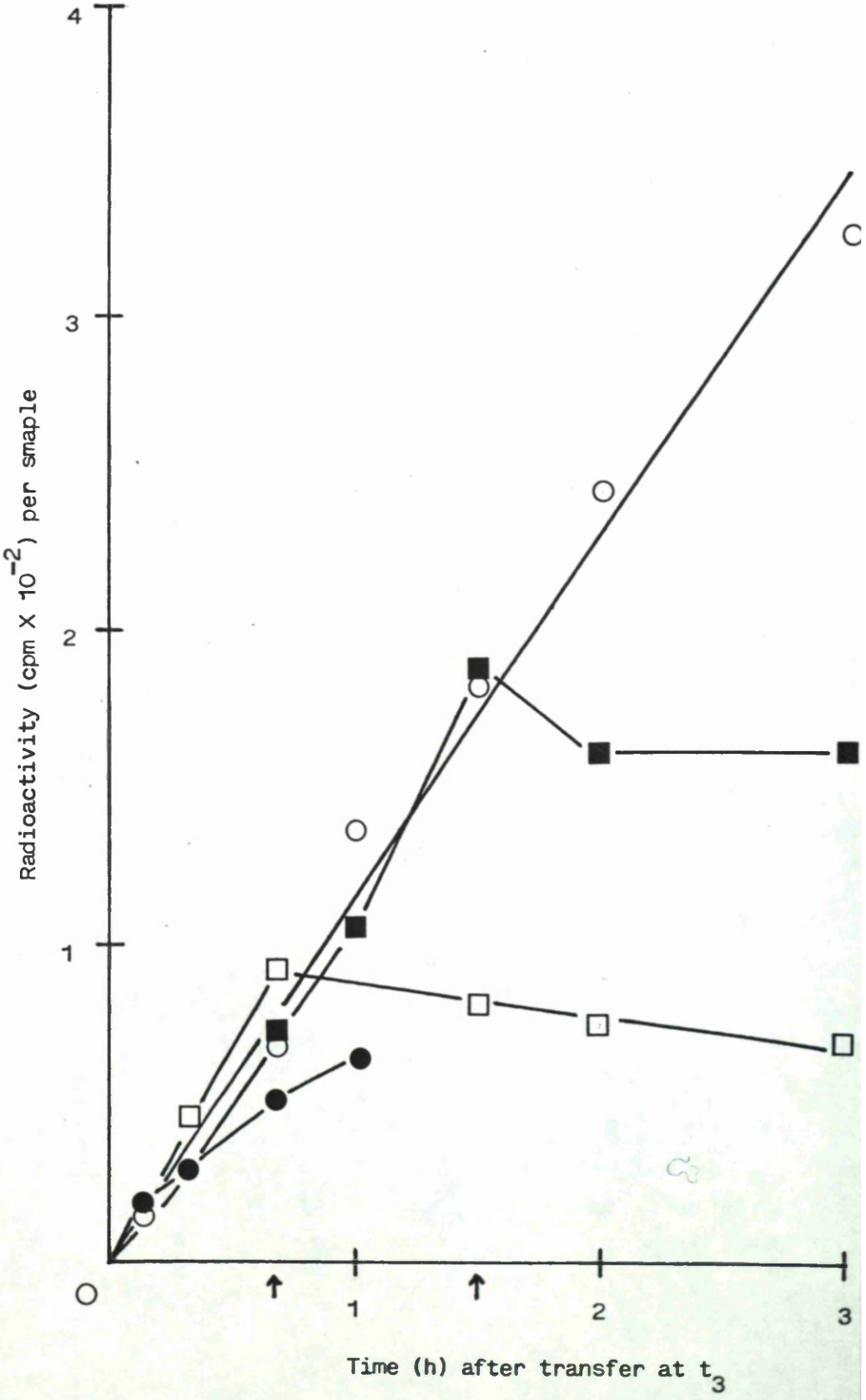

4
3
2
1
0
Radioactivity (cpm X 10^{-2}) per smaple
1
2
3
Time (h) after transfer at t_3

Figure 33. DNA synthesis after transfer of cells from BUdR to TdR medium.

Strain but-32 was grown and resuspended in 40 ml media containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). Three hours after resuspension, at t_3, portions (10 ml) of the cultures were removed. The cells from each portion were collected on a membrane filter, washed with SM and suspended in SM (10 ml) with TdR (8.5 μgml^{-1}) + $[^{14}C]$- TdR (0.025 $\mu Ciml^{-1}$). Chloramphenicol (100 μgml^{-1}) was added to one culture (●) immediately on transfer, and HPUra (50 μgml^{-1}) added at 45 min (□) and 90 min (■) after transfer, for two further cultures. The remaining 10 ml was untreated (0). At intervals after the initial transfer samples were removed for measurement of incorporation of radioactivity into acid-insoluble material (see Materials and Methods, section VIII). The symbol ↑ refers to the times of HPUra addition.

TABLE 19. Recovery from BUdR inhibition of sporulation

Strain but-32 was grown and resuspended in 100 ml media containing TdR (1 μgml^{-1})+BUdR(15 μgml^{-1}). At t_{zero} a sample (10 ml) was removed, the cells collected on a membrane filter, washed with SM and resuspended in SM (10 ml) with TdR (8.5 μgml^{-1})+HPUra (50 μgml^{-1}). At t_3, eight 10 ml samples were filtered and suspended in SM, one of which contained TdR (8.5 μgml^{-1})+HPUra(50 μgml^{-1}), one with no added TdR, and the others, TdR (8.5 μgml^{-1}). HPUra (50 μgml^{-} was added to five of the latter samples at intervals after transfer to TdR medium. The level of sporulation in all samples was determined at t_{20}.

Treatment	Spores $ml^{-1}(X10^{-4})$ at t_{20}
Growth and resuspension in TdR + BUdR medium	22
Cells transferred to TdR medium + HPUra at t_{zero}	2
Cells transferred at t_3 to medium with:	
a) No TdR	6
b) TdR	500
c) TdR + HPUra	4
d) TdR: HPUra added after 10 min.	5
e) TdR: HPUra added after 20 min.	8
f) TdR: HPUra added after 40 min.	7
g) TdR: HPUra added after 60 min.	17
h) TdR: HPUra added after 90 min.	61

C. Recovery from BUdR inhibition of sporulation

Using the non-isogenic derivative, But(32), it was shown that recovery from inhibition of sporulation by BUdR can be obtained if the analogue is removed from resuspension medium and replaced by TdR (Coote, 1977). However, if DNA synthesis in but-32 is prevented by starvation for TdR or treatment with HPUra (50 µgml the recovery in sporulation capacity on removal of BUdR is not obtained (Table 19). The results of this experiment also showed the dependence of recovery on continued DNA replication, as additic of an inhibitory concentration of HPUra to cells at intervals after transfer at t_3 to medium with TdR showed that the cells only began to regain their capacity to sporulate after about 60 min.

The 60 min delay was not caused by a lag in DNA synthesis in cells exposed to BUdR for 3h and then resuspended with TdR alone as DNA synthesis proceeded normally, i.e. at a similar rate to that in cells suspended in TdR alone from t_{zero}, after transfer from BUdR to TdR medium at t_3, ceasing immediately HPUra was added (Fig. Chloramphenicol was added to one culture at the time of transfer to inhibit initiation of DNA replication. By 1h after the transfer of cells from BUdR to TdR medium in the presence of chloramphenicol the overall rate of incorporation of $\left[^{14}C\right]$- TdR was decreasing relative to the control rate. An attempt was made at this point to wash out the chloramphenicol to determine if further initiation of DNA replication in the absence of BUdR was required for recovery

Fig. 32

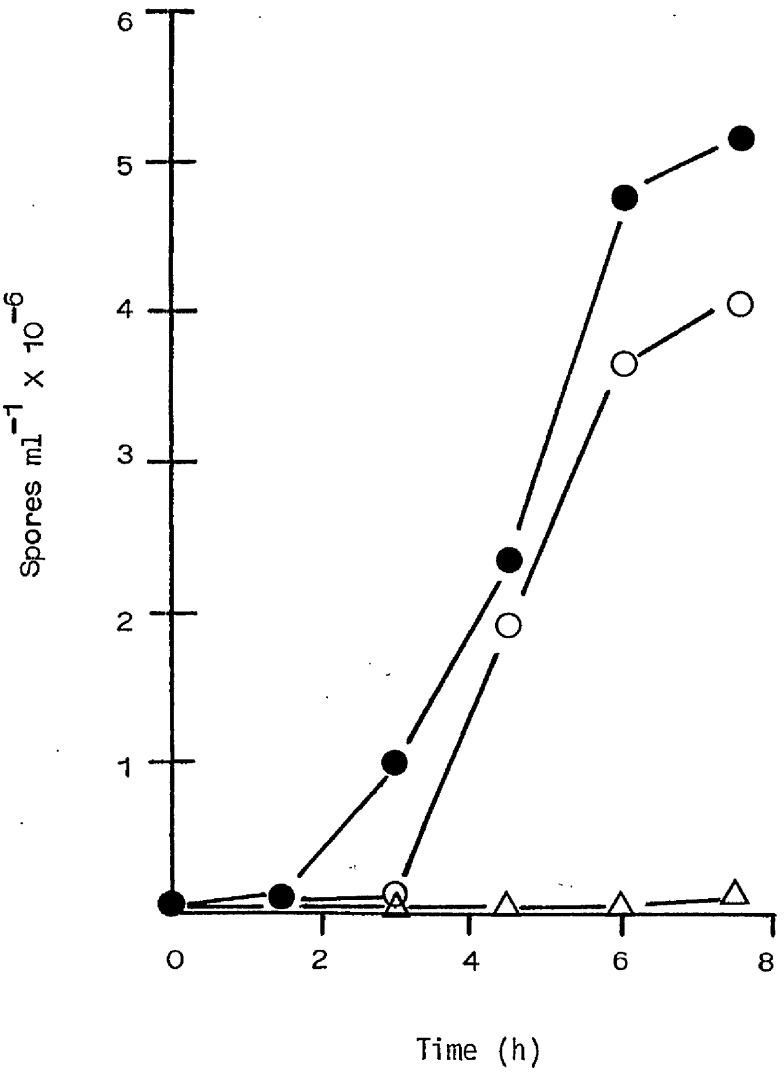


Figure 32. Escape of strain but-32 from HPUra and BUdR inhibition of sporulation.

This experiment was identical to the previous one (Fig. 31), but with different sampling times over a longer period of sporulation. In addition, 10 ml portions of each culture were removed during sporulation for marker-frequency analysis of DNA replication (Fig. 39).

In this experiment BUdR reduced the level of sporulation to 0.4% of the control value in TdR alone, when added at t_{zero}.

Culture (i) treated with HPUra (O) or BUdR (●); culture (ii) treated with BUdR (△).

Fig. 31

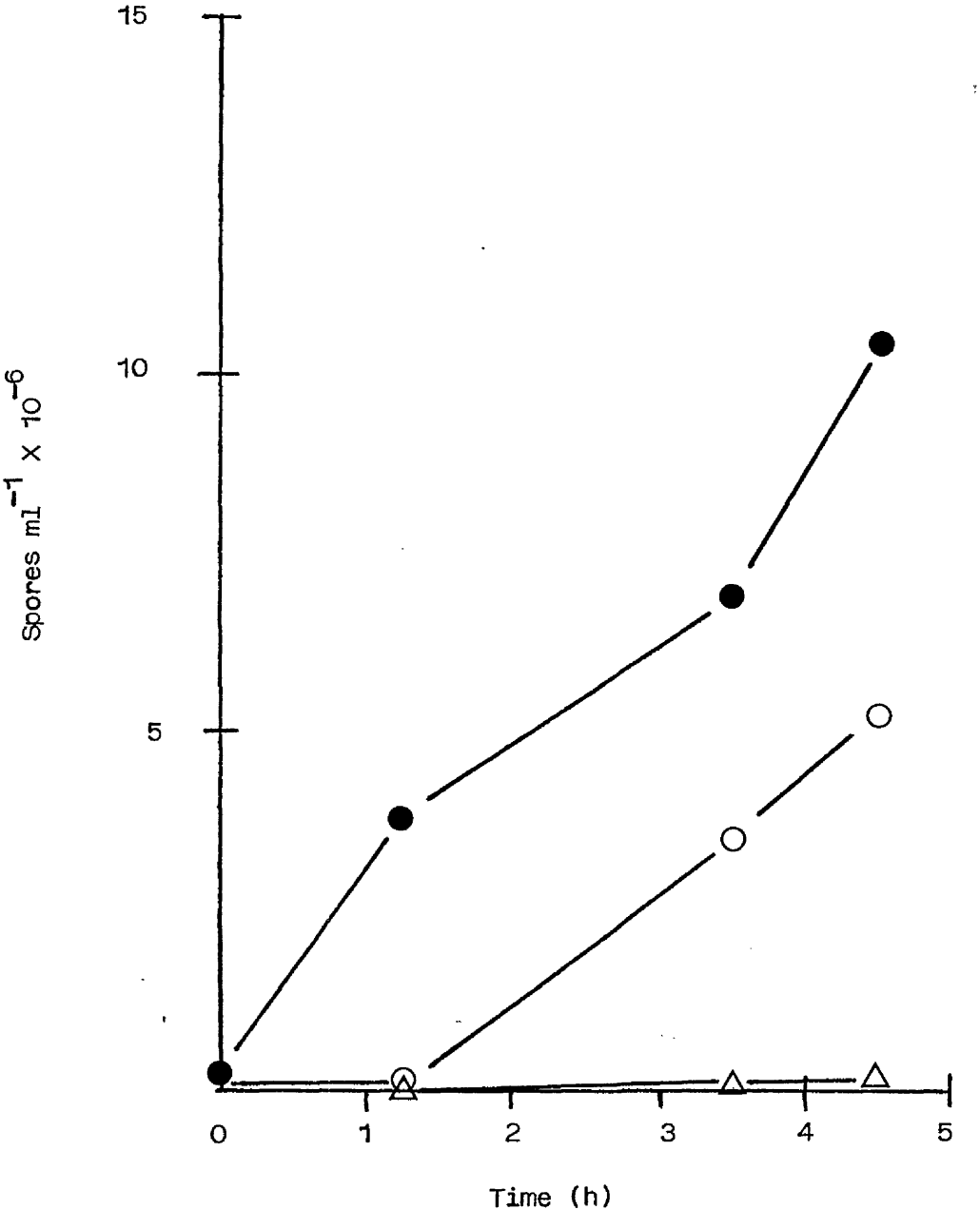


Figure 31. Escape of strain but-32 from HPUra and BUdR inhibition of sporulation.

The cells were grown and resuspended in 200 ml media containing (i) TdR (8.5 μgml^{-1}), or (ii) TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). At intervals after resuspension two 10 ml aliquots were removed from culture (i), one treated with HPUra (50 μgml^{-1}) (O) and the other cells transferred to 10 ml SM + TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●), as described in the legend to Fig. 27. At the same sampling times portions (10 ml) were removed from culture (ii) and HPUra added at 50 μgml^{-1} (△). In this experiment BUdR reduced the level of sporulation to 0.3% of the control value in TdR alone when added at t_{zero}.

Fig. 30

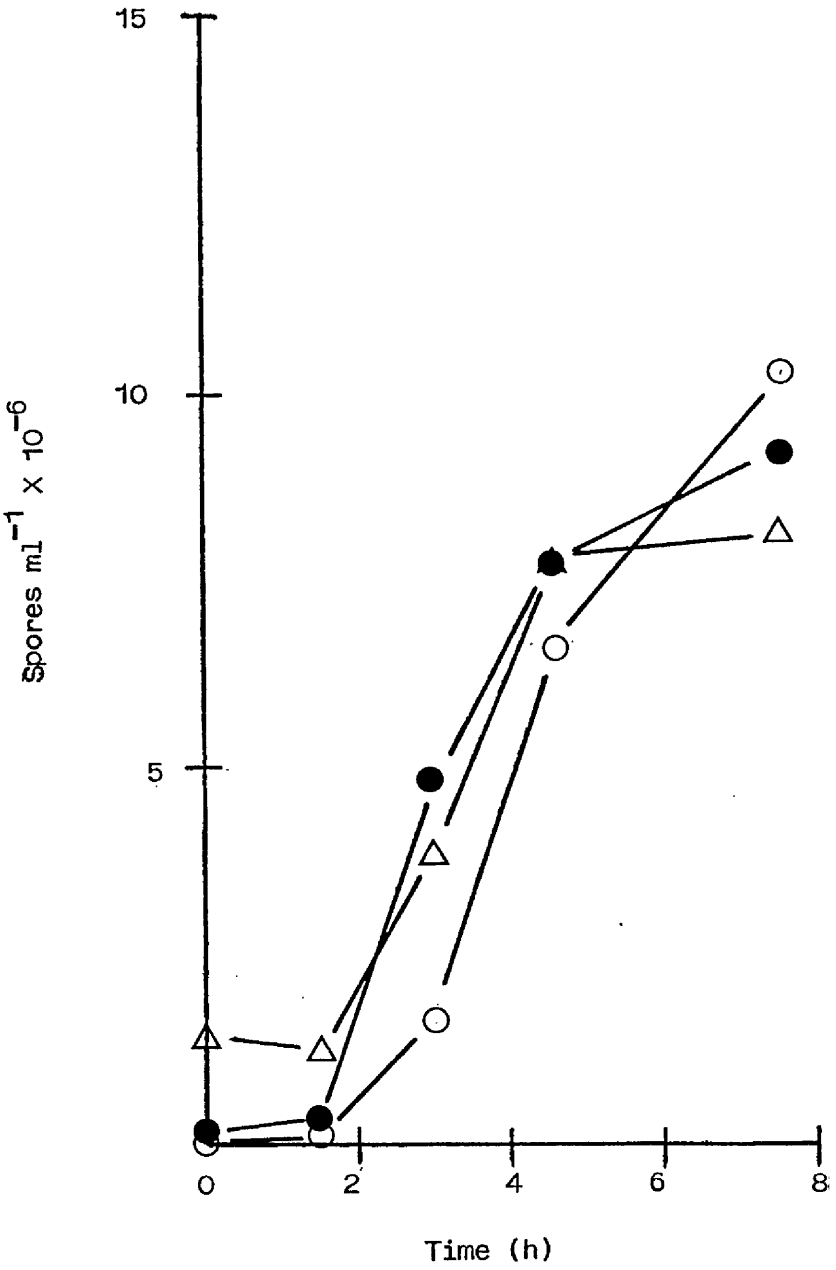


Figure 30. Escape of strain but-32 from HPUra and BUdR inhibition of sporulation.

The cells were grown and resuspended in 200 ml media containing TdR (8.5 μgml^{-1}). At intervals after resuspension, three 10 ml portions were removed. One portion was treated with HPUra (50 μgml^{-1}) (0), another with HPUra (0.5 μgml^{-1}) (△), and the cells of the third portion transferred as before (see legend to Fig. 27) to SM (10 ml) containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●). The level of sporulation was determined at t_{20}. In this experiment BUdR reduced the level of sporulation to 1%, HPUra (0.5 μgml^{-1}) to 13% and HPUra (50 μgml^{-1}) to 0.02% of the control level respectively when added at t_{zero}.

Fig. 29

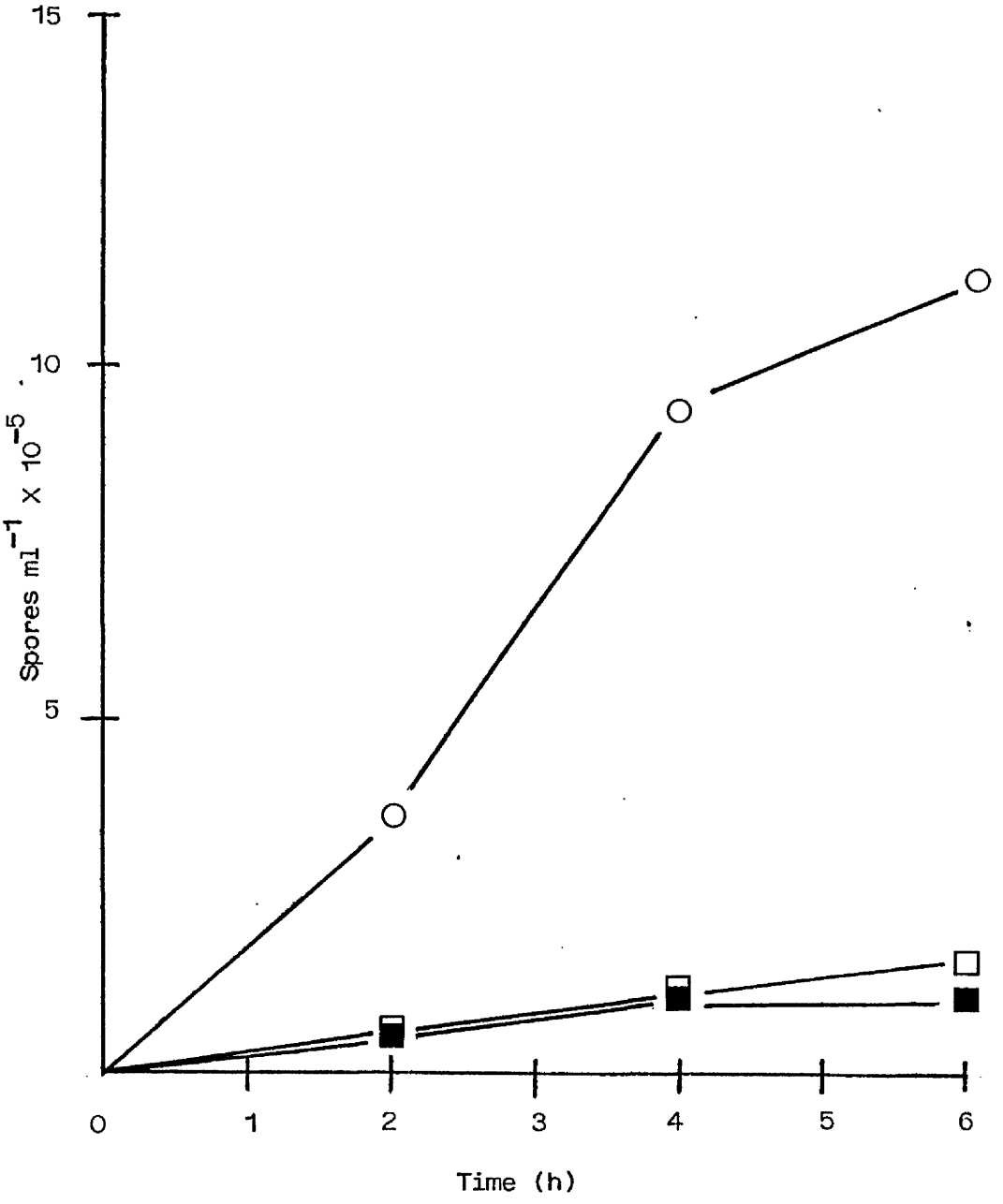


Figure 29. Escape of strain but-32 from HPUra inhibition of sporulation.

The cells were grown and resuspended in 50 ml media containing (i) TdR (8.5 μgml^{-1}) (0), (ii) TdR (8.5 μgml^{-1}) + HPUra (0.5 μgml^{-1}) (■) or (iii) TdR (8.5 μgml^{-1}) + HPUra (0.75 μgml^{-1}) (□). At intervals after resuspension a portion (10 ml) was removed from each culture and treated with HPUra (50 μgml^{-1}). The level of sporulation was determined at t_{20}.

could have been attributed to an effect on growth. HPUra at a concentration of 0.5 μgml^{-1} inhibited sporulation to 10% of the control level without affecting the growth rate (Fig. 29), and a concentration of 0.75 μgml^{-1} inhibited sporulation to a similar extent. At both concentrations escape of but-32 from inhibition of sporulation by the high HPUra concentration was delayed.

In a further experiment, a comparison was made of the escape of but-32 from inhibition of sporulation by HPUra (50 μgml^{-1} BUdR, and a sub-inhibitory concentration of HPUra (0.5 μgml^{-1}) (Fig. 30). The escape curves of the latter two were similar and preceded escape from the higher HPUra concentration, although the presence of HPUra (0.5 μgml^{-1}) from t_{zero} allowed a greater level of sporulation than BUdR, 13% of the control rate compared to a value of 1% for BUdR-treated cells.

In addition, like the effect of 0.5 μgml^{-1} HPUra, cells grown and resuspended with BUdR also exhibited delayed escape of sporulation from inhibition by the higher HPUra concentration (Fig's. 31 and 32). The escape observed in these two experiments did not result in a large increase in spore incidence, but it was still detectable. Therefore, these results suggested that BUdR acts to delay termination of DNA replication during sporulation.

Fig. 28

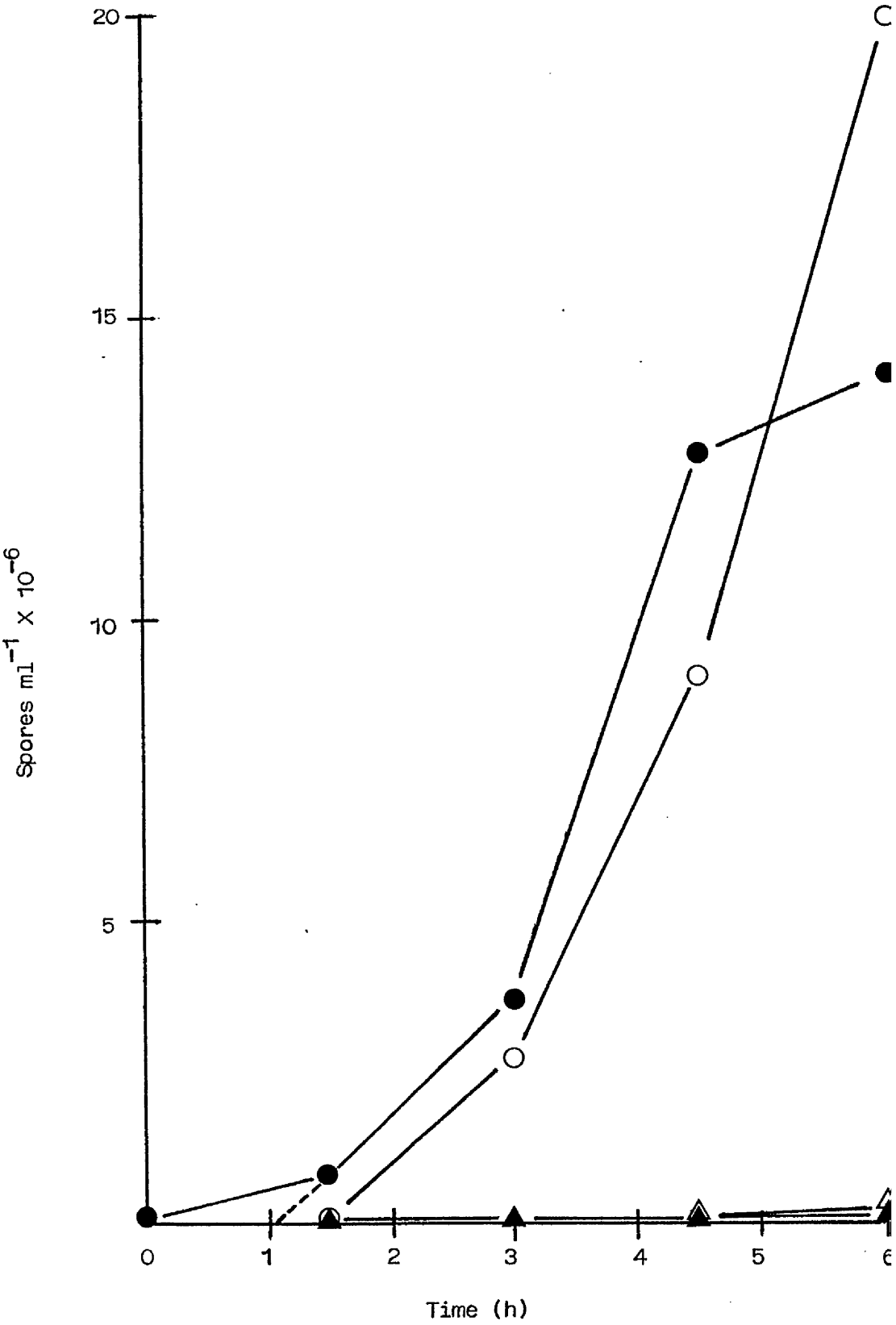


Figure 28. Escape of strain but-32 from HPUra and BUdR inhibition of sporulation.

The cells were grown and resuspended in 120 ml media containing (i) TdR (8.5 μgml^{-1}) and (ii) TdR (8.5 μgml^{-1}) + HPUra (1 μgml^{-1}). At intervals after resuspension portions (10 ml) were removed from each culture and treated with HPUra (50 μgml^{-1}). In addition, the cells in 10 ml portions of both cultures were resuspended with TdR (1 μgml^{-1}) plus BUdR (15 μgml^{-1}), as described in the legend to Fig. 27. Culture (i) treated with HPUra (0) or BUdR (●). Culture (ii) treated with HPUra (△) or BUdR (▲). The level of sporulation was determined at t_{20}. Cells exposed to HPUra (1 μgml^{-1}) or BUdR from t_{zero} sporulated to 2% and 0.04% respectively of the level in control cells with TdR alone.

Figure 35. DNA synthesis after transfer of cells from BUdR to TdR medium.

Strain but-32 was grown and resuspended in 50 ml media containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + $\left[^{14}C\right]$ - TdR (0.025 $\mu Ciml^{-1}$) (■). At t_{zero}(0), t_3 (●), t_4 (△) and t_5 (□) portions (10 ml) of the culture were removed, the cells collected on a membrane filter, washed with SM and suspended in SM (10 ml) containing TdR (8.5 μgml^{-1}) + $\left[^{14}C\right]$ - TdR (0.025 $\mu Ciml^{-1}$). At intervals samples were removed for measurement of radioactivity incorporated into acid-insoluble material (see Materials and Methods, section VIII).

Fig. 35

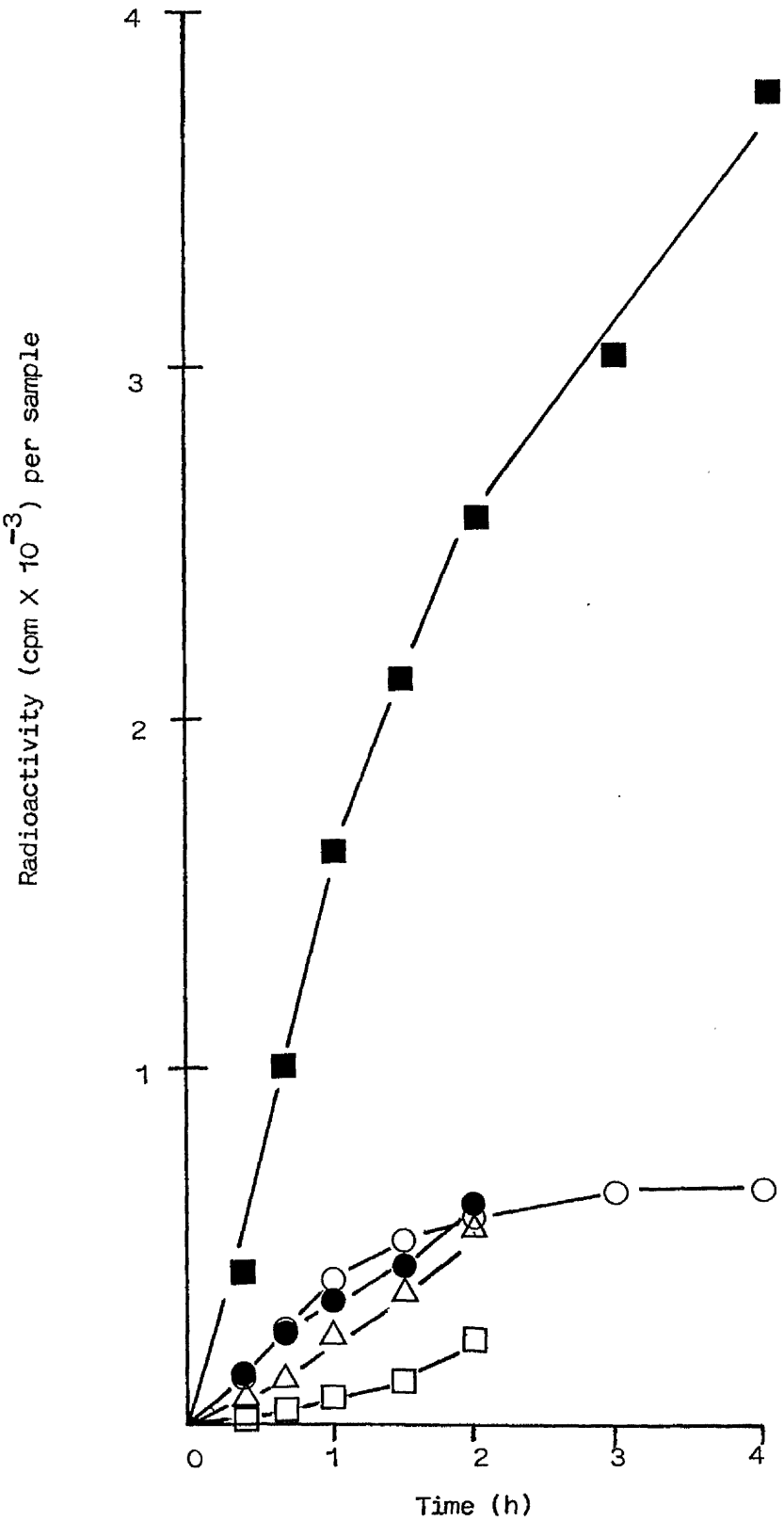
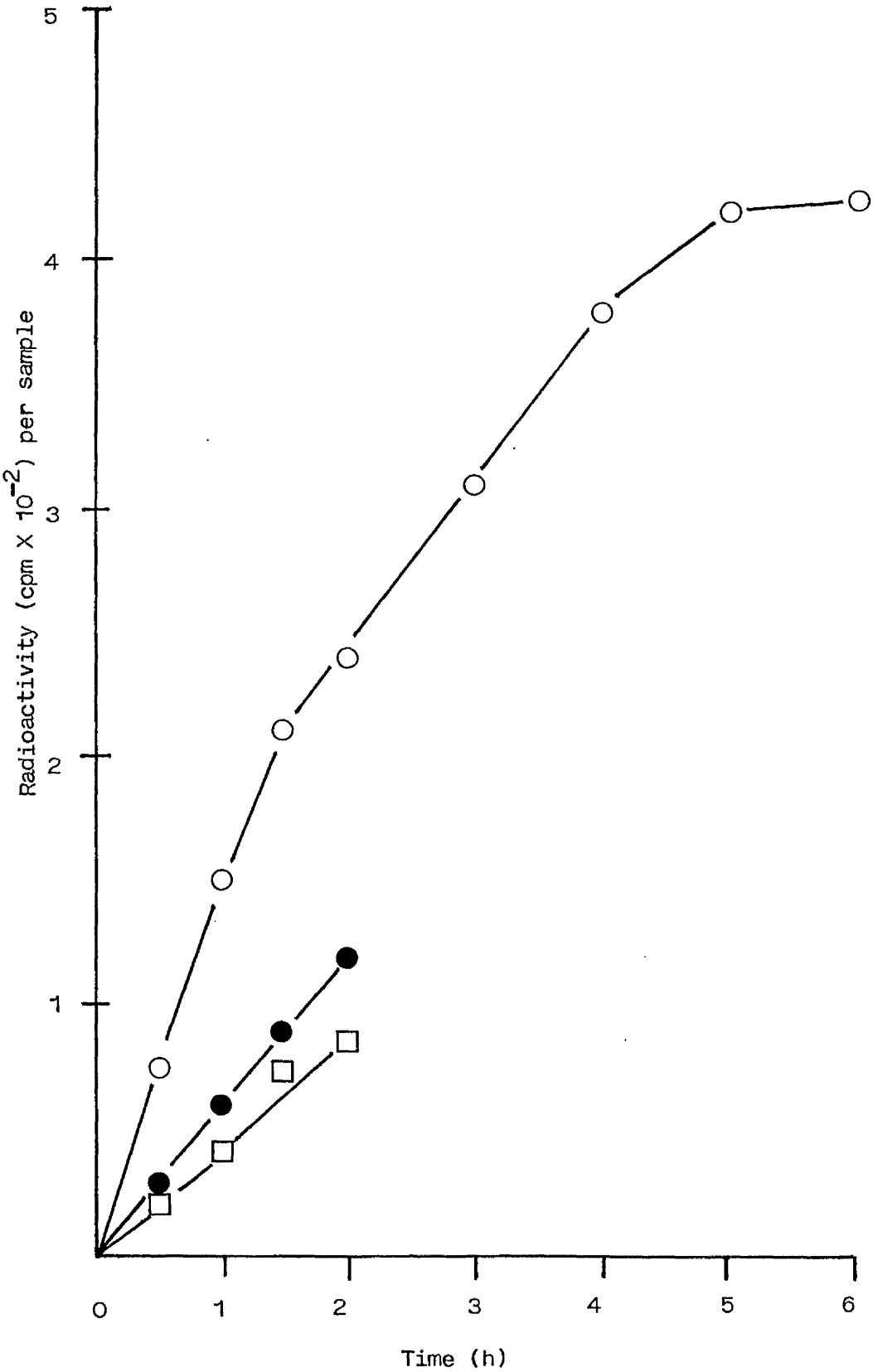


Figure 36. DNA synthesis after transfer of cells from BUdR to TdR medium.

Strain but-32 was grown and resuspended in 50 ml media containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). At t_{zero}(0), t_3 (●) and t_7 (□) portions (10 ml) of the culture were removed, the cells collected on a membrane filter, washed with SM and suspended in SM (10 ml) + TdR (8.5 μgml^{-1}) + $\left[^{14}C\right]$ - TdR (0.025 $\mu Ciml^{-1}$). At intervals after resuspension samples were removed for measurement of radioactivity incorporated into acid-insoluble material (see Materials and Methods, section VIII).

Fig. 36



t_{zero} had become constant by t_5. This is some 2-3h later than in other experiments in which cells were exposed to TdR alone. (Fig's 34 and 35).

An examination of the effect of the period of exposure of cells to BUdR on subsequent recovery of sporulation capacity and viability at the time of transfer from BUdR to TdR medium (Table 20) showed that the cells had the capacity to sporulate after removal of BUdR at any time up to t_8, but the capacity declined slightly from t_4 to t_8. By t_8, the number of viable cells was reduced slightly. Also, when HPUra (50 $\mu g ml^{-1}$) was added at intervals after transfer from BUdR to TdR medium, it appeared that some cells began to escape the effect of HPUra on sporulation even after transfer at t_8, indicating that DNA replication occurred after transfer at t_8, and some cells had terminated DNA replication between 45 and 90 min after this time. Thus, the recovery of cells from BUdR inhibition tended to decline the longer the cells were exposed to the analogue, and this correlated to some extent with a decreased rate of DNA replication on transfer to TdR medium.

Therefore, it was conceivable that BUdR might be preventing termination of DNA replication in the majority of cells, thereby preventing successful sporulation. However, replication appeared to proceed normally in cells exposed to BUdR at various times after initiation of sporulation (Fig. 37). The rate of incorporation of $\left[^{14}C\right]$- TdR continued in a linear manner up to

TABLE 20. Recovery from BUdR inhibition of sporulation.

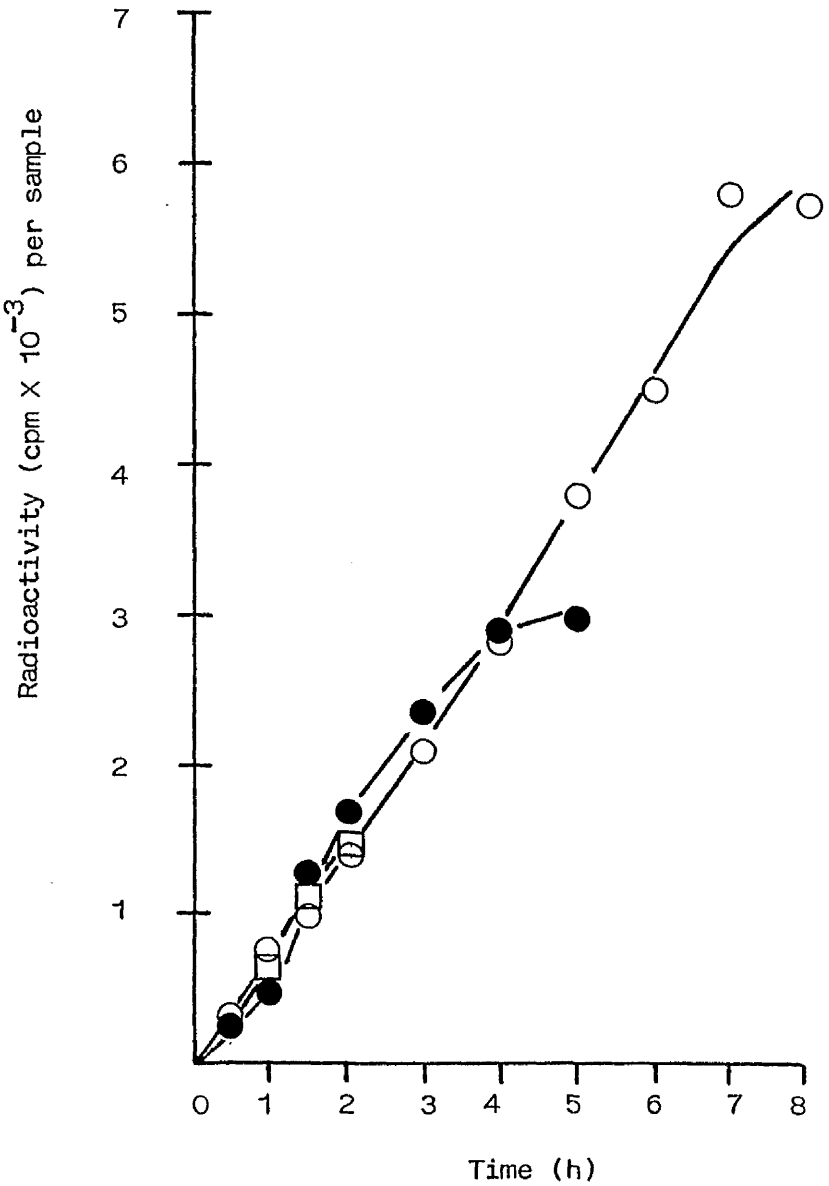
Strain but-32 was grown and resuspended in 300 ml media containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). At intervals, samples (10 ml) were removed, the cells collected on a membrane filter, washed with SM and suspended in SM (10 ml) + TdR (8.5 μgml^{-1}). At t_3, t_5 and t_8 two additional samples were transferred into the same medium, HPUra (50 μgml^{-1}) added to one 45 min after the transfer, and to the other 90 min after transfer. At the time of transfer the cultures were sampled for a viable cell count. The level of sporulation was determined at t_{20}.

Determination	Colony - forming units $ml^{-1}X10^{-5}$ at indicated time (h) after shifting cell sample to TdR medium					
	t_3	t_4	t_5	t_6	t_7	t_8
Viable cells at time of transfer	950	720	670	970	1080	250
Spores at t_{20}	17	52	29	19	12	10
Spores at t_{20} : HPUra added 45 min after transfer	1.3		1.4			0.7
Spores at t_{20}:HPUra added 90 min after transfer	4.1		4.7			1.9

Figure 37. DNA synthesis after transfer from TdR to BUdR medium.

Strain but-32 was grown and resuspended in 50 ml media containing TdR (8.5 μgml^{-1}). At t_{zero}(0), t_3 (●) and t_6(□) samples (10 ml) were removed, the cells collected on a membrane filter, washed in SM and suspended in SM containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + $\left[^{14}C\right]$- TdR (0.025 μCml^{-1}). At intervals, samples were removed for measurement of radioactivity incorporated into acid-insoluble material (see Materials and Methods, section VIII).

Fig. 37


Radioactivity (cpm X 10^{-3}) per sample
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Time (h)

t_7 in cells given BUdR from t_{zero}, whereas in cells given TdR alone from t_{zero} the rate levelled off at a much earlier time (about t_5 in Fig. 36, t_3 in Fig.'s 34 and 35). Cells exposed to BUdR after 3h with TdR continued replication for a further 3 to 4h at a near-linear rate and, surprisingly, cells exposed to BUdR after 6h with TdR, when replication had virtually ceased (Fig.'s 34, 35 and 36), also incorporated $\left[^{14}C\right]$- TdR at a linear rate for 2h. This latter result might suggest that BUdR can promote initiation of DNA replication.

D. Marker-frequency analysis of DNA replication during sporulation

It seemed clear that DNA replication during sporulation of but-32 was not adversely affected by BUdR. This was confirmed by marker-frequency analysis (Fig.'s 38 and 39). In these experiments DNA was prepared from cell samples taken at intervals after initiation of sporulation in the presence of BUdR, and the ratio of pur^+ to met^+ transformants compared to that of cells initiated to sporulate with TdR alone.

During sporulation the pur^+/ met^+ ratio of DNA from cells in TdR alone fell over an initial 90 min period as more and more cells terminated DNA replication (Fig. 38). Thereafter the ratio did not alter significantly up to $t_{7.5}$ (Fig. 39). However, for

Figures 38 and 39. Marker-frequency analysis of DNA replication during sporulation of strain but-32.

In separate experiments the cells were grown and resuspended in media (100 ml) containing TdR (8.5 μgml^{-1}) (○), or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●). Immediately (t_{zero}), and at intervals thereafter, samples (10 ml) were removed from each culture at the times indicated and DNA extracted (see Materials and Methods, section VIA).

Strain Mu8u5u16 was used as a recipient for transformation, and pur^+ and met^+ transformants were selected separately (see Materials and Methods, section VIB). The values presented in Fig.'s 38 and 39 are the means of three separate transformations for each DNA sample.

Fig. 38

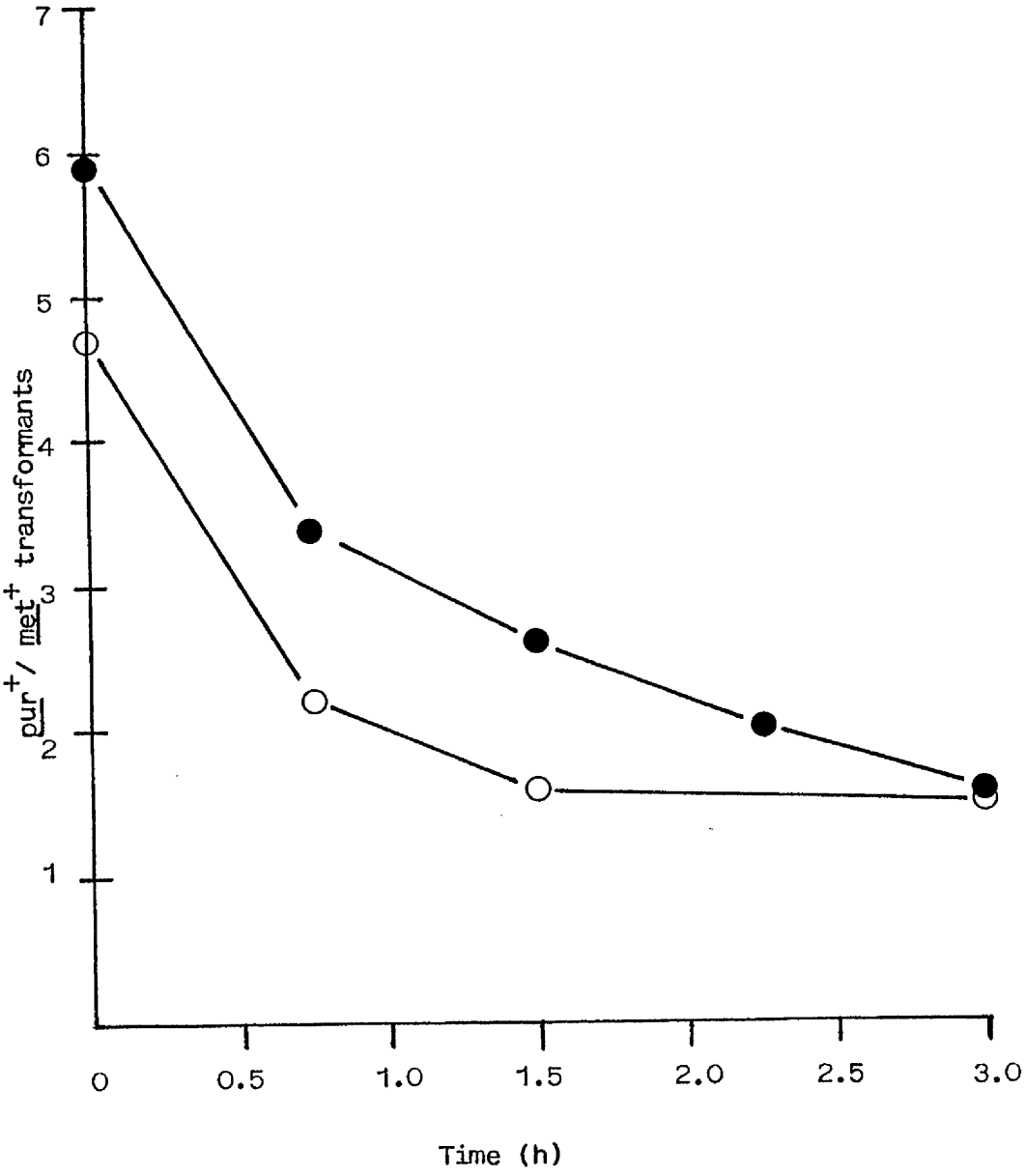
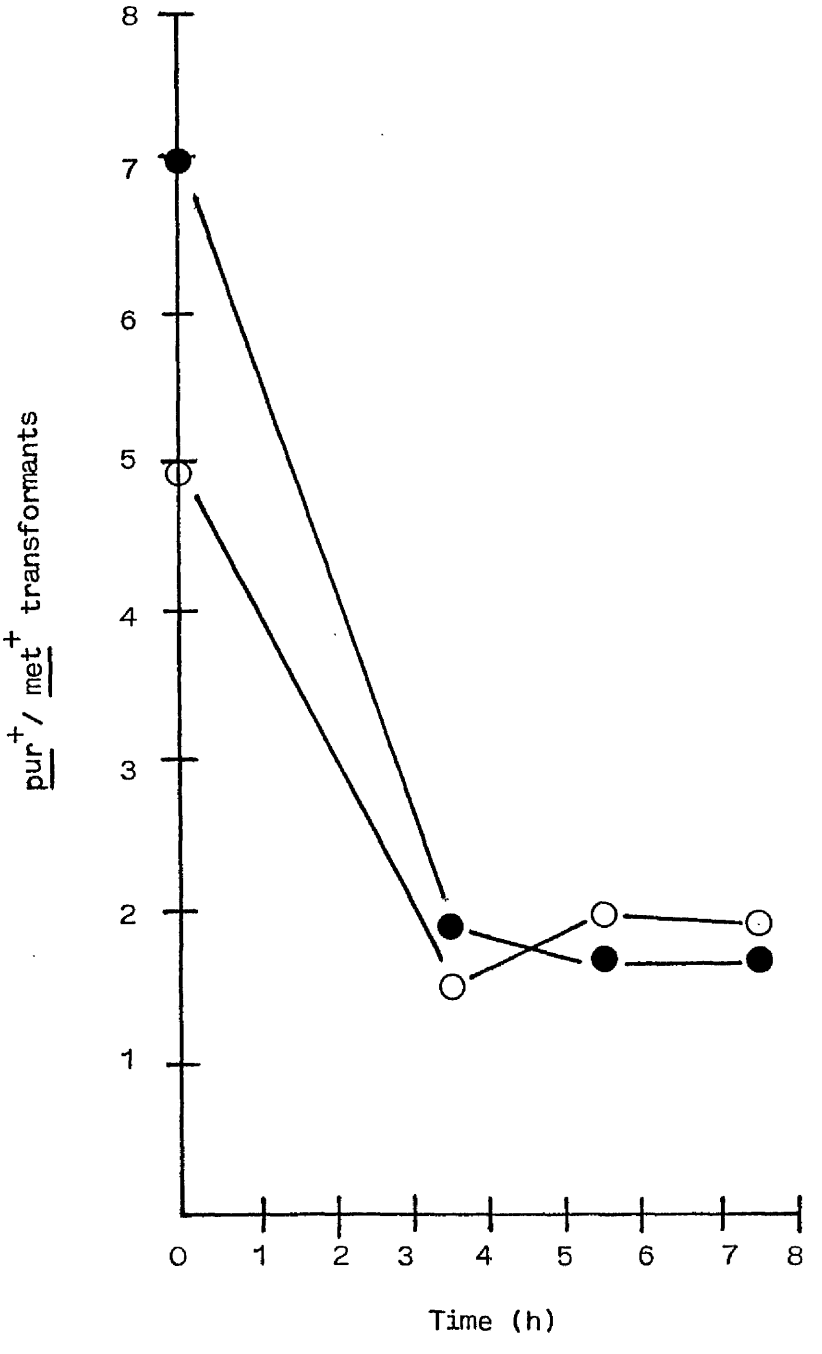


Fig. 39



cells in medium containing BUdR the ratio became constant at a later point, i.e. around 3h after initiation, and again remained relatively constant up to $t_{7.5}$. The difference may be accounted for by the higher *pur*$^+$/ *met*$^+$ ratio of BUdR-treated cells at the start of the sampling period, although even taking this into consideration the *pur*$^+$/ *met*$^+$ ratio for BUdR-treated cells should perhaps have fallen to the constant value earlier than t_3. In neither experiment did the ratios fall to the value of 1.38 obtained for *but-32* spore DNA.

This result therefore suggested that in the presence of BUdR, replication required for sporulation was terminated in most of the cells, but at a later time during sporulation than in TdR alone.

However, when strain *but-32* is subjected to the starvation stimulus there is a natural asynchrony in the cell population, and so the marker ratios obtained represented an average of cells in different stages of DNA replication. Although there appeared to be an effect of BUdR on the *pur*$^+$/ *met*$^+$ marker ratios of *but-32* during sporulation, it was impossible to determine from these results whether the delay in the fall of the ratio during sporulation in BUdR medium was due to an effect on the rate of replication, or to reinitiation. If the latter was true then the average marker ratios at each point would be increased, and this effect, when superimposed on termination, would appear as a slower rate

of decrease in the *pur*$^+$/ *met*$^+$ ratios.

Therefore, it was hoped to clarify the interpretation by using a system where initiation of DNA replication during starvation was prevented. Rather than using chloramphenicol to achieve this, with the problem of totally removing the drug when required, it was decided to use a mutant strain temperature-sensitive (ts) for initiation of DNA replication, i.e. *Ts134* (*trp C*, *thy A*, *thy B*, *dnaB(Ts)134*). If DNA replication in this strain could be synchronized during growth at the permissive temperature, and the cells initiated to sporulate at the restrictive temperature, the precise effect of BUdR on the rate of termination of DNA replication during sporulation could be determined. Mandelstam and Higgs (1974) used a temperature of 45^{o}C to inhibit initiation of new rounds of DNA replication during growth of this strain, but Callister *et al* (1977) reported some initiation of replication at 45^{o}C which was completely inhibited at 47^{o}C.

Fig. 40 illustrates the ts nature of DNA replication during growth of this strain. At 35^{o}C, the permissive temperature, DNA replication proceeded, but if a portion of the cells were removed to 47^{o}C, the restrictive temperature, the cells ceased DNA synthesis after approximately 60–90 min, presumably due to completion of existing rounds of DNA replication without further initiation. At 47^{o}C the rate of DNA replication in the strain from which *Ts134* was derived, *B. subtilis* (*trp C*, *thy A*, *thy B*), the original parent

Figures 40 and 41. The effect of temperature on DNA synthesis and doubling time during growth of B. subtilis (trpC, thyA, thyB) and a mutant derivative temperature-sensitive (ts) for initiation of DNA replication, B. subtilis (trpC, thyA, thyB, dnaB(Ts)134).

Both strains were grown at 35°C in CH medium (20 ml) with TdR (8.5 $\mu g ml^{-1}$) and $[^{14}C]$ - TdR (0.025 $\mu Ci ml^{-1}$). At an E_{600nm} of approximately 0.5, each culture was halved, one portion of each shifted to a shaking water-bath (Gallenkamp Ltd,) at 47°C, the other half left at 35°C. (↑)denotes the time of temperature shift. Throughout the experiment samples were removed for measurement of incorporation of $[^{14}C]$-TdR into acid-insoluble material (Fig. 40), and the growth rates monitored by extinction at 600nm (Fig. 41) (see below)

Symbol	Strain	Temperature (°C)	Doubling time (min)
○	parent	35	48
●	parent	47	30
□	Ts134	35	60
■	Ts134	47	45

Fig. 40

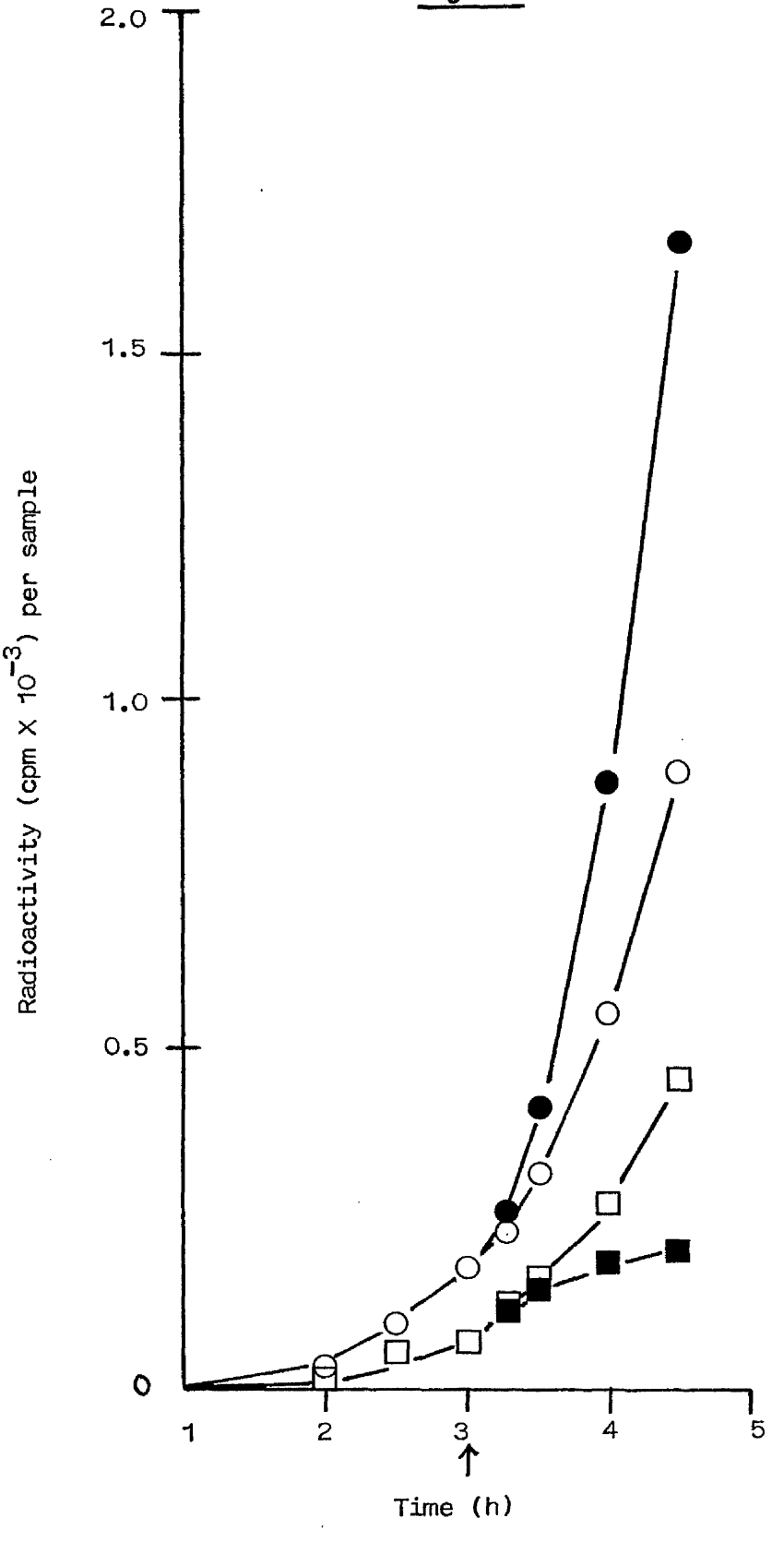
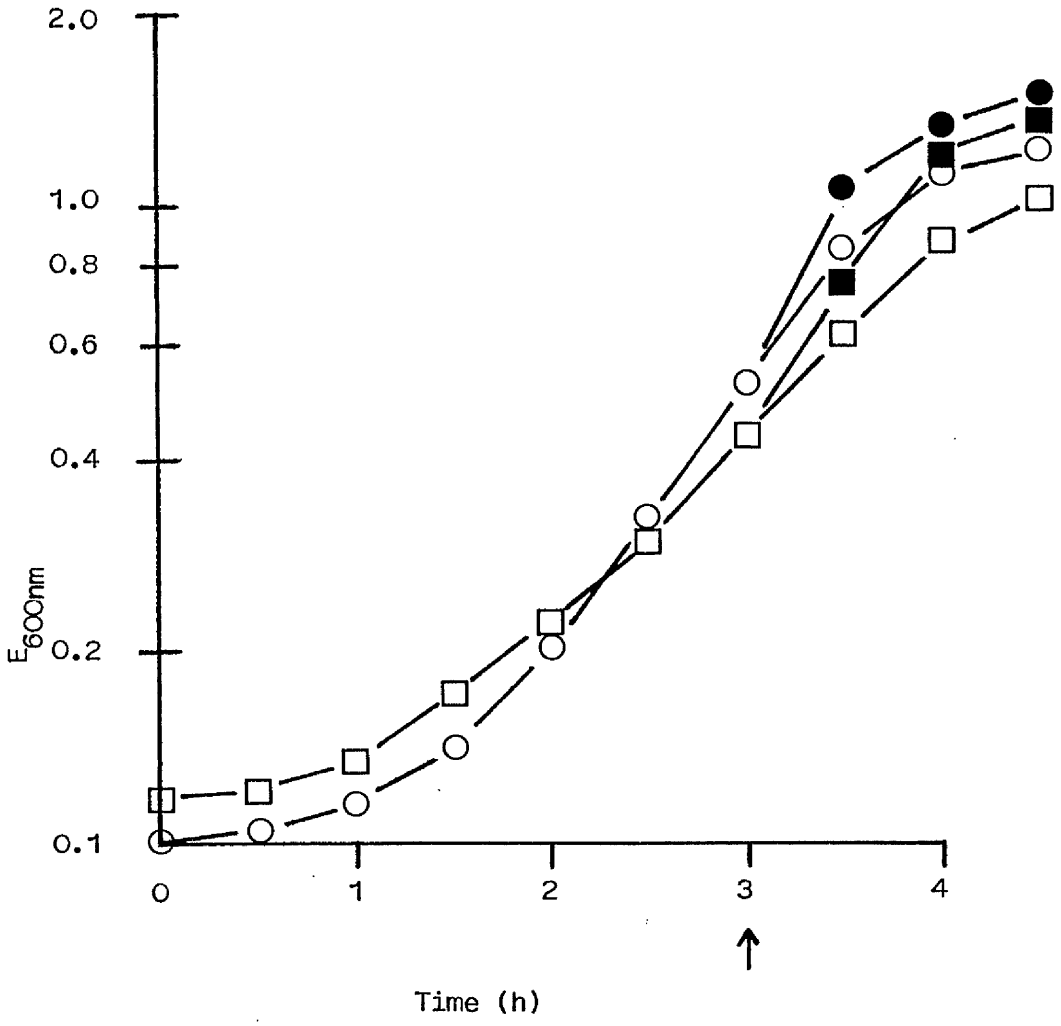


Fig. 41



strain in this work, was slightly greater than at 35°C.

The difference between the rates of [^{14}C]- TdR incorporation of these strains at 35°C may be a general effect caused by the slower rate of growth of Ts134 at this temperature (Fig. 41). In both strains the rate of growth was increased at 47°C, showing that even without continued DNA synthesis Ts134 could still grow under these conditions.

In a separate experiment (Fig. 42), cells of the ts strain were reincubated at 35°C for 15 min after a 90 min period at 47°C, to determine if DNA replication could be reinitiated during this time. Dilution of the culture at 47°C with fresh medium at 25°C was necessary to simultaneously decrease the E_{600nm} of the culture to approximately 0.6, and to bring the temperature down to about 35°C. The level of incorporated radioactivity had increased by 15 min at this temperature and, because DNA replication had terminated by 90 min at 47°C, this must have been due to reinitiation of DNA replication, presumably in a synchronous manner. Growth medium was used as this strain did not sporulate well if the cells were resuspended in SM immediately after completing their chromosomes in rich medium. This was also observed by Mandelstam and Higgs (1974), and was probably due to lack of reinitiation of DNA replication in the starvation medium (see Fig. 47).

Figure 42. Reinitiation of DNA replication in strain Ts134 at the permissive temperature.

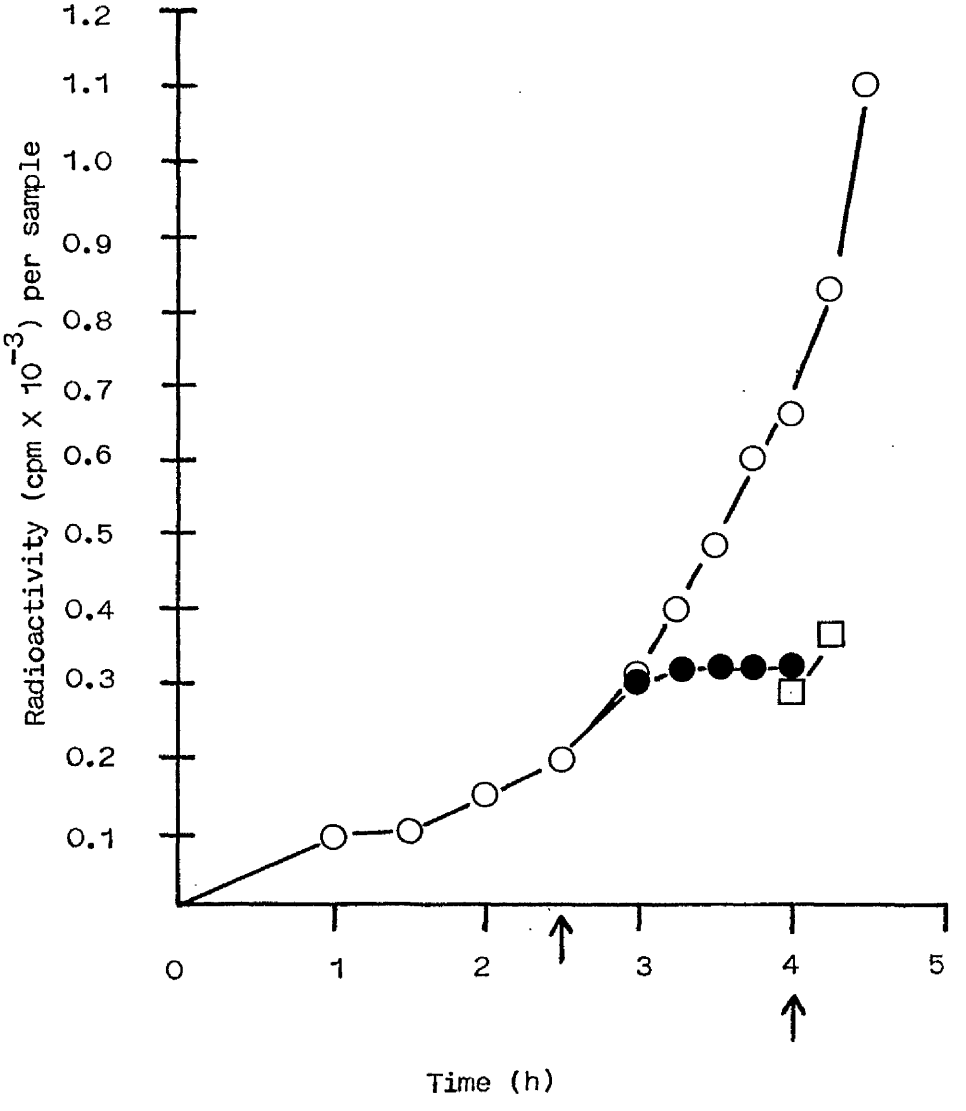
Strain Ts134 was grown at 35°C in CH medium (50 ml) plus TdR (8.5 $\mu g ml^{-1}$) and [^{14}C] - TdR (0.025 $\mu Ci ml^{-1}$) to an E_{600nm} of approximately 0.5. The culture was then halved, one half shifted to 47°C, the other half left at 35°C. Incubation was continued for 90 min, when the cells at 47°C were diluted to an E_{600nm} of 0.6 using fresh CH medium kept at room temperature, plus an appropriate amount of [^{14}C] - TdR. Incubation was continued at 35°C for a further 15 min, together with the control culture already at 35°C. Throughout the experiment samples were removed for measurement of incorporation of [^{14}C] - TdR into acid-insoluble material. ↑ denotes the times of temperature shifts.

○ Ts134; 35°C

● Ts134; 47°C

□ Ts134; 47°C, shifted back to 35°C

Fig. 42


1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Radioactivity (cpm X 10^{-3}) per sample
0
1
2
3
4
5
Time (h)

In another two experiments (Table 21) the procedure outlined in Fig. 42 was used to synchronously reinitiate DNA replication in Ts134. After the 15 min reinitiation period at 35°C in growth medium, the cells were then initiated to sporulate in SM at 47°C. It was assumed that DNA replication already initiated would continue to completion in the starvation medium, but any reinitiation would not occur. The level of sporulation in the presence of BUdR and HPUra at two concentrations (0.5 μgml^{-1} and 50 μgml^{-1}) at this temperature was low compared to a control culture at 35°C, but sporulation was also inhibited at 47°C in TdR alone. This result could have occured either because the cells required a whole round of chromosome replication in resuspension medium to successfully sporulate, i.e. initiation of replication was required in starvation medium for successful sporulation, or simply that at 47°C the cells were metabolically unable to undergo sporulation. The latter is probably true, as the parent strain, treated in an identical manner as Ts134, failed to sporulate to a detectable level (i.e. at a 10^{-2} dilution) in TdR alone at 47°C, whereas it sporulated well at 35°C (Table 21).

The pattern of incorporation of $[^{14}C]$- TdR into acid-insoluble material after suspension of cells in SM at 47°C in the presence of BUdR closely followed that in TdR (8.5 μgml^{-1}) (Fig. 43), but by the end of the sampling period the E_{600nm} values for these cultures, as well as for one with TdR (1 μgml^{-1}) had dropped by half, indicative of a loss of viability (see legend to Fig. 43).

TABLE 21. Ts134 sporulation at the restrictive temperature after synchronizing DNA replication during growth.

The parent strain and Ts134 were each grown in CH medium (120ml) + TdR (8.5 μgml^{-1}). Synchronized initiation of DNA replication of the ts strain was achieved using the method of a previous experiment (Fig 42.). After reinitiation of replication at 35° C for 15 min, six volumes (each 20 ml) of cells were centrifuged, and the pellets suspended in SM (20ml) at 47° C, with the additions shown, except for one control culture with TdR (8.5 μgml^{-1}) left at 35° C. The level of sporulation was determined at t_{20}. The parent strain was treated in an identical fashion as Ts134. Figures in parentheses give the sporulation levels for a similar experiment, where only the ts strain was used, and the effect of HPUra (0.5 μgml^{-1}) was not tested.

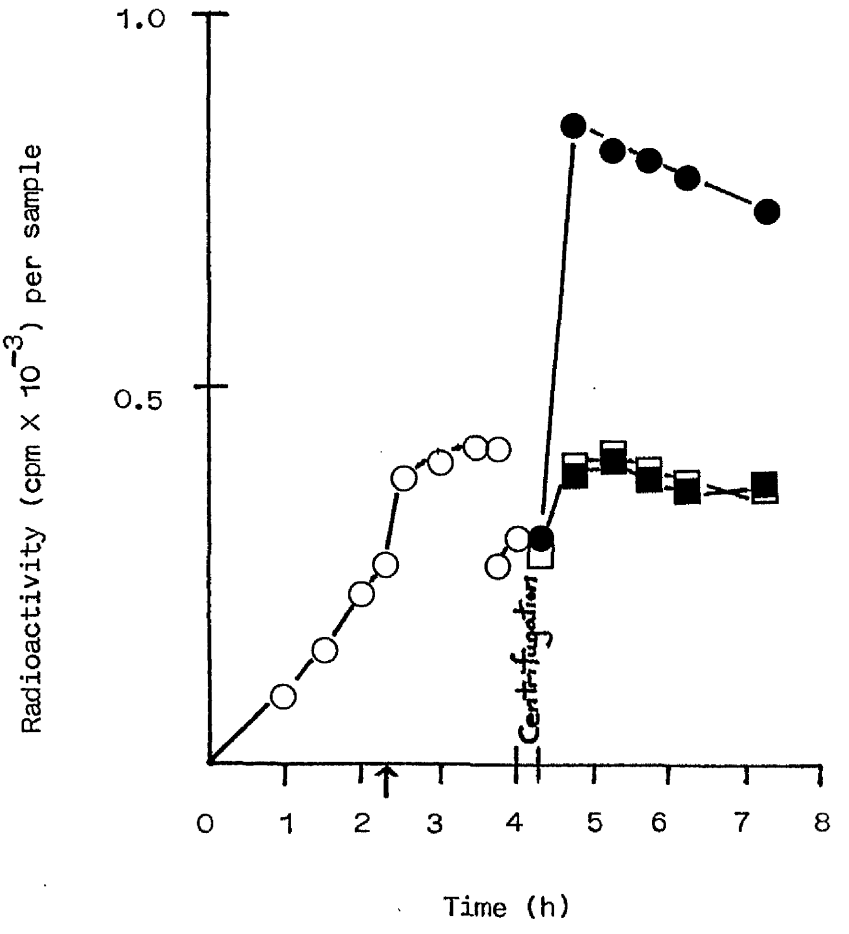
Additions to SM	Temperature (°C)	Spores $ml^{-1} X10^{-6}$ at t_{20}	
		parent	Ts134
TdR (8.5 μgml^{-1})	35	44	151 (44)
TdR (8.5 μgml^{-1})	47	0	0.46 (0.84)
TdR (8.5 μgml^{-1}) +HPUra (0.5 μgml^{-1})	47	0	0.10
TdR (8.5 μgml^{-1}) + HPUra (50 μgml^{-1})	47	0	0.34 (0.91)
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	47	0	0.28 (0.65)
TdR (1 μgml^{-1})	47	0	0.23 (1.0)

Figure 43. The rate of termination of DNA replication during Ts134 sporulation at the restrictive temperature.

Strain Ts134 was grown at 35°C in CH medium (150 ml) with TdR (8.5 μgml^{-1}) + $[^{14}C]$- TdR (0.025 $\mu Ciml^{-1}$) (○). DNA replication was synchronized in this medium using the procedure outlined in a previous experiment (Fig. 42). After the 15 min reinitiation period at 35°C, the cells were centrifuged in three 50 ml volumes, and the pellets suspended at 47°C in SM (50 ml) containing TdR (8.5 μgml^{-1}) (□), TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (■) or TdR (1 μgml^{-1}) (●). $[^{14}C]$- TdR (0.025 $\mu Ciml^{-1}$) was added to all three cultures. These were sampled at intervals after resuspension for measurement of incorporation of radioactivity into acid-insoluble material. The values for the E_{600nm} of the three cultures at the time of resuspension (t_{zero}) and the end of the sampling period (t_3) are given below. (↑) denotes the time of the shift of growing cells from 35°C to 47°C.

Additions to SM	E_{600nm}	
	t_{zero}	t_3
TdR (8.5 μgml^{-1})	0.64	0.25
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	0.72	0.33
TdR (1 μgml^{-1})	0.70	0.33

Fig. 43


1.0
0.5
Radioactivity (cpm X 10^{-3}) per sample
Centrifugation
0
1
2
3
4
5
6
7
8
Time (h)

Therefore, the rapid cessation of further $[^{14}C]$ - TdR incorporation observed for these three cultures after an initial increase at 47°C in SM was probably due to a viability effect rather than a consequence of the phenotype of Ts134.

Therefore, it was concluded that 47°C was an unsuitable temperature for sporulation, and two similar experiments were carried out, in which the cells were allowed to sporulate at 35°C (Table 22). BUdR and HPUra (50 μgml^{-1}) inhibited sporulation of this strain, but in one experiment the level with TdR (1 μgml^{-1}) was relatively low compared to a culture with TdR at 8.5 μgml^{-1}. In the other experiment the level of sporulation at a TdR concentration of 1 μgml^{-1} was much higher, and almost up to the control level.

Allowing the cells to sporulate at 35°C meant that reinitiation of DNA replication could not be prevented if it was occurring, and any effect of BUdR on the rate of decrease in the pur^+/ met^+ ratios during sporulation could still be interpreted as either a decreased rate of DNA replication, or reinitiation of replication. Nevertheless, it was hoped that by using this procedure any reinitiation in the presence of BUdR could be visualised more easily in a marker-frequency analysis.

After synchronizing DNA replication during growth of Ts134, and initiating sporulation at 35°C in TdR alone, the

TABLE 22. Sporulation of Ts134 at 35° C, after synchronizing DNA replication during growth.

Strain Ts134 was grown at 35° C in CH medium (100 ml) + TdR (8.5 μgml^{-1}). DNA replication was synchronized as described for a previous experiment (Fig.42). After reinitiation of DNA replication at 35° C, the culture was divided into five equal portions, the cells harvested by centrifugation and initiated to sporulate in SM (20 ml) containing the additions shown. One culture, containing TdR (8.5 μgml^{-1}) was incubated at 47° C, whereas the others were incubated at 35°C. The level of sporulation in all cultures was determined at t_{20}. Figures in parentheses are the sporulation levels in another experiment in which the effect of HPUra (50 μgml^{-1}) was not tested.

Additions to SM	Temperature (°C)	Spores ml^{-1} ($X10^{-6}$) at t_{20}
TdR (8.5 μgml^{-1})	47	0.49 (0.25)
TdR (8.5 μgml^{-1})	35	58 (44)
TdR (8.5 μgml^{-1}) + HPUra (50 μgml^{-1})	35	0.82
TdR (1 μgml^{-1})	35	2.64 (38)
TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	35	1.50 (0.9)

pur$^+$/ *met*$^+$ ratio fell over an initial 3h period (Fig. 44), and thereafter did not alter significantly. However, in BUdR-treated cells the ratio did not completely level off during the course of this experiment. This was a similar result to that obtained using strain *but-32* (Fig. 38), but in this case the ratio in TdR alone took a longer time to reach the basal level. The reason for this could have been that even using this procedure not all the cells had initiated replication during the 15 min reinitiation period. Therefore, during centrifugation and after resuspension at 35^{o}C initiation was still occuring in many cells. Indeed, the *pur*$^+$/ *met*$^+$ ratio for DNA prepared from cells sampled at the end of the 15 min period of reinitiation in growth medium was only 1.56 (average of three determinations), suggesting that little initiation had occurred by this time.

The clear difference between the effects of BUdR and HPUra (0.5 $\mu g ml^{-1}$) on the ratios might suggest a different mode of action, i.e. BUdR may not be slowing down the rate of DNA synthesis, which was achieved using a subinhibitory concentration of HPUra, or at least it was not slowing down the rate to the same extent as HPUra.

In a separate experiment, cell samples were taken for preparation of DNA during both growth and sporulation, and the *pur*$^+$/ *met*$^+$ ratio measured (Fig. 45). In this experiment, after

Figure 44. Marker-frequency analysis of DNA replication during Ts134 sporulation, after synchronizing replication during growth.

Strain Ts134 was grown at 35°C in CH medium plus TdR (8.5 $\mu g ml^{-1}$) in three parallel 100 ml cultures. DNA replication was synchronized as in a previous experiment (Fig. 42). At the end of the reinitiation period of 15 min, a sample (10 ml) was removed from each culture, for preparation of DNA for marker-frequency analysis. The remaining cells of each culture were centrifuged, and the cells resuspended in SM (100 ml) + TdR (8.5 $\mu g ml^{-1}$) (○), TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) (●) or TdR (8.5 $\mu g ml^{-1}$) + HPUra (0.5 $\mu g ml^{-1}$) (▲). At intervals during sporulation 10 ml aliquots were removed from each culture for extraction of DNA for marker-frequency analysis. The pur^+/met^+ ratios presented are the mean values for three separate transformations for each DNA sample.

The levels of sporulation in the presence of BUdR and HPUra (0.5 $\mu g ml^{-1}$) were 2.9×10^6 and 1.0×10^6 spores ml^{-1} respectively, compared to a value of 52×10^6 in TdR alone.

Fig. 44

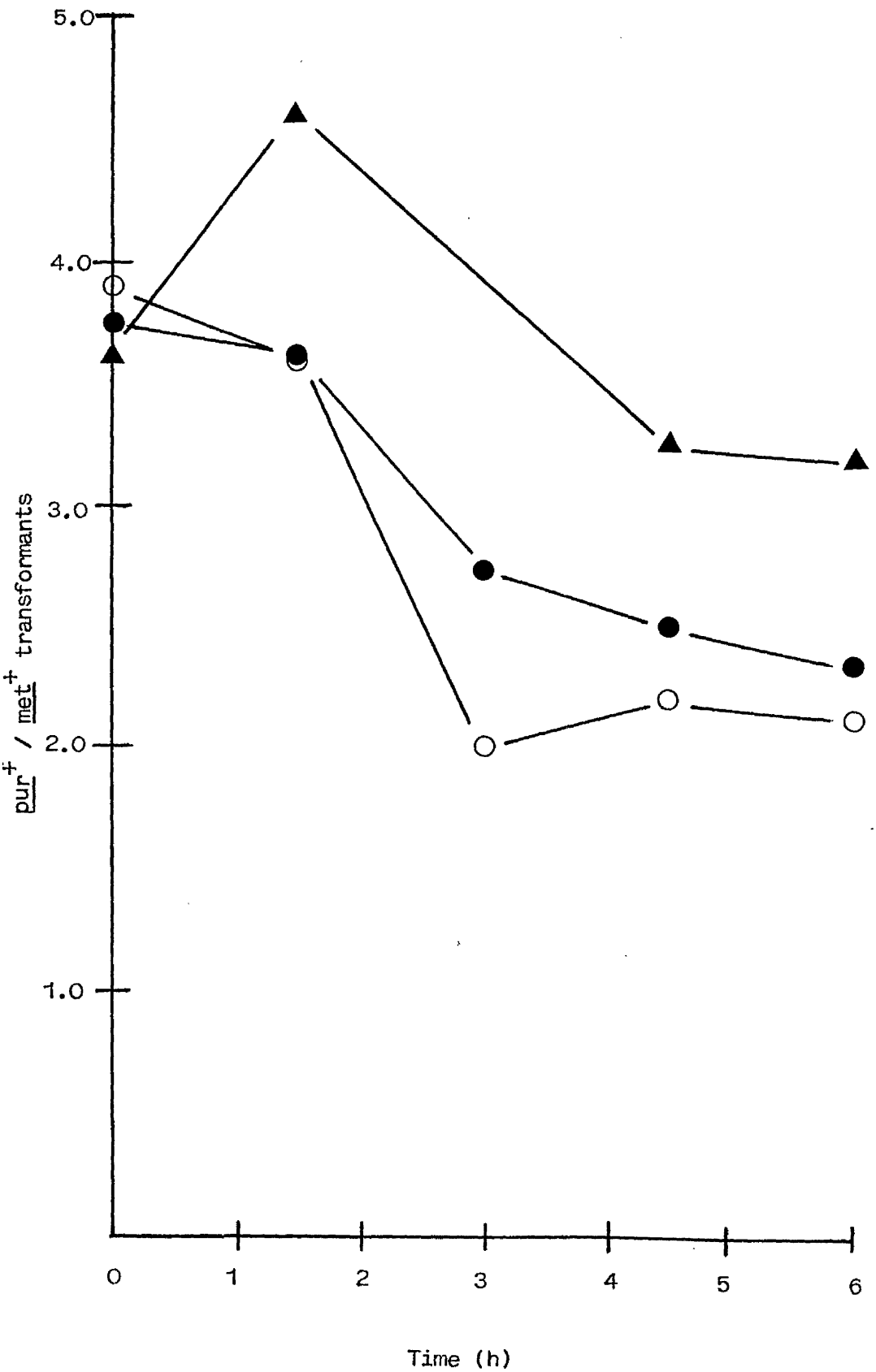


Figure 45. Marker-frequency analysis of DNA replication during Ts134 sporulation, after synchronizing DNA replication during growth.

The procedure outlined in the previous experiment (Fig. 44) was followed, except that samples (10 ml) were also removed during growth for extraction of DNA for marker frequency analysis. In addition, the cells were only grown at $35^{o}C$ to an E_{600nm} of 0.2, before shifting the cells to $47^{o}C$, and the reinitiation period at $35^{o}C$ was increased from 15 min to 45 min. $\left[^{14}C\right]$- TdR (0.025 $\mu Ciml^{-1}$) was present throughout the experiment in all cultures for measurement of DNA synthesis (see Fig. 46). The ratios presented for cells during growth are the mean values for samples of three parallel cultures (one transformation for each). The ratios for cells during sporulation are the mean values for three separate transformations.

In this experiment the level of sporulation in the presence of BUdR and HPUra was 0.2 and 1.3×10^{6} spores ml^{-1} respectively, compared to a value of 12.8×10^{6} in TdR alone.

Fig. 45

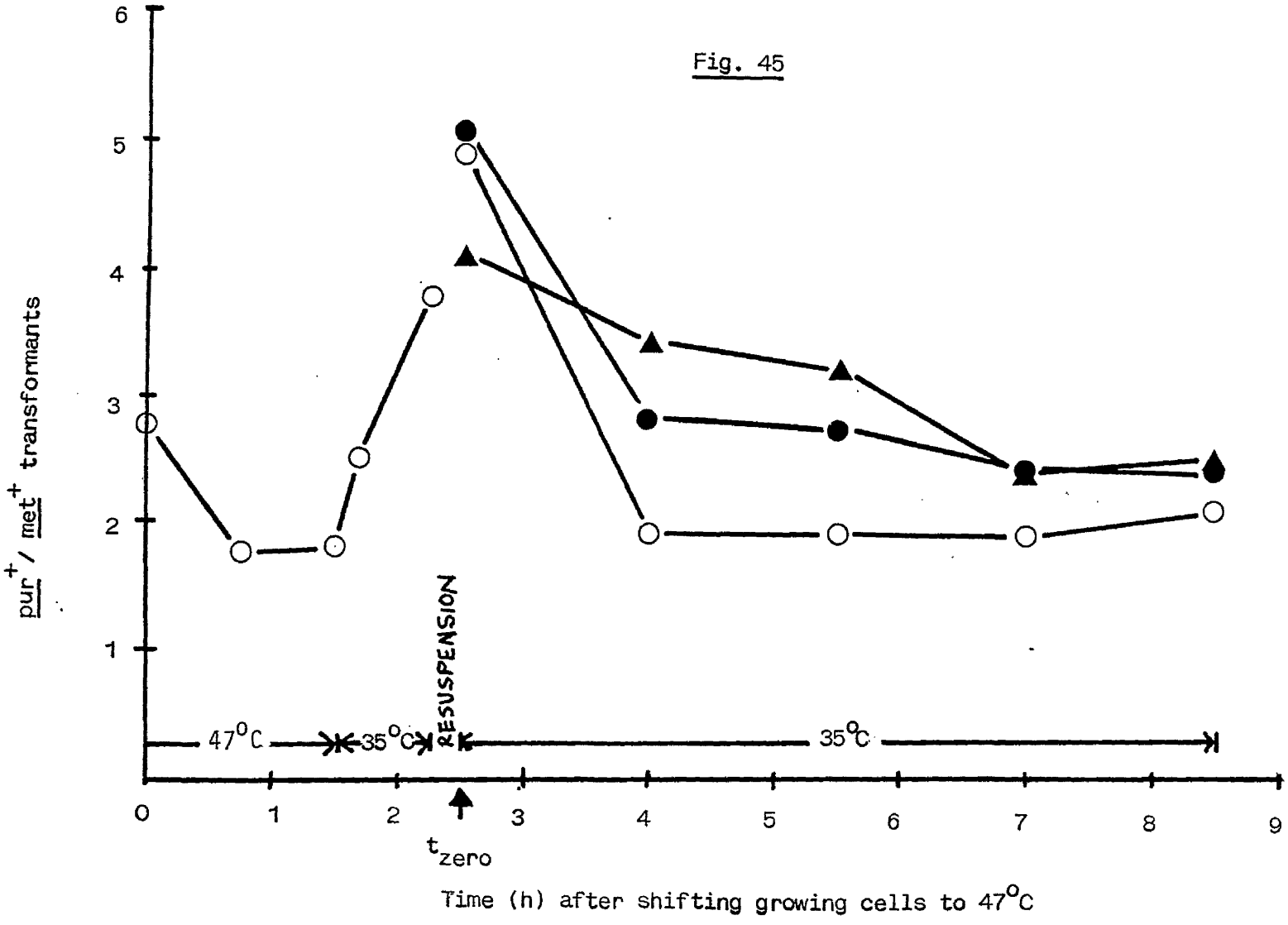

6
5
4
3
2
1
pur+ / met+ transformants
47°C
35°C
RESUSPENSION
35°C
0
1
2
3
4
5
6
7
8
9
tzero
Time (h) after shifting growing cells to 47°C

Figure 46. Incorporation of $\left[^{14}C\right]$ - TdR into acid-insoluble material during growth and sporulation of Ts134 in the previous experiment (Fig. 45).

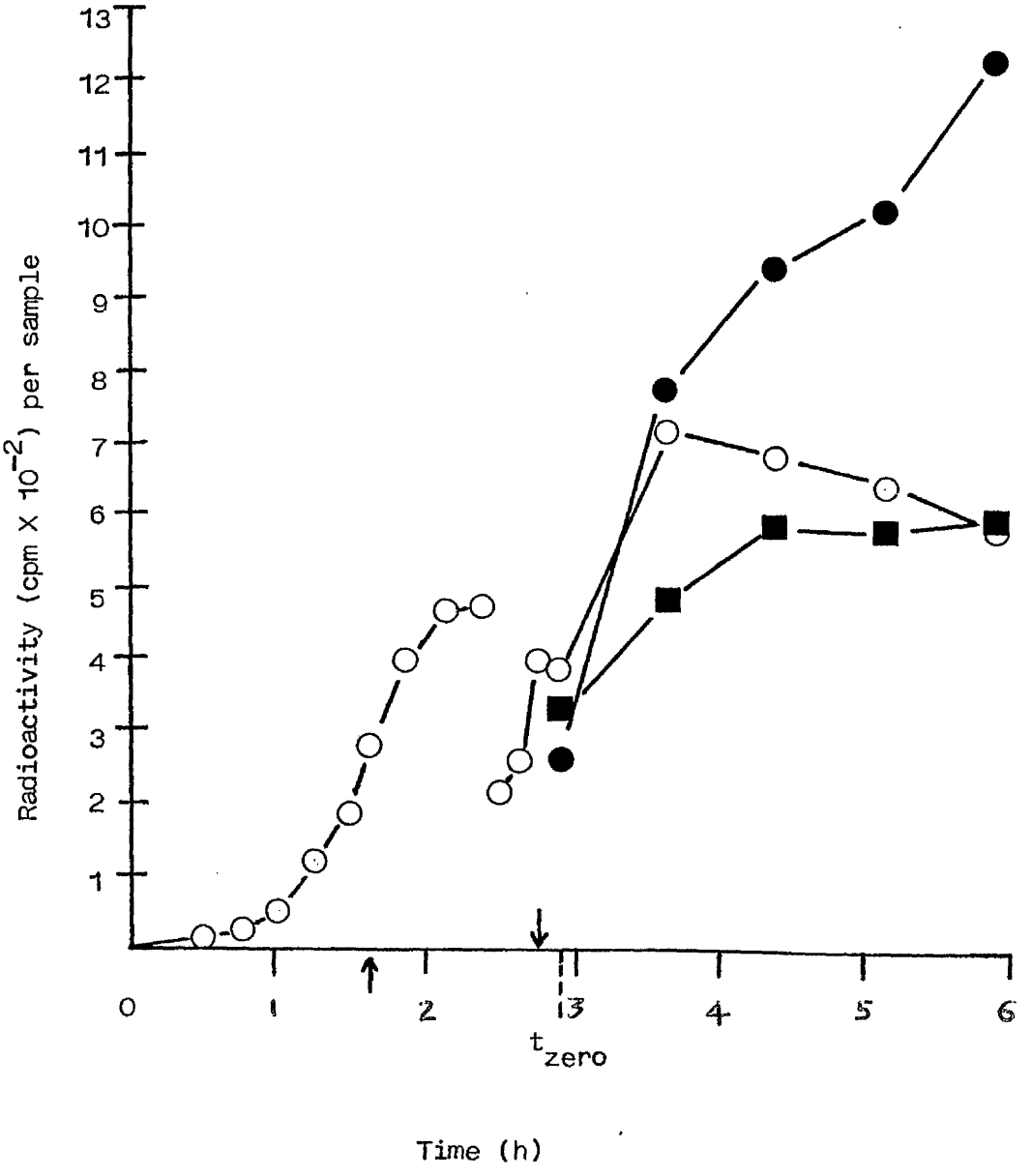
The incorporation of $\left[^{14}C\right]$ - TdR into acid-insoluble material of samples taken during the previous experiment was measured. ↑ denotes the time of shift from 35°C to 47°C. ↓ denotes the end of the reinitiation period at 35°C. The mean cpm for samples taken from the three cultures during growth are presented.

(○) CH medium or SM containing TdR (8.5 μgml^{-1})

(●) SM + TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})

(■) SM + TdR (8.5 μgml^{-1}) + HPUra (0.5 μgml^{-1})

Fig. 46


Radioactivity (cpm X 10^{-2}) per sample
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
t_{zero}
Time (h)

completion of existing rounds of replication at 47°C, the cells were incubated at 35°C over a period of 45 min to ensure that most cells initiated DNA replication in growth medium. The procedure was further modified by initially only growing cells at 35°C to an E_{600nm} of 0.2, an early point in exponential growth, before shifting the cells to 47°C, to ensure that the reduction in the rate of DNA synthesis at 47°C was due to the ts mutation, and not to the effect of cells entering the stationary phase of growth.

$\left[^{14}C\right]$ - TdR incorporation into acid-insoluble material was measured throughout the experiment (Fig. 46), and if the data in these two figures are considered together it can be seen that the marker ratios decreased and DNA synthesis ceased during growth at 47°C, due to completion of existing rounds of DNA replication already initiated before the temperature increase. At this point the cells were almost at the end of exponential growth (E_{600nm} of 0.8). After dilution to 35°C, the marker ratio quickly increased and DNA synthesis resumed as reinitiation of replication occured. After resuspension in SM at 35°C the $\underline{pur}^+$/ $\underline{met}^+$ ratio in TdR alone fell to a constant value by at least 1½h after initiation, after which no further DNA synthesis was measured. In the presence of a subinhibitory concentration of HPUra the rate of decrease of the $\underline{pur}^+$/ $\underline{met}^+$ ratio was delayed, and DNA synthesis was only completed by approximately 3h after initiation of sporulation. However, in the presence of BUdR the $\underline{pur}^+$/$\underline{met}^+$

ratio reached half of its initial value by 1½h after resuspension, and then only slowly decreased until the end of the sampling period (t_6), by which time DNA synthesis had still not terminated.

These results suggested a difference between the modes of action of BUdR and HPUra (0.5 μgml^{-1}) resulting in inhibition of sporulation. However, it was still not possible to determine definitely whether BUdR inhibition was due to a reduction in the rate of DNA synthesis or reinitiation of replication during sporulation, measured as a delay in termination of DNA replication. The different pattern in the fall of the marker ratios obtained by reducing the rate of DNA synthesis with a subinhibitory HPUra concentration to that obtained with BUdR suggested the latter possibility might be the correct one.

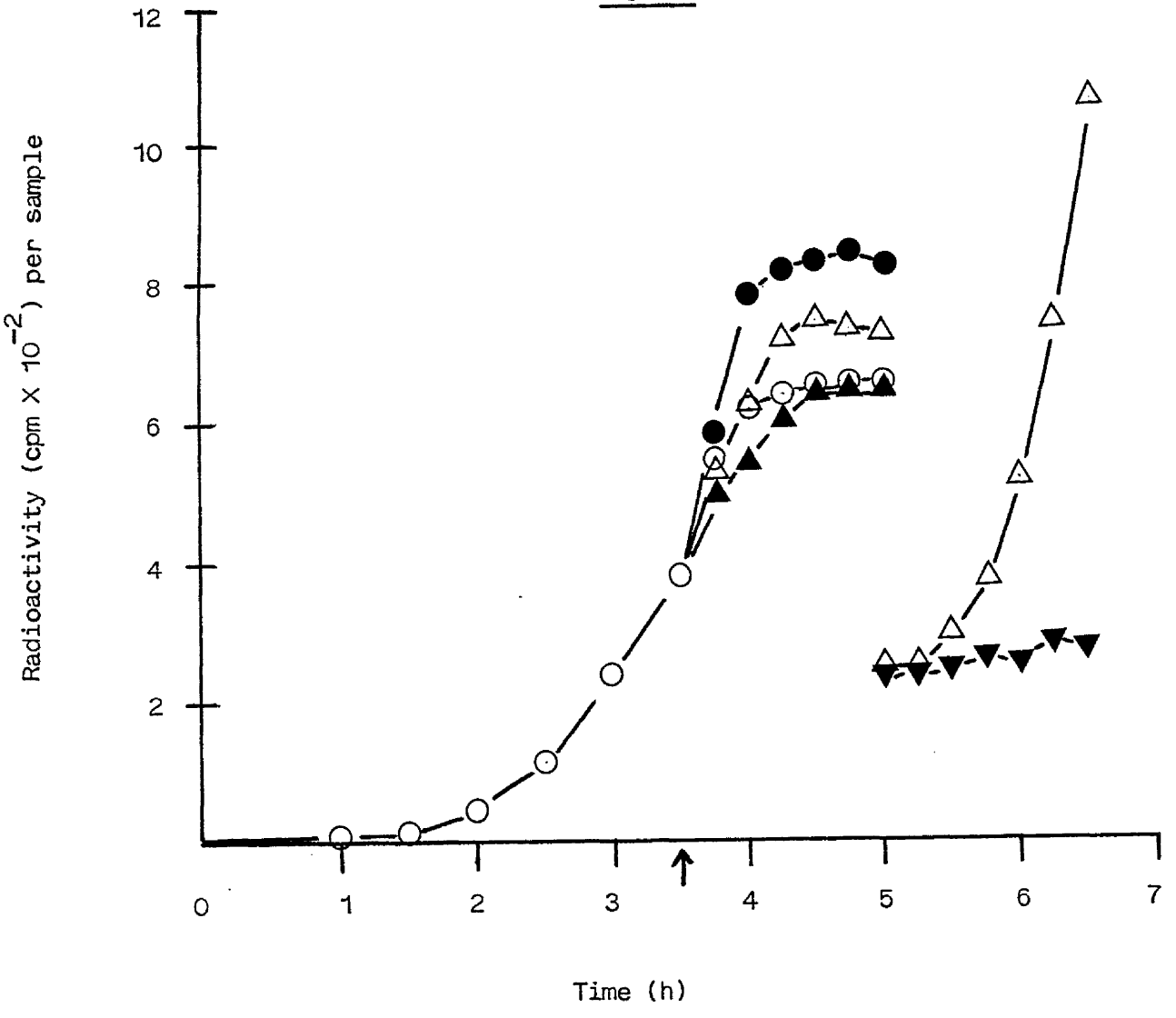
E. Termination of DNA replication

Direct measurement of the rate of termination of DNA replication during sporulation of Ts134 at the non-permissive temperature, by monitoring incorporation of $\left[^{14}C\right]$ - TdR into acid-insoluble material, was not considered possible due to the effect of the temperature on sporulation (Table 21). However, the effect of BUdR on the rate of termination of DNA replication during growth of this strain at 47°C was determined (Fig. 47).

Figure 47. Incorporation of $\left[^{14}C\right]$ - TdR into acid-insoluble material during growth and sporulation of Ts134.

Strain Ts134 was grown at 35°C in CH medium (100 ml) with TdR (8.5 μgml^{-1}) + $\left[^{14}C\right]$ - TdR (0.025 $\mu Ciml^{-1}$) to an E_{600nm} of 0.5. The cells from three 20 ml volumes were harvested by centrifugation and resuspended at 47°C in fresh, pre-warmed CH medium (20 ml) containing TdR (8.5 μgml^{-1}) (○), TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (●) or TdR (8.5 μgml^{-1})+ HPUra (0.5 μgml^{-1}) (▲). Incubation was continued for 90 min at 47°C. ↑ denotes the time of temperature-shift. The remaining 40 ml of the culture was also incubated at 47°C for 90 min (△), halved, and the cells in each half were centrifuged. One half was resuspended at 35°C in an appropriate volume of CH medium + TdR (8.5 μgml^{-1}) + $\left[^{14}C\right]$-TdR (0.025 $\mu Ciml^{-1}$) (△), and cells from the other half suspended in the same volume of SM plus the same additions (▽), so that the E_{600nm} of each was 0.6. Incubation was continued at 35°C for a further 90 min. Samples were removed throughout the experiment for measurement of incorporation of radioactivity into acid-insoluble material.

Fig. 47


Radioactivity (cpm X 10^{-2}) per sample
12
10
8
6
4
2
0
1
2
3
4
5
6
7
Time (h)

Compared to cells in TdR alone there was a negligible effect of BUdR on the time of termination of replication. Decreasing the rate of DNA synthesis by addition of a subinhibitory HPUra concentration delayed termination by perhaps only 15 min. Incidentally, using the same cells in a parallel experiment, it was noted that whereas cells in TdR alone that had terminated DNA replication at 47°C could reinitiate DNA replication at 35°C in growth medium by 30 min, no reinitiation was observed during the sampling period (2h) when the cells were transferred directly to resuspension medium at 35°C after completing replication of their chromosomes at 47°C.

These results had been obtained using Ts134, a derivative of the original parent strain sensitive to the inhibitory effect of BUdR on growth, and as such, it could be argued that any effects of BUdR on DNA replication during sporulation were not specific to the sporulation process, but due to other deleterious effects which also affected growth. A better comparison of the effect of BUdR on sporulation of but-32 and Ts134 could be made using a BUdR-tolerant derivative of Ts134. However, the procedure routinely used for preparation of competent cells of B. subtilis (method 2, Materials and Methods, section VI) did not result in efficient transfer of BUdR-tolerance to Ts134 due to the poor competence of this strain, as even transformation of this strain from trp^- to trp^+ was very inefficient. Nevertheless, some BUdR-tolerant cells were grown from the few colonies which

appeared on minimal agar supplemented with tryptophan (10 μgml^{-1}) + TdR (1 μgml^{-1}) + BUdR (20 μgml^{-1}), after incubation of Ts134 cells with but-32 DNA.

After further characterisation of these colonies, two BUdR-tolerant, TdR-and tryptophan-requiring strains, which failed to grow at 45°C on nutrient agar, were chosen for future use. These strains, termed Ts134(but-1) and Ts134(but-11), grew as well as but-32 on BUdR-minimal medium supplemented with tryptophan, but even but-32 failed to grow at 47°C on nutrient agar, although it could grow at 45°C, hence the use of 45°C rather than 47°C to indicate which strains were temperature-sensitive (see above).

Whereas growth of but-32 and Ts134(but-1) in liquid medium were relatively unaffected at a TdR : BUdR ratio of 1 : 15 μgml^{-1}, compared to cultures with TdR alone, growth of Ts134(but-11) and Ts134 was inhibited, although the tolerant strain did grow exponentially, albeit slowly (Table 23).

In addition, the ts nature of DNA replication in these strains was examined (Fig. 48). Although the rates of DNA synthesis in both But derivatives of Ts134 decreased to almost zero by about 60 min during growth at 47°C, the level of incorporation of $\left[^{14}C\right]$ - TdR in Ts134(but-11) was much lower than that of

TABLE 23. The effect of BUdR on growth and sporulation of two BUdR - tolerant derivatives of Ts134.

Strains Ts134, Ts134(but-1), Ts134(but-11) and but-32 were grown separately at 35° C in CH medium (20 ml) containing TdR (8.5 μgml^{-1}) or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). The rates of growth (doubling times) were measured until an E_{600nm} of 0.7 was reached, when the cells were resuspended in SM (20 ml) with TdR (8.5 μgml^{-1}) or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}). The level of sporulation was determined at t_{20}.

Strain	Additions to medium			
	TdR (8.5 μgml^{-1})		TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1})	
	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}	Doubling time (min)	Spores ml^{-1} ($X10^{-6}$) at t_{20}
Ts134*	66	16	240 → ∞	-
but-32	45	14	56	1.8
Ts134(but-1)	66	48	66	4.8
Ts134(but-11)	63	40	96	0.8

*strain Ts134 grew poorly in BUdR medium and could not be resuspended in SM.

Figure 48. Incorporation of $[^{14}C]$ - TdR into acid-insoluble material during growth of two BUdR-tolerant derivatives of Ts134.

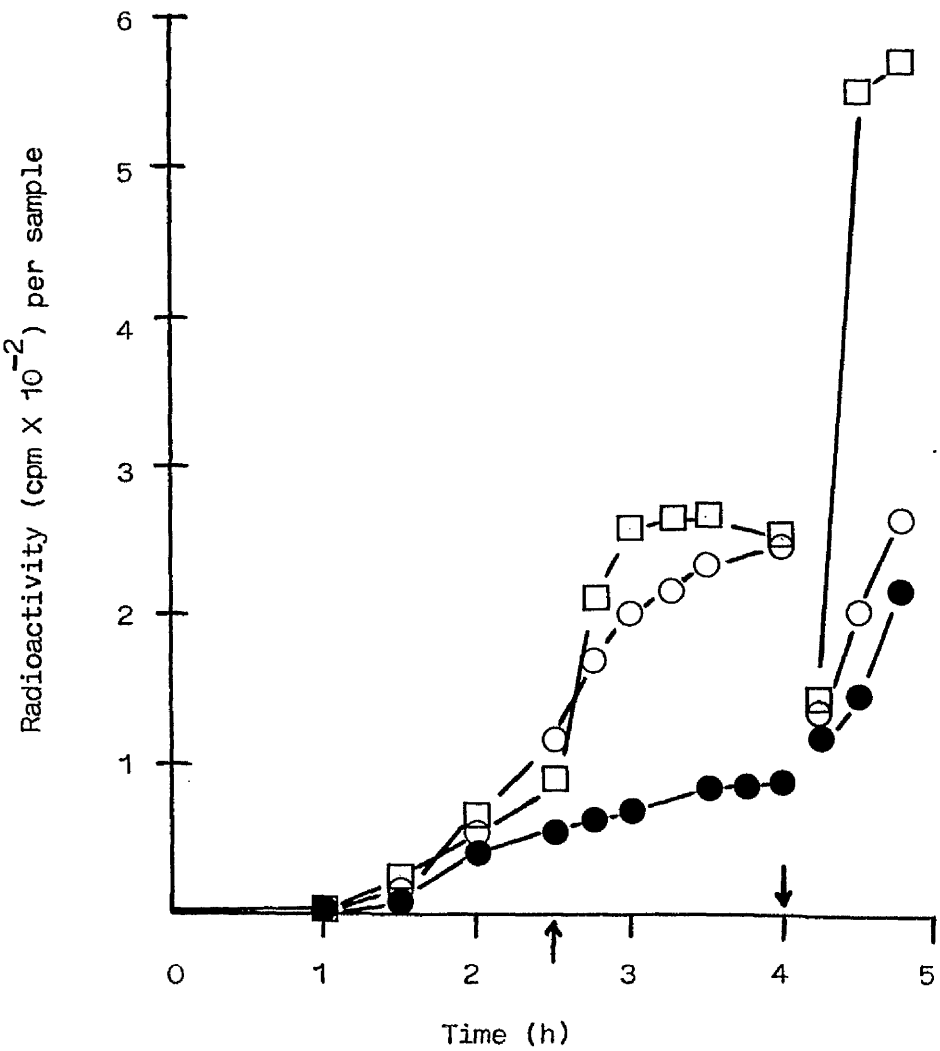
Ts134(but-1), Ts134(but-11) and Ts134 were grown at 35°C in CH medium (20 ml) containing TdR (8.5 μgml^{-1}) plus $[^{14}C]$ - TdR (0.025 $\mu Ciml^{-1}$) to an E_{600nm} of 0.25. At this point (↑) the cultures were transferred to a shaking water-bath at 47°C and incubated for 90 min. After this period (↓) the cultures were diluted back to 35°C using fresh CH medium plus TdR (8.5 μgml^{-1}) and an appropriate amount of $[^{14}C]$TdR , and incubation continued for a further 45 min. Throughout the experiment samples were removed for measurement of incorporation of radioactivity into acid-insoluble material.

(○) Ts134

(□) Ts134(but-1)

(●) Ts134(but-11)

Fig. 48



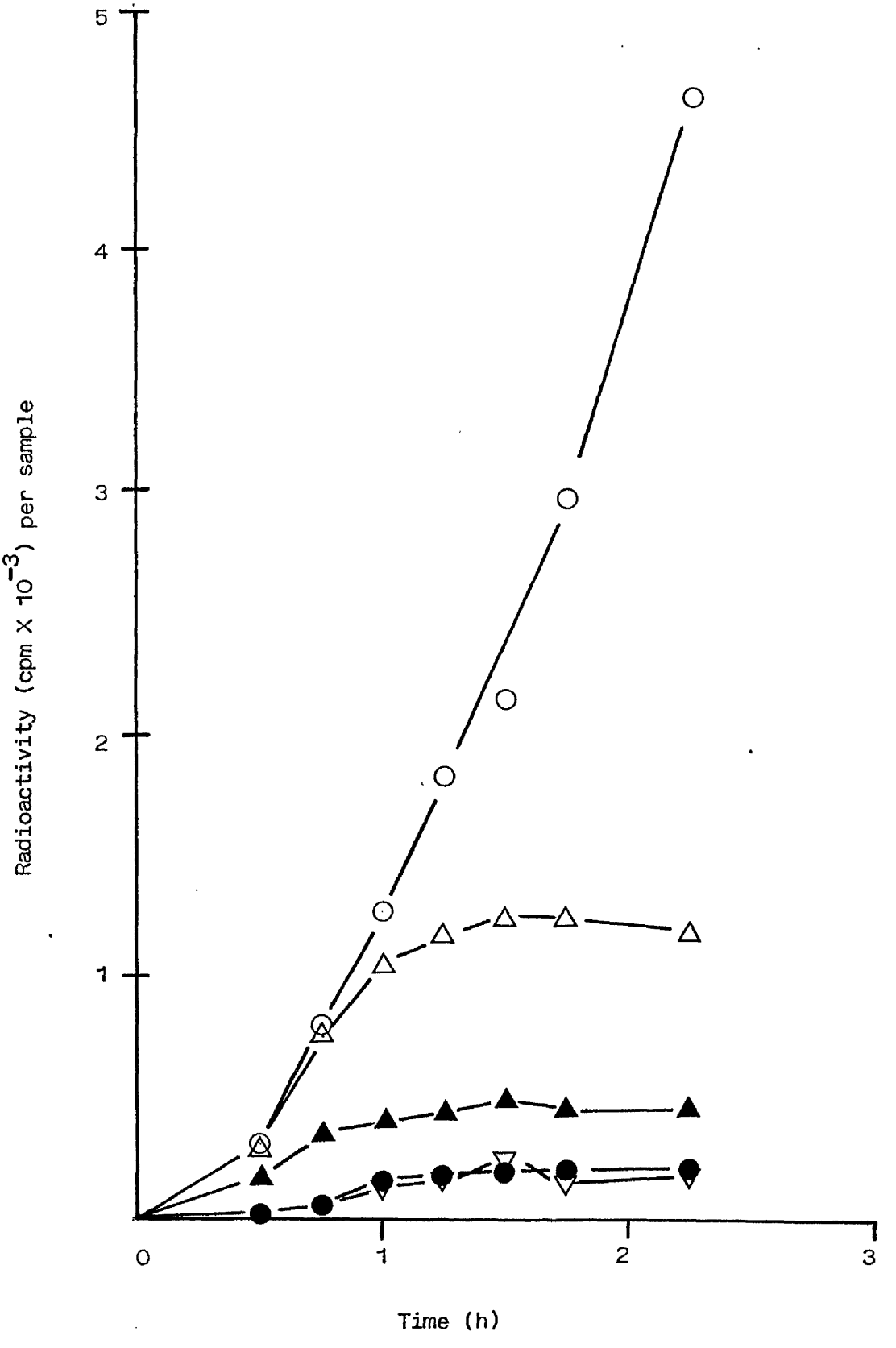
Ts134(but-1) or Ts134. All three strains, however, exhibited reinitiation of DNA replication when subsequently reincubated at 35°C for 30 min, but Ts134(but-1) apparently reinitiated much quicker than the other two strains.

Therefore, because strain Ts134(but-1) exhibited a level of BUdR-tolerance comparable with that of strain but-32, and the temperature-sensitivity of initiation of DNA replication was comparable with that of Ts134, it was used in an experiment testing the effect of BUdR on the rate of termination of DNA replication during growth at the restrictive temperature (Fig. 49). Compared to cells in TdR alone, BUdR did not appear to significantly delay termination of DNA replication at 47°C, although the levels of radioactivity incorporated in this experiment were low. Surprisingly, the incorporation of labelled TdR in BUdR medium did not follow more closely the rate in TdR (1 $\mu g ml^{-1}$) alone, unlike the pattern of incorporation for strain but-32 (see Fig's 10 and 16), but the rate was still higher than in TdR (8.5 $\mu g ml^{-1}$) alone. Therefore, there may have been some exclusion of BUdR from the DNA of Ts134(but-1), although not to the same degree as in but-32. However, it was not possible to further investigate this strain due to lack of time. For the same reason it was not possible to use this strain for marker-frequency analysis of DNA replication during sporulation in the presence of BUdR after synchronization of DNA replication during growth.

Figure 49. The effect of BUdR on the rate of termination of DNA replication during growth of Ts134(but-1) at the restrictive temperature.

Strain Ts134(but-1) was grown at 35°C in CH medium (100 ml) with TdR (8.5 μgml^{-1}) to an E_{600nm} of 0.25. The cells from five 20 ml portions of this culture were harvested separately by filtration through membrane filters, and 4 portions were resuspended at 47°C in fresh pre-warmed CH medium (20 ml) containing TdR (8.5 μgml^{-1}) (●), TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) (▲), TdR (1 μgml^{-1}) (△), and TdR (8.5 μgml^{-1}) + HPUra (0.5 μgml^{-1}) (▽). The fifth sample was resuspended at 35°C in fresh pre-warmed CH medium (20 ml) containing TdR (8.5 μgml^{-1}) (○). [methyl-^{3}H] TdR (0.5 $\mu Ciml^{-1}$) was added to all cultures, and at intervals the cultures were sampled for incorporation of radioactivity into acid-insoluble material.

Fig. 49



Sargent (1980 b) described a method for measuring the rate of termination of DNA replication during sporulation, in which the chromosome terminus of B. subtilis (thy A, thy B, trp C), the original parent strain, was pulse-labelled with [^{3}H] - TdR, and the cells then treated with HPUra at a concentration completely inhibiting further DNA synthesis. Therefore, the only labelled spores formed must have been from cells that had completed DNA replication during the pulse period. He found that the amount of radioactivity in purified spore samples, which had been labelled in the terminus region, reached a broad peak when the cells were pulse-labelled and treated with HPUra about 2h after the start of sporogenesis.

An attempt was made to label the chromosome terminus of but-32 using this method, but in two experiments the level of sporulation in all samples in TdR alone was about 10-fold less than that obtained by Sargent (1980 b), using B. subtilis (thy, trp). Consequently the levels of radioactivity in spores were only slightly above background levels, and the effect of BUdR could not be measured satisfactorily.

F. CsCl density gradient analysis of DNA replication during sporulation

Finally, the degree of incorporation of BUdR into DNA during growth and sporulation in the presence of the analogue

was examined by CsCl pycnography. A previous report suggested that DNA extracted from spores formed in the presence of the analogue contained no BUdR, even using the BUdR-tolerant strain But(32) (Adams and Wake, 1980). Therefore, if it could be shown that BUdR was incorporated into DNA during sporulation, but was subsequently absent from the few spores formed in SM containing BUdR, then it could be concluded that the inhibitory effect of BUdR on sporulation must be a direct result of its incorporation into DNA.

BUdR was incorporated into DNA during growth of but-32 (Fig. 21). It was necessary to include in all gradients two reference DNA samples (labelled with an alternative radioisotope) representing substituted and unsubstituted DNA, as well as the test DNA, as the isodensity position of samples from any one DNA preparation varied slightly between gradients. This procedure was used for all gradients presented in this section.

Strain but-32 was grown in TdR alone but initiated to sporulate in the presence of BUdR. 4h after initiation, 17% of the DNA was in a completely heavy form, 66% in an intermediate form, and 17% in a completely light form (Fig. 50). However, the DNA prepared from the few spores formed in this medium appeared entirely of intermediate density (Fig. 51). When, in the same experiment, the cells were grown in the presence of BUdR but

Figures 50-52. CsCl density gradient analysis of incorporation of BUdR into but-32 DNA during sporulation.

Strain but-32 was grown at 35°C in CH medium (300 ml) containing either TdR (1 μgml^{-1}), or TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), plus $\left[^{14}C\right]$- TdR (0.017 $\mu Ciml^{-1}$). The cells which had been grown in TdR alone were harvested at an E_{600nm} of 0.7 by centrifugation, and were initiated to sporulate in SM (300 ml) containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}) + $\left[^{14}C\right]$- TdR (0.017 $\mu Ciml^{-1}$) (culture 1). Those cells grown in the presence of BUdR were initiated to sporulate in SM (300 ml) + TdR (1 μgml^{-1}) + $\left[^{14}C\right]$- TdR (0.017 $\mu Ciml^{-1}$) (culture 2). Three 10 ml samples were removed from culture 1 at t_4 during sporulation for DNA extraction to give enough material for several gradients (Materials and Methods, section VIA). At t_{20} the levels of sporulation were determined and DNA extracted from the purified spores (Materials and Methods, section VIA). Culture 1 had 3.88×10^6 spores ml^{-1}, and culture 2 had 16×10^6 spores ml^{-1}.

A TdR concentration of only 1 μgml^{-1} was used instead of 8.5 μgml^{-1} to increase the relative levels of radioactivity in the spore DNA. 300 ml volumes were used to obtain a large number of spores for subsequent extraction of DNA.

In all cases the buoyant densities of the samples were directly compared with those of two reference DNA samples,

one of light density, the other heavy density, run on the same gradient, but labelled with a different isotope to that labelling the sample (Materials and Methods, section VIIB). In Figures 50–52 the two reference DNA samples were labelled with [methyl-^{3}H] - TdR.

The profile in each figure was repeated in at least one other CsCl gradient. The differences in the peak levels of radioactivity of the two reference DNA samples was due to alterations in the volumes applied to the gradient.

Fig. 50 DNA from cells at t_4 in culture 1 (cells grown in TdR and resuspended in BUdR medium plus [^{14}C] - TdR).

Fig. 51 Spore DNA (culture 1)

Fig. 52 Spore DNA (culture 2; cells grown in BUdR and resuspended in TdR medium plus [^{14}C] - TdR).

—— [^{14}C] - DNA

----- [^{3}H] - DNA (reference samples)

Fig. 50

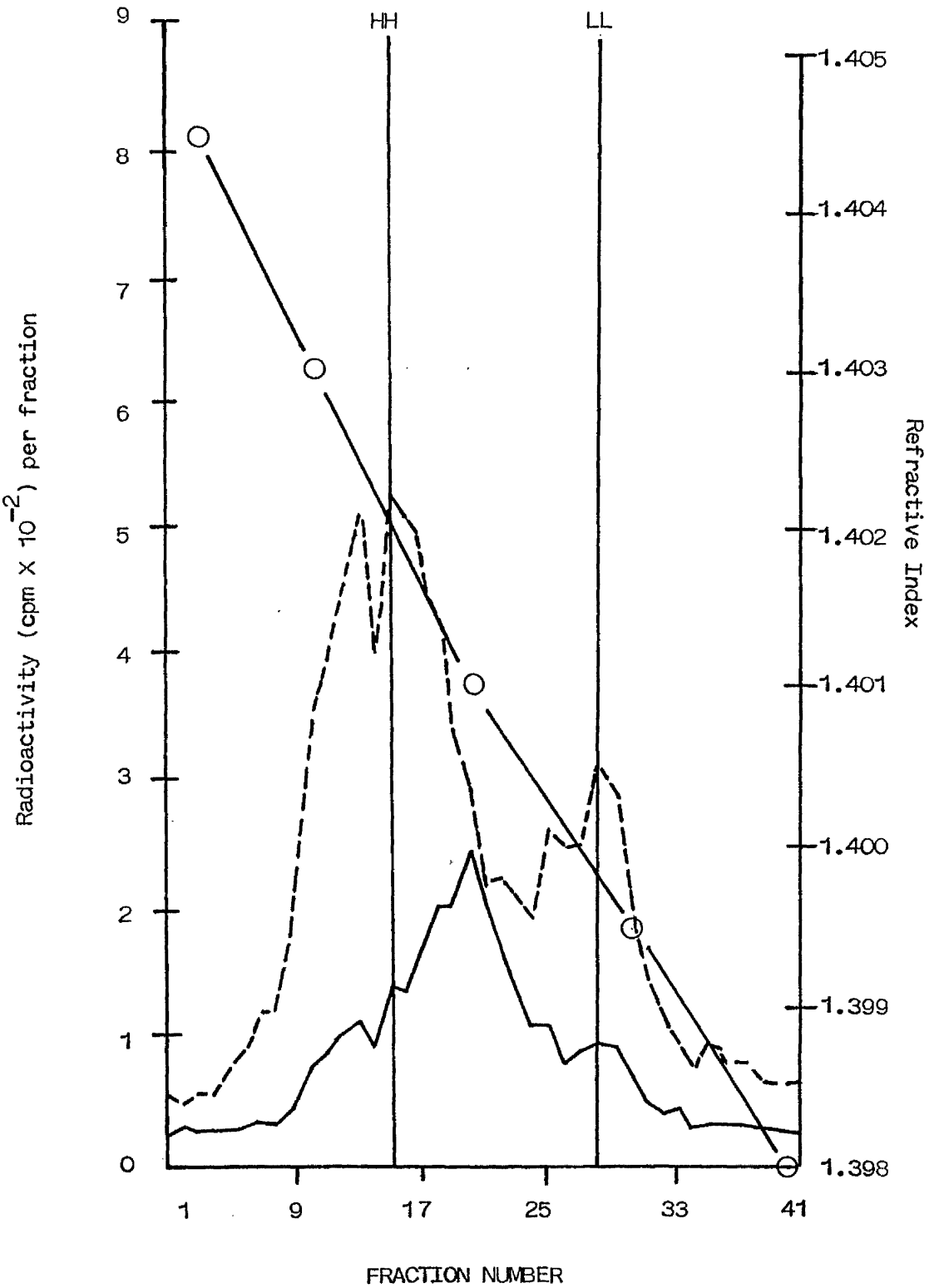

HH
LL
9
8
7
6
5
4
3
2
1
0
1.405
1.404
1.403
1.402
1.401
1.400
1.399
1.398
1
9
17
25
33
41
Radioactivity (cpm X 10^{-2}) per fraction
Refractive Index
FRACTION NUMBER

Fig. 51

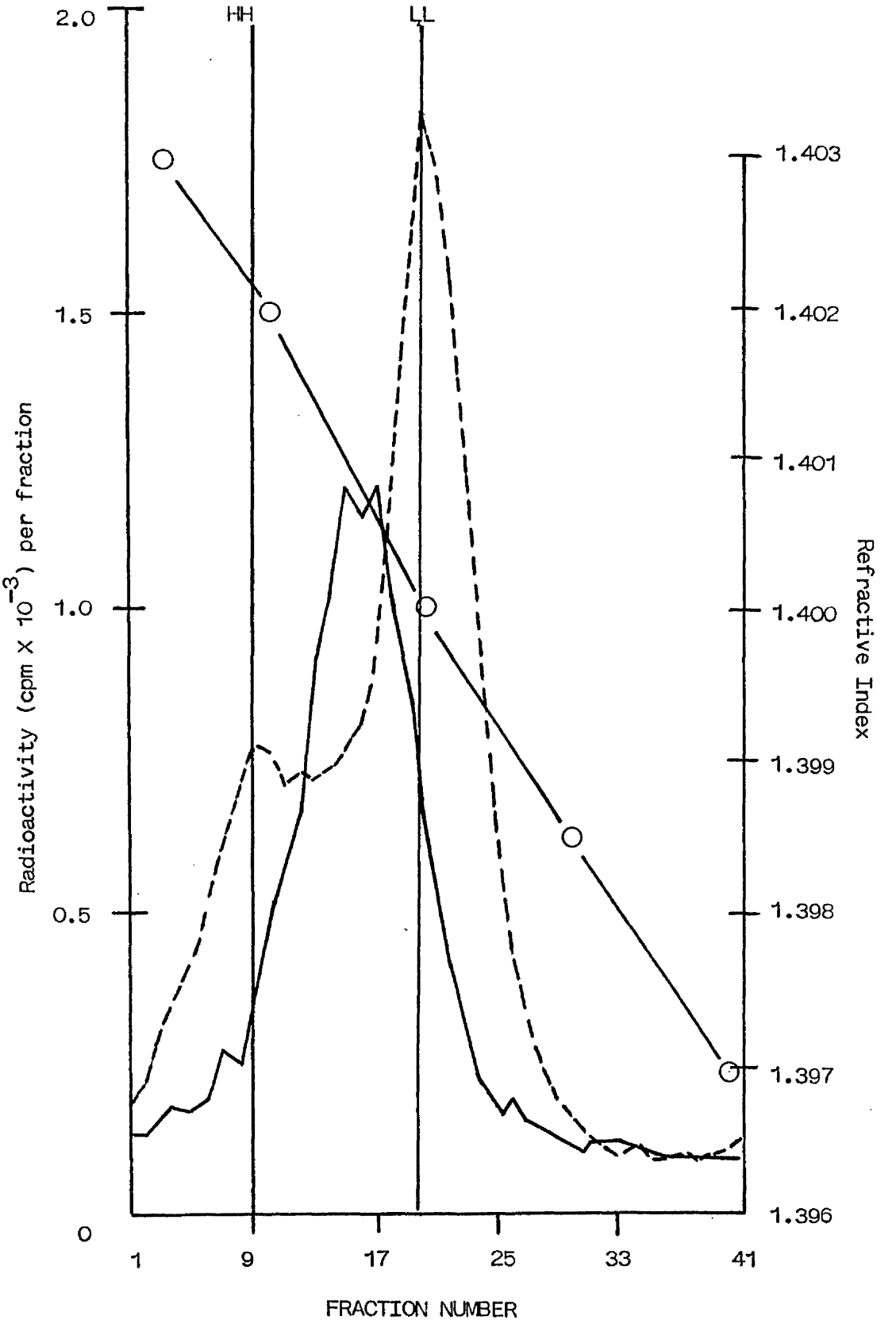
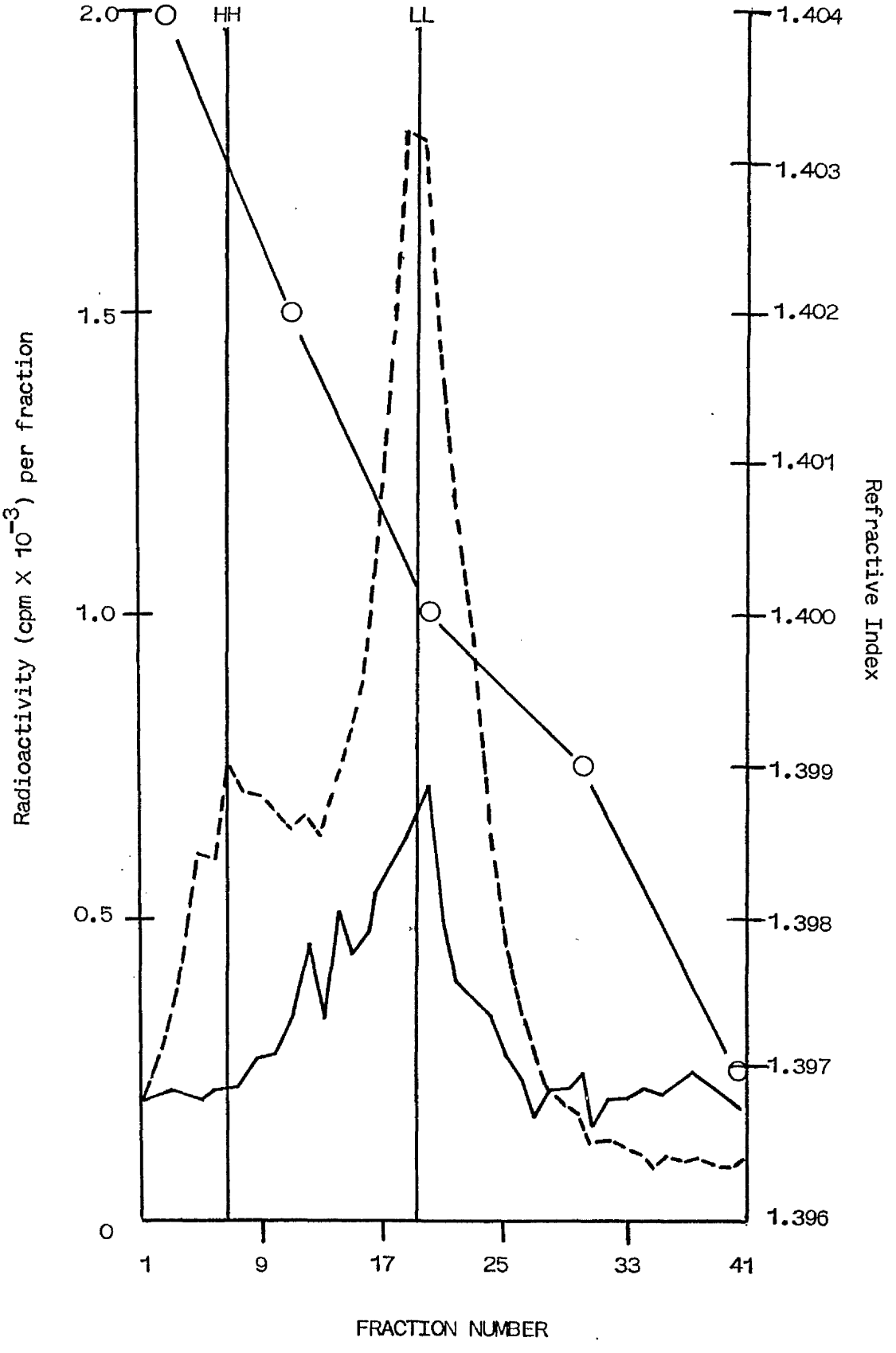

HH
LL
2.0
1.5
1.0
0.5
0
1.403
1.402
1.401
1.400
1.399
1.398
1.397
1.396
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
1
9
17
25
33
41
FRACTION NUMBER

Fig. 52


2.0
1.5
1.0
0.5
0
HH
LL
1.404
1.403
1.402
1.401
1.400
1.399
1.398
1.397
1.396
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
1
9
17
25
33
41
FRACTION NUMBER

initiated to sporulate in TdR alone, the relative level of sporulation increased and the spore DNA appeared mainly in the light density form, with a smaller peak of intermediate density (Fig. 52). In a similar experiment, the DNA was radioactively-labelled with [methyl-^{3}H] - TdR instead of [^{14}C] - TdR to increase the specific activity. When the cells were grown in the presence of TdR but initiated to sporulate in BUdR medium, 7h after initiation (when DNA replication was still in progress (Fig. 56)), 42% of the DNA was in the heavy form, 41% in intermediate form and 17% still in the light density form (Fig. 53). DNA replication in TdR alone had terminated by t_4 (Fig. 56). Again the DNA prepared from the few spores formed in the presence of BUdR was entirely of intermediate density (Fig. 54). This therefore suggests that incorporation of BUdR into both strands of DNA prevented sporulation. Also, the absence of any light density DNA suggests that a full round of DNA replication in resuspension medium is required for a spore to be made. Also again, spore DNA extracted from cells grown in the presence of BUdR, but initiated to sporulate in its absence, appeared to be mainly in the light density form, with a shoulder of intermediate density (Fig. 55). No heavy density form was detected, which again indicated that a full round of DNA replication in the starvation medium was required for sporulation. If the composition had been known of a DNA sample prepared from cells taken at the point of resuspension in SM + TdR, after growth in CH + TdR + BUdR, it would have been possible to compare it with

Figures 53-55. CsCl density gradient analysis of incorporation of BUdR into but-32 DNA during sporulation

Strain but-32 was grown and resuspended in media identical to that used in the previous experiment (Fig.'s 50-52), but [methyl-^{3}H] - TdR (0.5 μCiml^{-1}) was used instead of [^{14}C] - TdR to radioactively label the DNA. In this experiment four 10 ml samples were removed from culture 1 during sporulation at t_7 to give enough material for several gradients. At t_{20} the levels of sporulation were determined and DNA extracted from purified spores.

In addition, an extra control culture (culture 3) was grown in parallel with the other two, but the radioactive label was [^{14}C] - TdR (0.025 μCiml^{-1}). In this case the cells were grown and resuspended in media (300 ml) + TdR (1 μgml^{-1}). The DNA extracted from purified spores at t_{20} was used as the light density reference DNA in Fig.'s 53-55. Heavy density reference DNA labelled with [^{14}C] - TdR was prepared as described in Materials and Methods, section VIIB.

Samples were also removed for measurement of incorporation of radioactivity into acid-insoluble material (Fig. 56). The levels of sporulation in this experiment were: culture 1, 6.1 X 10^6 spores ml^{-1}; culture 2, 136 X 10^6 spores ml^{-1}, and culture 3, 146 X 10^6 spores ml^{-1}.

The buoyant densities of the samples were compared to

those of heavy and light reference DNA samples as in the previous experiment (Fig.'s 50-52).

Fig. 53 A representative gradient of DNA from cells at t_7 in culture 1 (cells grown in TdR and resuspended in BUdR medium + $[^3H]$- TdR).

Fig. 54 A representative gradient of spore DNA (culture 1).

Fig. 55 A representative gradient of spore DNA (culture 2; cells grown in BUdR and resuspended in TdR medium + $[^3H]$- TdR).

——— $[^3H]$- DNA

----- $[^{14}C]$- DNA (reference samples)

Fig. 53

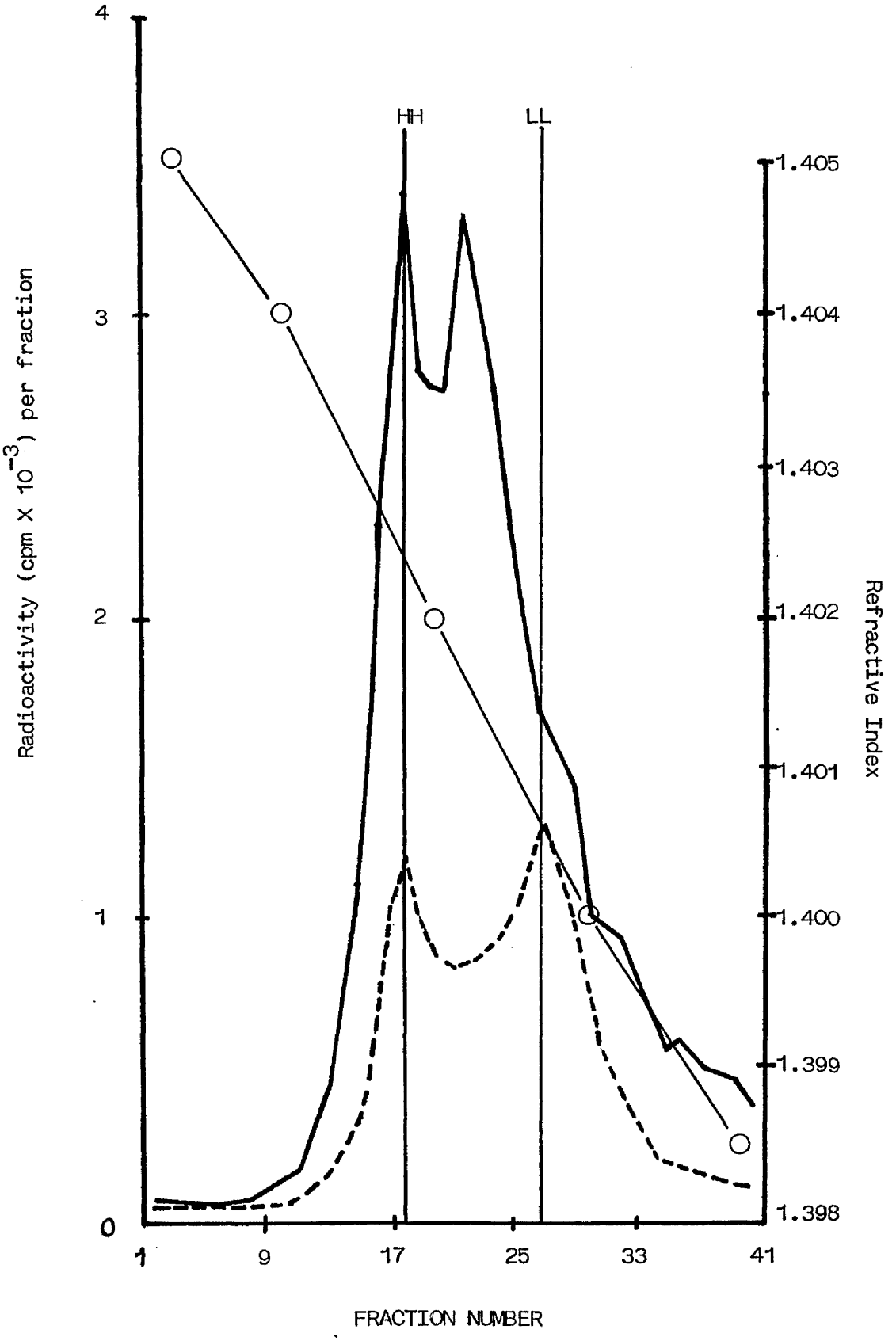

HH
LL
4
3
2
1
0
1
9
17
25
33
41
1.405
1.404
1.403
1.402
1.401
1.400
1.399
1.398
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
FRACTION NUMBER

Fig. 54

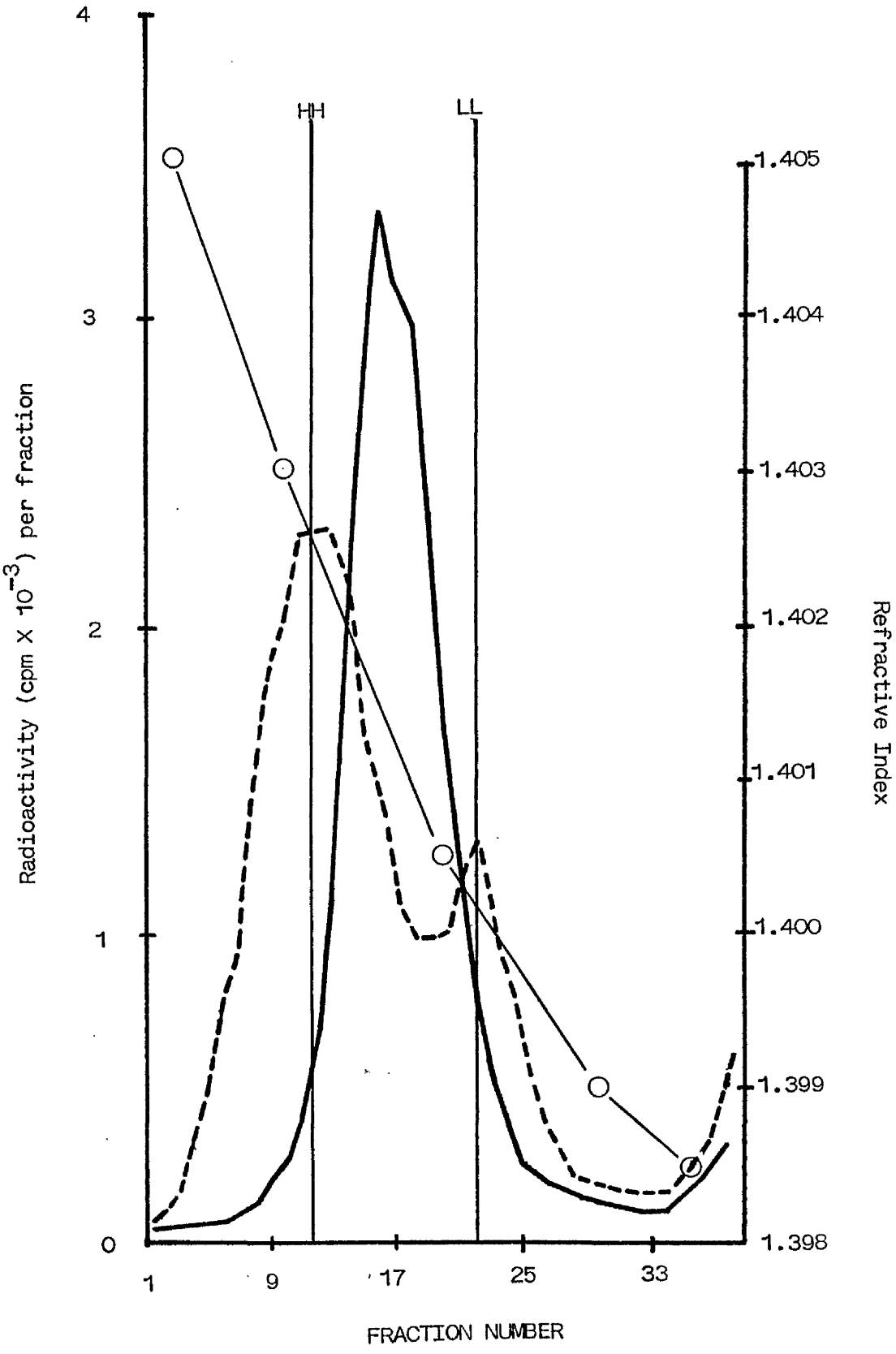

4
3
2
1
0
HH
LL
1.405
1.404
1.403
1.402
1.401
1.400
1.399
1.398
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
1
9
17
25
33
FRACTION NUMBER

Fig. 55

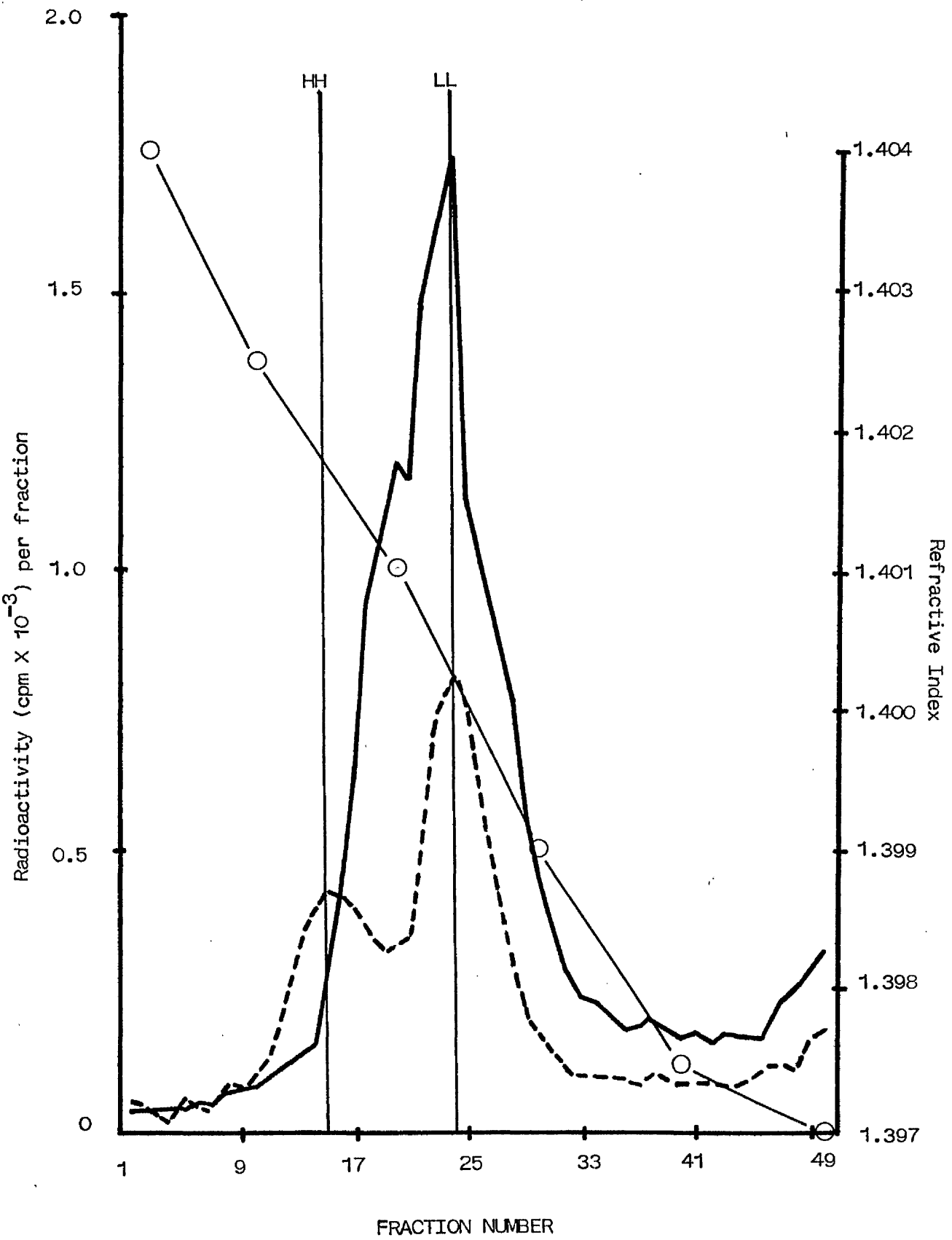

2.0
1.5
1.0
0.5
0
HH
LL
1.404
1.403
1.402
1.401
1.400
1.399
1.398
1.397
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
1
9
17
25
33
41
49
FRACTION NUMBER

Figure 56. Incorporation of [methyl-^{3}H] - TdR or [^{14}C] - TdR into acid-insoluble material during but-32 sporulation.

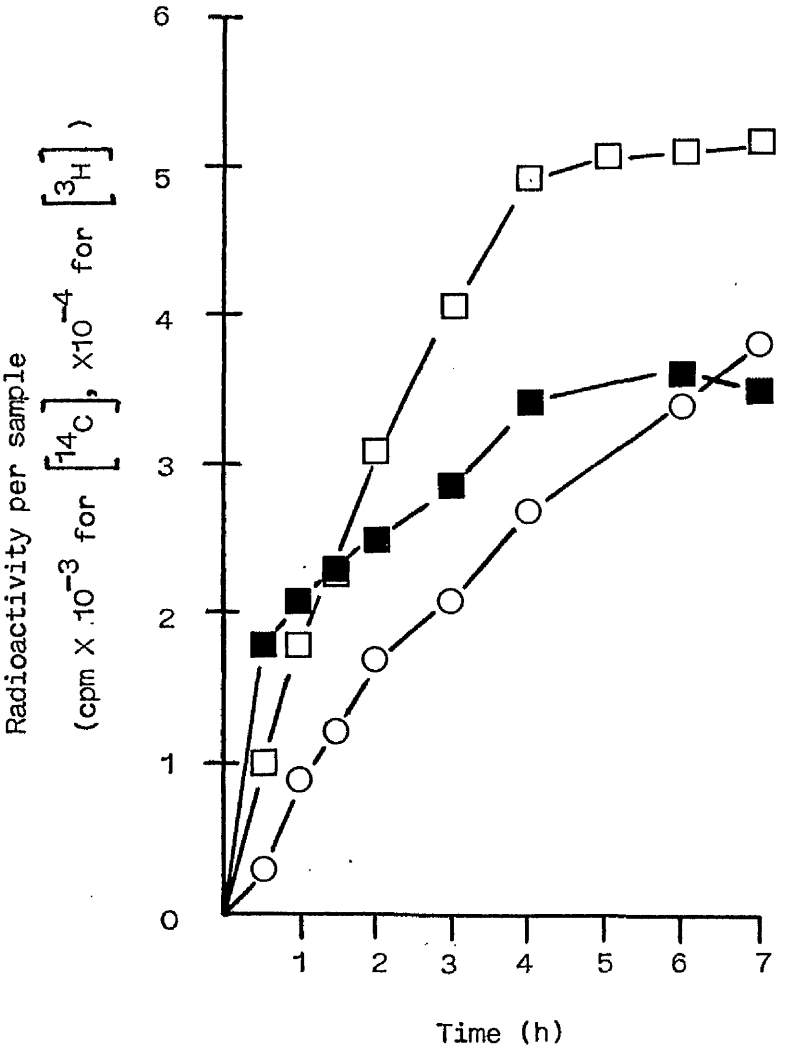
The cell samples removed from the three cultures in the previous experiment (Fig.'s 53-55) were measured for incorporation of radioactivity into acid-insoluble material.

○ Culture 1; cells grown in TdR and resuspended in BUdR medium + [^{3}H] - TdR

□ Culture 2; cells grown in BUdR and resuspended in TdR medium + [^{3}H] - TdR

■ Culture 3; cells grown in TdR and resuspended in TdR medium + [^{14}C] - TdR

Fig. 56


Radioactivity per sample
(cpm X 10^{-3} for [^{14}C], X10^{-4} for [^{3}H])
0
1
2
3
4
5
6
1
2
3
4
5
6
7
Time (h)

the known composition of the spore DNA formed from these cells (Fig. 55). From such a comparison it might have been possible to conclude, firstly, whether initiation of DNA replication specifically required for sporulation occurs in a sporulation medium, and secondly, how many rounds of replication occur in SM before a spore is made. However, no sample was taken at the point of resuspension. Even if it had been taken, it might still have been difficult to consider the two conclusions above as the cells would only have been grown for about four generations in BUdR growth medium (they had been grown overnight in TdR medium), and it is unlikely that by the end of this time that the DNA of this tolerant strain would have been completely labelled in both strands by BUdR.

It was therefore decided to improve the experiment by growing strain but-32 overnight in CH medium + TdR + BUdR + [^{3}H]-TdR, reinoculating the cells into fresh medium of the same composition, and growing them to mid-exponential phase. The cells were then transferred to SM + TdR alone (plus [^{3}H] - TdR), a sample taken for DNA extraction at t_{zero} (Fig. 57), and incubation continued until t_{20}, when the level of sporulation was determined, and the DNA extracted from purified spores. In this experiment the level of sporulation was similar to that obtained for cells grown and resuspended in TdR medium (see legends to Fig.'s 57-59, and 53-55).

Figures 57-59. CsCl density gradient analysis of DNA replication during sporulation of strain but-32.

Five cultures of strain but-32 were grown overnight in CH medium (20 ml) containing TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + [methyl-^{3}H] - TdR (0.5 $\mu Ci ml^{-1}$ final concentration). Each culture had been inoculated with a different number of growing cells to ensure that at least one culture contained cells in mid-exponential phase after overnight incubation. A sample (5 ml) was taken for extraction of DNA from the overnight culture containing cells in mid-exponential phase, and the cells in the remainder of the culture (15 ml) were used to inoculate 300 ml CH medium containing TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) + [methyl-^{3}H] - TdR (0.5 $\mu Ci ml^{-1}$). These cells were grown to an E_{600nm} of 0.7 and initiated to sporulate in SM (300 ml) plus TdR (1 $\mu g ml^{-1}$) and [methyl-^{3}H] - TdR (0.5 $\mu Ci ml^{-1}$). Immediately after the resuspension of cells in this medium, three 10 ml samples were removed from the culture for DNA extraction, to give enough material for several gradients (see Materials and Methods, section VIA). Incubation of the culture was continued, and at t_{20} the level of sporulation was determined. DNA was then extracted from purified spores (Materials and Methods, section VIA). The level of sporulation in this experiment was 138 X 10^6 spores ml^{-1}, a value close to the control value for cells grown and sporulated in TdR alone (see legend to Figs 53-55).

The [^{14}C] - labelled heavy and light density reference DNA samples used for the gradients below were the same preparations used for the previous experiment (Fig.'s 53-55).

Figure 57. A representative gradient of DNA from cells grown overnight in BUdR medium, and regrown to mid-exponential phase in fresh BUdR medium.

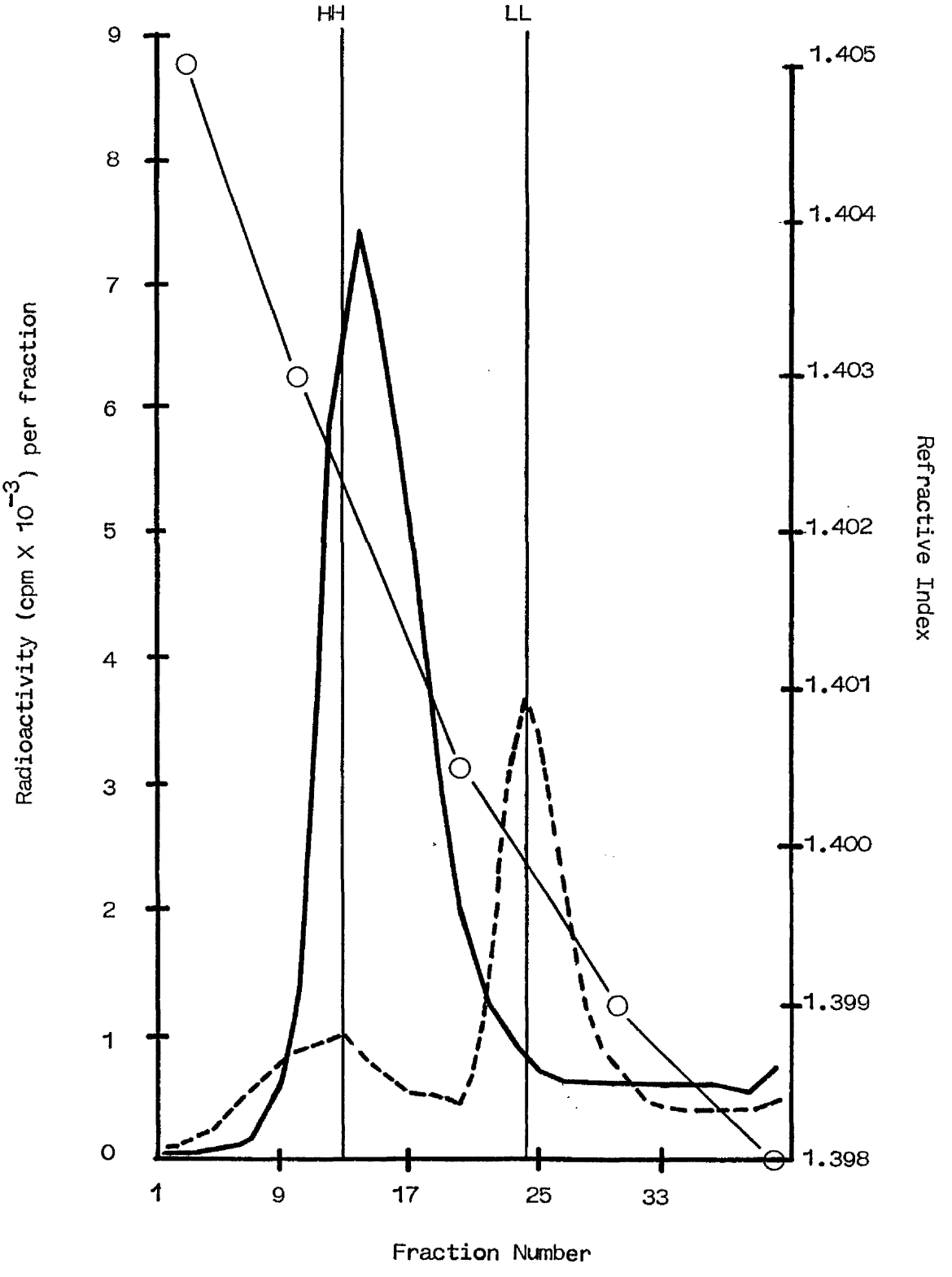
Figure 58. A representative gradient of DNA extracted from the cells grown overnight in BUdR medium (see legend to Fig. 57).

Figure 59. A representative gradient of DNA prepared from spores formed from the cells grown in BUdR medium (see Fig. 57) and initiated to sporulate in TdR medium.

——— [^{3}H] DNA

------ [^{14}C] DNA (reference samples)

Fig. 57


HH
LL
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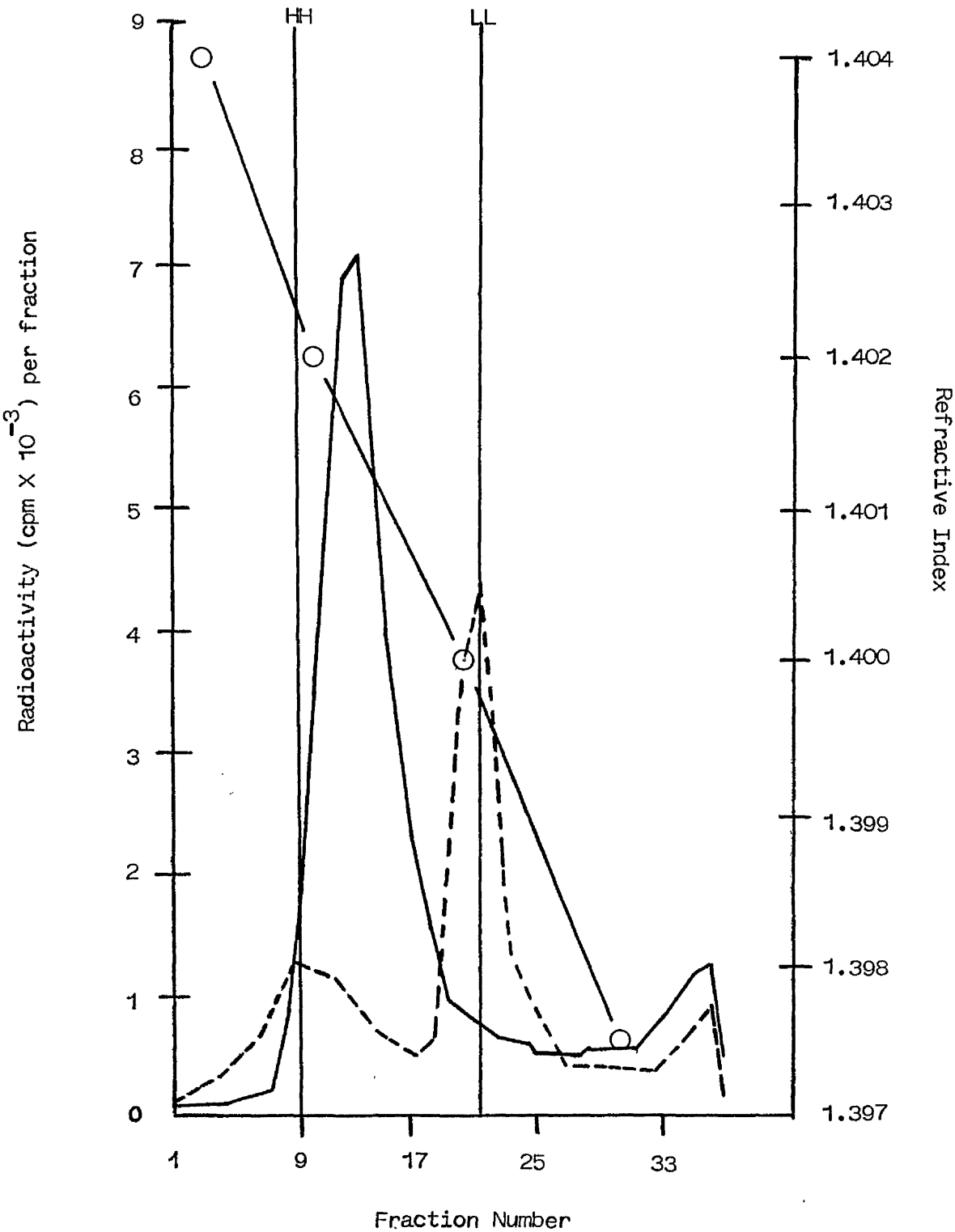
1.405
1.404
1.403
1.402
1.401
1.400
1.399
1.398
1
9
17
25
33
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
Fraction Number

Fig. 58


HH
LL
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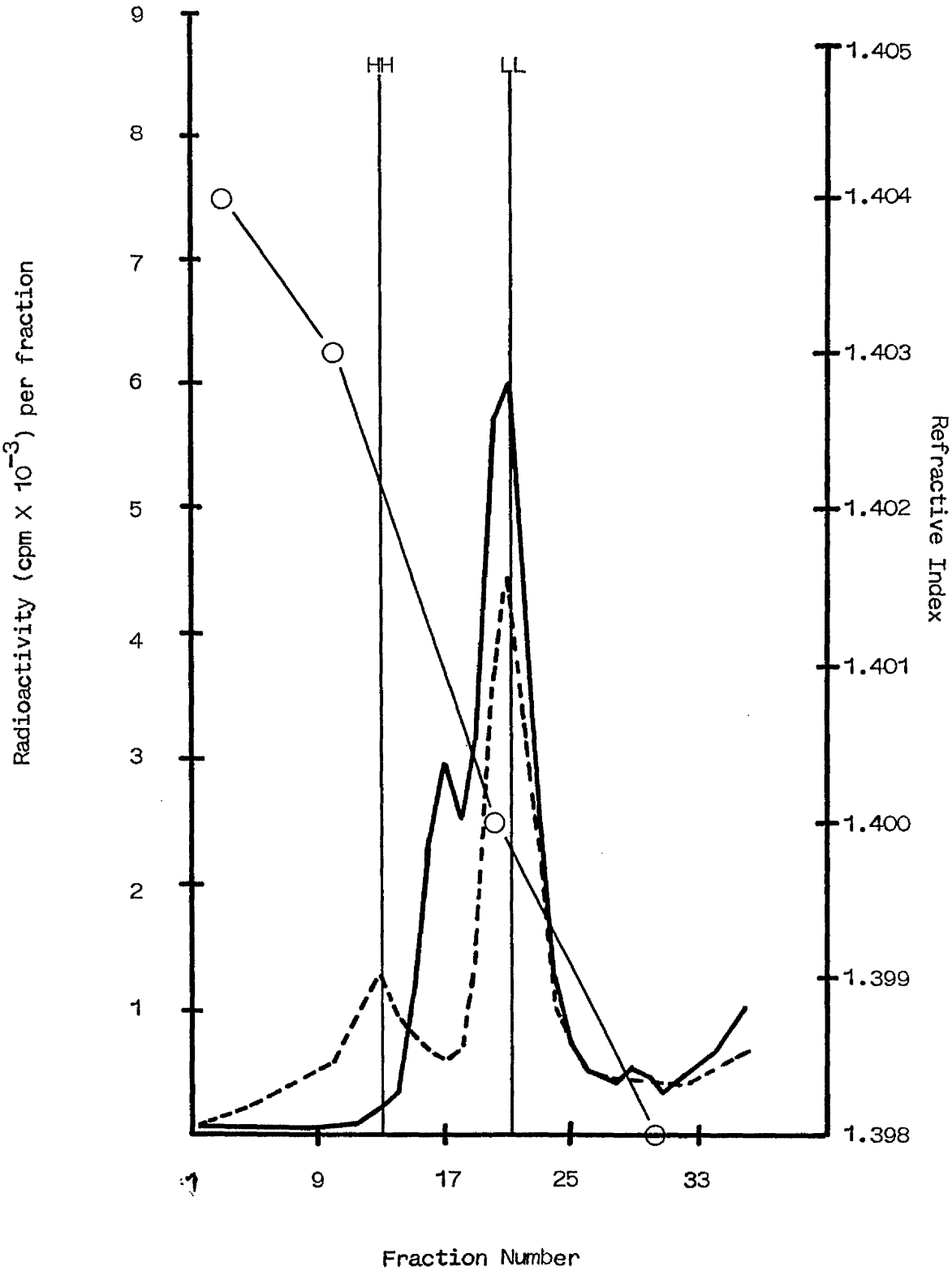
8
7
6
5
4
3
2
1
0
1.404
1.403
1.402
1.401
1.400
1.399
1.398
1.397
1
9
17
25
33
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
Fraction Number

Fig. 59


HH
LL
9
8
7
6
5
4
3
2
1
1.405
1.404
1.403
1.402
1.401
1.400
1.399
1.398
1
9
17
25
33
Radioactivity (cpm X 10^{-3}) per fraction
Refractive Index
Fraction Number

The buoyant density of the DNA from cells at t_{zero}, after growth overnight and for four further generations in BUdR medium, almost corresponded to that of the heavy-heavy reference DNA (Fig. 57). The slight difference between the peak buoyant densities of these two samples was probably due to the stage during growth in BUdR medium at which the cells were sampled. The reference heavy-heavy DNA was prepared from cells grown into stationary phase in BUdR medium, whereas the test DNA was prepared from cells grown only to mid-exponential phase overnight, followed by a further 3h of growth to mid-exponential phase in an identical medium (cells grown overnight in BUdR medium to stationary phase did not start to grow by 3h after inoculation into fresh BUdR medium, probably due to a prolonged lag phase). It may be that the reference DNA contained slightly more BUdR than the test sample because the cells in this case were grown into stationary phase, by which time most of the TdR in the medium had possibly been metabolized by the cells, leaving only BUdR to be incorporated. Exponentially-growing cells of this strain probably preferentially incorporate TdR over BUdR (see section II), so that DNA labelled in both strands from cells in stationary phase has a slightly increased buoyant density compared to that of DNA labelled in both strands from cells during exponential growth. Supportive evidence for this conclusion was that the peak buoyant density of DNA prepared from cells grown overnight in BUdR medium to mid-exponential phase was lower than that of the reference

heavy-heavy DNA (Fig. 58).

The DNA extracted from spores formed from the cells grown in BUdR medium, but initiated to sporulate in TdR alone, appeared mainly of light-light density (70%), with a second peak of intermediate density corresponding to 30% of the total DNA (Fig. 59). The absence of any heavy-heavy density DNA from this sample again suggested that a full round of replication in a sporulation medium, initiated from the replication origin, is required for normal sporulation. The possible reasons for the distribution of spore DNA into 30% heavy-light form and 70% light-light form, from the 100% heavy-heavy form of DNA at the point of initiation of sporulation, will be discussed later (see Discussion).

Finally, the results of over 30 gradients established the average light-light, intermediate and heavy-heavy buoyant densities at 1.704, 1.715 and 1.729 gml^{-1}, respectively. The latter two values correspond to 10% and 22% substitution respectively of BUdR for TdR residues in intermediate and heavy-heavy density DNA. Since the intermediate form contains one unsubstituted strand, this strain, under these conditions, substituted 22% of its TdR residues in DNA with BUdR.

DISCUSSION

I. The BUdR-tolerance phenotype, and the effect of BUdR on growth of B. subtilis.

The isolation of TdR-requiring strains differing in the relative degree of tolerance to the analogue during growth suggested that classes of BUdR-tolerance exist. The basis for these differences was not investigated, but may have been a result of either mutations conferring tolerance being situated in different genes, or within one gene. Genetic mapping of the mutations conferring tolerance in these strains would indicate which of these alternatives is the most likely. The technique in this case would be difficult, and would involve the construction of a selection of strains, each harbouring a different auxotrophic marker in addition to the unlinked thy A and thy B mutations, and transformation of these strains, using DNA isolated from each tolerant strain. If this was achieved, the percentage of co-transfer of the auxotrophic markers with the tolerance mutation(s) from each strain would reflect the physical distance between the loci on the chromosome, i.e. their linkage relationship. If the tolerance mutations were found to be scattered at various positions around the chromosome, then the classes of tolerance exhibited by these strains would be a consequence of the altered expression of different genes. If mutations in different genes gave rise to similar phenotypes, it might suggest that more than one target exists for the inhibitory action of BUdR on growth. However, if the mutations were all linked

to one auxotrophic marker, it would be more likely that differences in the level of tolerance between strains were a result of the altered expression of one particular gene.

It is also possible that a mutation conferring tolerance to BU also confers some degree of tolerance to BUdR, since transfer of the BU-tolerance mutations from strains VUB112 and But 23.1 into a thy A, thy B genetic background produced two derivatives which grew as well as strain but-32 on solid medium containing TdR (1 $\mu g ml^{-1}$) + BUdR (20 $\mu g ml^{-1}$). However, they did not apparently exhibit a comparable degree of tolerance to the BUdR-tolerant strains when grown in liquid minimal medium (Table 7), although strain but-23.1 appeared less sensitive to BUdR when grown in rich medium, yet was still more sensitive than strain but-32 (Table 8). There may therefore be a mechanism operating in BU-tolerant strains which also allowed some growth in medium containing BUdR. Conversely, it also appeared that the BUdR-tolerance mutation but-32 allowed this strain to grow in medium containing TdR + BU at a higher rate than the BUdR-sensitive parent strain (Table 12). However, the parent strain grew exponentially in this medium, albeit slowly, whereas its growth was almost completely inhibited in medium containing TdR + BUdR (Table 14). The effect of BU on growth of these strains in the presence of thymine (instead of TdR) was not tested, but might be expected to be relatively stronger if, as it has been suggested,

thymine and TdR-requiring strains preferentially incorporate pyrimidine nucleosides over the free pyrimidine bases (Cohen and Barner, 1956). Laird and Bodmer (1967) noted that the preference for thymine over BU was greater than the preference for TdR over BUdR in a thymine-requiring strain of B. subtilis. Therefore, it might be expected that TdR could relieve the inhibitory effect of BU on growth of the BUdR-sensitive parent strain to a greater degree than thymine.

Inhibition of growth of the parent strain by BUdR was probably not due to an effect of limiting TdR concentration in medium containing TdR (1 μgml^{-1}) plus BUdR (15 μgml^{-1}), as this strain grew exponentially with TdR at 0.5 μgml^{-1}. However, it is possible that competition with TdR by BUdR for uptake into the cell could have lowered the intracellular TdR concentration to a level which could not allow a normal rate of growth.

The possibility was excluded that the basis for the inhibitory action of BUdR on growth of the parent strain was via incorporation into RNA, since BUdR had little effect on the rate of incorporation of $\left[^3H\right]$ - uridine into acid-insoluble material (Fig. 12B). Also, excess uridine in the medium did not affect the incorporation of $\left[^3H\right]$ - BUdR into acid-insoluble material (Fig. 11B).

In some eukaryotic cells the addition of deoxycytidine

(dCyd) to the medium has been shown to overcome the toxic effect of BUdR (Meuth and Green, 1974; Davidson and Kaufman, 1978). It has been suggested that BUdR-triphosphate inhibits the enzyme ribonucleotide reductase, thus starving the cells of dCyd residues and effectively inhibiting DNA synthesis. In addition, resistance to BUdR may be associated with resistance to inhibition of ribonucleotide reductase by BUdR-triphosphate. However, when equal concentrations (15 $\mu g ml^{-1}$) of BUdR and dCyd were included in medium with TdR (1 $\mu g ml^{-1}$), growth of the parent strain was still prevented (Fig. 13). Therefore, inhibition of ribonucleotide reductase by BUdR was probably not the basis for its action. When dCyd was added in excess, however, the relative inhibitory effect of BUdR on growth of both the parent strain and but-32 was enhanced. The basis for this effect may be related to the suggestion that the enzyme thymidine kinase, which uses 5-halogenated deoxyuridines as substrates as well as TdR, is activated by dCTP (Okazaki and Kornberg, 1964). Addition of dCyd to these cells may therefore have increased the intracellular dCTP levels so that in a low concentration of TdR (1 $\mu g ml^{-1}$) relatively more BUdR was incorporated, compared to cells grown without dCyd. Therefore, a greater intracellular concentration of BUdR, leading to a greater degree of incorporation of the analogue into DNA of both strains, may have increased inhibition of growth.

The sensitive parent strain incorporated more radiolabelled

BUdR into acid-insoluble material than strain but-32 in medium containing TdR (1 μgml^{-1}) plus BUdR (7.5 μgml^{-1}) (Fig.'s 10 and 11). However, in medium with TdR and BUdR at concentrations of 1 and 15 μgml^{-1}, respectively, after growth for about 2 generations in excess TdR, there was little difference between strains in the rate of incorporation of $[^3H]$- BUdR into both whole cells and acid-insoluble material (Fig.'s 14 and 15). The difference between the results of these two experiments could be explained by the difference in the concentration of unlabelled BUdR in the medium. In the second experiment increased competition with unlabelled BUdR may have reduced the relative degree of incorporation of the radiolabelled analogue into whole cells of the parent strain. However, if the tolerant strain only had the capacity to take up a limited amount of BUdR in medium containing TdR (1 μgml^{-1}), less $[^3H]$- BUdR than expected may have been taken up by these cells at the higher BUdR concentration.

The existence of a mechanism in the tolerant strain which in effect preferentially excludes BUdR was further suggested by the results of experiments in which the effect of BUdR on the rate of incorporation of $[^{14}C]$- TdR into whole cells and acid-insoluble material was tested (Fig.'s 16, 17, 18, 19 and 20). For both whole cells and acid-insoluble material, the incorporation of radiolabelled TdR in the presence of BUdR followed more closely that of TdR (8.5 μgml^{-1}) for the parent strain, and

TdR (1 $\mu g ml^{-1}$) for the tolerant strain. This suggested that expression of the tolerance mutation was at the cell surface, resulting in the preferential uptake of TdR over BUdR, and that possibly the overall rate of DNA synthesis was decreased by BUdR to a greater extent in the parent strain than for but-32. However, incorporation of unlabelled BUdR competing with radiolabelled TdR could have accounted for at least some of the apparent reduction in the measured rates of $[^{14}C]$- TdR incorporation. The degree of substitution of BUdR for TdR during growth of these strains was measured by CsCl pycnography (Fig. 21). The results showed that, after exposure of growing cells to BUdR, but-32 DNA incorporated more $[^{14}C]$- TdR than DNA isolated from the parent strain grown under identical conditions. This showed clearly that strain but-32 was preferentially utilizing TdR over BUdR. The increase in buoyant density of DNA prepared from the parent strain and but-32 in this experiment represented 15% and 6% substitution of BUdR for TdR residues respectively after 45 min growth in BUdR medium. Since 45 min is probably less than the time taken for a complete round of chromosome replication under these conditions (Results, section III, and Discussion, section II), this DNA probably represented DNA of intermediate density, i.e. with one strand completely unsubstituted, and the other strand with its TdR residues partially substituted by BUdR. Therefore, the true values for the percentage substitution of TdR residues by BUdR under these experimental conditions are

30% for the parent strain, and 12% for the tolerant strain. The value of 12% is lower than the value of 22% obtained after growth of the But strain for at least 15 generations with TdR and BUdR at 1 and 15 μgml^{-1} respectively (Fig.'s 50-55, and 57-59). The low value may have been due to the short period of exposure to the analogue, and perhaps some endogenous TdR remaining after growth in medium with excess TdR. Preferential incorporation of BUdR over TdR by this strain may have resulted in incorporation of less BUdR into DNA during exponential growth with TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), than for cells grown over a much longer period in BUdR medium, when most of the extracellular TdR may have been utilized, leaving only BUdR to be incorporated.

From a comparison of the rates of incorporation of radiolabelled TdR into acid-insoluble material of both strains grown in medium containing TdR (1 μgml^{-1}) alone, or with TdR (1 μgml^{-1}) plus BUdR (15 μgml^{-1}), and correcting for the level of substitution of BUdR for TdR in DNA, it should be possible to determine if BUdR decreased the rate of DNA synthesis in these strains. When this comparison was made (using data from Fig.'s 22A and 23A), the rate of incorporation of $[^{14}C]$--TdR during exponential growth of the But strain in medium with TdR (1 μgml^{-1}) plus BUdR (15 μgml^{-1}) was approximately 60% of the rate in TdR (1 μgml^{-1}) alone. A corresponding value of 30% was obtained for the parent

strain. The 40% reduction (in BUdR medium) of the rate of [^{14}C]-TdR incorporation into acid-insoluble material by but-32 could not be totally accounted for by substitution of BUdR for 12% of TdR residues in DNA. Therefore, using these values, under these experimental conditions, BUdR may have decreased the rate of DNA replication of strain but-32 by about 28%.

BUdR appeared to exert a stronger inhibitory effect on the rate of incorporation of [^{14}C]-TdR into the parent strain, and reduced the rate of DNA synthesis by 40% of the rate in cells with TdR alone, after correction for BUdR substitution in DNA. However, this effect could have been attributed to the slower rate of growth of these cells in BUdR medium. An alternative method of calculating the relative rates of DNA replication of both strains, under conditions in which the strains were exposed to BUdR after growth in excess TdR, was to compare the peak levels of of radioactivity of cell lysates of both strains extracted under identical conditions and centrifuged to equilibrium in a CsCl concentration gradient (Fig. 21). For samples removed 45 min after transfer to medium with BUdR, the peak value for the parent cell lysate was 36% of the peak value for the but-32 lysate (Fig. 21, G & H). Therefore, assuming that the rates of overall DNA synthesis in the control cultures of both strains were identical, the peak cpm value for the lysate of the parent strain, grown in BUdR medium, corresponded to 36% of the rate of incorporation of [^{14}C] - TdR into acid-insoluble material of but-32,

grown in BUdR medium. The rate for but-32 in this medium was itself 60% of the control rate in TdR (1 $\mu g ml^{-1}$) alone (see earlier). Therefore, 36% of 60%, i.e. approximately 20%, is the calculated value for the relative rate of DNA synthesis, compared to that of a control culture in TdR alone, for the parent strain grown in BUdR medium. Only 30% of the difference between this value, 20%, and 100% for the control rate can be accounted for by substitution of BUdR for TdR in DNA of the parent strain. Therefore, there may have been as much as a 50% reduction in the normal rate of DNA replication when the parent strain was exposed to BUdR. Thus, the tolerance mutation in but-32 presumably allowed cells to maintain a much higher rate of DNA synthesis in medium containing BUdR than that exhibited by the parent strain, suggesting that the basis for inhibition of growth of the parent strain might be the marked reduction in the rate of DNA synthesis.

Nagley and Wake (1969) calculated that the duration of the first round of DNA replication after germination of thymine-requiring spores in medium containing BU + thymine was more than double the value obtained in medium containing only thymine. They concluded that dichotomous replication, not obtained in thymine medium, was due solely to the decreased rate of DNA synthesis induced by BU. Marker-frequency analysis of chromosome replication during growth of both the sensitive and But strains in this work indicated that BUdR did not markedly increase the average

pur$^+$/ met$^+$ ratios, compared to values for cells grown in TdR (8.5 μgml^{-1}) alone (Tables 9 and 10). The pur A16 marker is located near the origin of replication, and the met B5 marker is near the chromosome terminus. The ratio of these markers in purified DNA gives an indication of the average number of replication positions per chromosome (n), using the relationship pur$^+$/ met$^+$ ratio = 2^n (Sueoka and Yoshikawa, 1965). In addition, the chromosome replication time (C) can be calculated from the relationship n = C/r, where n is the average number of replication positions per chromosome, and r is the generation time of the bacteria (Ephrati - Elizur and Borenstein, 1971). Therefore, a doubling in the pur$^+$/ met$^+$ ratio is required to increase n by 1, which in turn increases the calculated chromosome replication time by one generation time, if the cells grow at the same rate. In one experiment (Table 10) the average marker-ratios for TdR (8.5 μgml^{-1})- and BUdR- grown cells, standardised with regard to spore DNA, were 2.7 and 2.9 respectively for but-32, and 2.5 and 2.5 respectively for the parent strain. For strain but-32, with generation times of 36 and 54 min in TdR- and BUdR media respectively, and n values of 1.4 and 1.5 (derived from the standardised pur$^+$/met$^+$ ratios), chromosome replication times of 50 and 81 min for TdR (8.5 μgml^{-1})- and BUdR- grown cells respectively are obtained. The parent strain had generation times of 36 and 114 min for TdR (8.5 μgml^{-1})- and BUdR- grown cells (until the E_{600nm} stopped increasing), and n values of 1.4 and 1.4 respectively, giving

chromosome replication times of 50 and 160 min for TdR- and BUdR- grown cells.

In a separate experiment (Table 9), using the *pur*$^+$/ *met*$^+$ values for cells in late exponential phase, n values of 2.1 and 2.0, and C values of 82 and 96 min respectively were obtained for strain *but-32* grown in TdR (8.5 $\mu g ml^{-1}$) or TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$). Values of 79 and 103 min respectively were obtained for the C values of the parent strain. In this experiment the generation times of strain *but-32* grown in TdR- and BUdR- medium were 39 and 48 min, respectively. This slight difference was a more typical result than that obtained in the other experiment (Table 10). Therefore, it appeared that BUdR slowed down the rate of replication in both strains, but to a much lesser extent in the tolerant strain. However, the *pur*$^+$/ *met*$^+$ values for the same DNA preparation appeared to vary between transformation experiments, probably due to using different batches of competent cells (Gillin and Ganesan, 1975), so that small changes in the calculated chromosome replication time might not be significant. Therefore, it was not possible to conclude firmly from these results that BUdR decreased the rate of chromosome replication in the tolerant strain, although it probably did so in the sensitive parent strain.

A subinhibitory concentration of HPUra (0.5 $\mu g ml^{-1}$) did not affect the growth rate of strain *but-32*, and had only

a slight inhibitory effect on growth of the parent strain, and did not appear to markedly increase the *pur*$^+$/*met*$^+$ marker ratios, compared to cells grown in TdR (8.5 μgml^{-1}) alone (Tables 9 and 10). However, this concentration of HPUra had a detectable inhibitory effect on the overall rate of DNA synthesis in both strains, measured by incorporation of $[^{14}C]$ - TdR into acid-insoluble material (Fig.'s 22 and 23). Therefore, the marker-frequency method, as used in these experiments, may not have been sensitive enough to detect a slight decrease in the overall rate of DNA synthesis. In addition, in one experiment (Table 10) the mean *pur*$^+$/*met*$^+$ ratios for DNA isolated from cells of both TdR-requiring strains grown in a low concentration of TdR, 1 μgml^{-1}, were almost double the values for cells grown in excess TdR, 8.5 μgml^{-1}. However, in a separate experiment (Table 11), there was no observed increase in the *pur*$^+$/*met*$^+$ ratio of DNA from cells of *but-32* grown at a low TdR concentration, compared to that in excess TdR. In both experiments, the growth rates of cells at both TdR concentrations were similar. The calculated chromosome replication times from the data in Table 10 were 88 and 96 min respectively for *but-32* and the parent strain grown in TdR (1 μgml^{-1}), compared to a value of 50 min for both strains in excess TdR. However, if the corresponding data from Table 11 are used, the chromosome replication times were 38 and 43 min respectively for *but-32* grown in TdR (1 μgml^{-1}) and TdR (8.5 μgml^{-1}), although this experiment was performed only once. This inconsistency highlighted the relative inaccuracy of the marker-

frequency method, as used in these experiments, for analysis of chromosome replication. Relatively small differences in the marker-ratios lead to large differences in the calculated chromosome replication times, if the generation time is constant. Therefore, the degree of variation observed between results of transformations, using the same DNA preparation with different batches of competent cells, may have led to errors in the calculated chromosome replication times. In spite of the possible inaccuracy of this method in giving absolute values for the average number of replication forks per cell, the results did yield some information.

The magnitude of the difference in the C values of the parent strain grown in BUdR or excess TdR is consistent with the effect of BU on the rate of replication in thymine-requiring strains (Nagley and Wake, 1969). BU induced multi-forked replication during outgrowth of germinating thymine-requiring spores of B. subtilis, and these workers attributed this to a BU-induced increase in the chromosome replication time of the first round of DNA replication from a control level in excess TdR of 62 min, to 160 min in medium containing thymine (2.5 $\mu g ml^{-1}$) + BU (25 $\mu g ml^{-1}$)

The apparently normal growth rates of the parent strain and but-32 at a low TdR concentration in one experiment (Table 10) may have been achieved by an increased rate of initiation of

DNA replication to offset a decrease in the rate of replication. This is in agreement with the effect of a limiting thymine concentration on the chromosome replication time during growth of thymine-requiring strains (Ephrati-Elizur and Borenstein, 1971).

It was also apparent, from another experiment (Table 9) that the pur$^+$/ met$^+$ ratios of DNA prepared from cells sampled at an early point during exponential growth were generally lower than the ratios for cells sampled at a later stage during exponential growth. The basis for the difference is unclear if the cells were growing exponentially at both sampling points (except perhaps for the parent strain in BUdR medium). It may be that at the early sampling point, just at the start of exponential phase, TdR-requiring strains undergo less dichotomous replication than at a later stage. There was also a slight increase in the pur$^+$/ met$^+$ ratios of both strains when the values obtained for DNA prepared from cells at the point of initiation of sporulation were compared with those from cells prior to the centrifugation step (Table 10). This increase was not as marked as that observed between early and late exponential phase cells.

The pur$^+$/ met$^+$ ratios in these experiments were standardised with regard to the values obtained for DNA extracted from purified spores of these strains. Values of 1.30 and 1.36 respectively were obtained for spore DNA of the parent and

but-32, compared to a value of 0.99 for DNA of spores of the wild-type. This difference is probably due to decreased efficiency of transformation around the met B locus when DNA from TdR-requiring strains is used for transformation (Callister and Wake, 1974; see Introduction). However, it could be argued that the number of met$^+$ transformants scored in these experiments may also have been lowered by discrimination against met$^+$, thy$^-$ transformants on selective media lacking TdR, as the met B marker is closely linked to the thy B locus. However, the thy B locus is one of two loci, thy A and thy B, coding for two thymidylate synthetases in B. subtilis (Neuhard et al, 1978), and mutations in both genes are required to create a thymine requirement. Furthermore, it has been reported that a thy A$^+$, thy B mutant contains wild-type levels of thymidylate synthetase (Wilson et al, 1966). Therefore, met$^+$ transformants with the genotype thy A$^+$, thy B should have grown as well as those with the genotype thy A$^+$, thy B$^+$ on medium lacking TdR. However, on selection plates lacking TdR, some met$^+$ colonies appeared to be smaller than others, but on plates supplemented with TdR the colonies were of uniform size. The smaller colonies may therefore have arisen from cells carrying the thy B marker, although this would not be expected if these strains have wild-type levels of thymidylate synthetase. In addition, the result of thisexperiment (Table 11) indicated that, when all selection plates were supplemented with TdR, the number of met$^+$ transformants, relative to pur$^+$ transformants, was approximately

doubled, compared to the number obtained on medium lacking TdR. If this effect is reproducible it might be important when the results of different laboratories are to be directly compared. Gillin and Ganesan (1975) added TdR to selective media, but still obtained *pur*$^+$/ *met*$^+$ ratios of >1 for DNA isolated from TdR-requiring strains. Therefore, it may be that the most probable reason for anomalous marker-ratios for DNA from TdR-requiring strains is the non-isogenicity around the *met B* locus.

To summarise, therefore, the evidence discussed in this section suggests that the tolerance phenotype of strain *but-32* may be the result of increased preferential uptake of TdR over BUdR when the analogue partially replaces TdR in the medium. The existence of classes of BUdR-tolerant mutants with varying degrees of tolerance to the analogue may therefore be a result of differences between strains in the relative exclusion of BUdR in favour of TdR uptake, although this was not tested during the course of this work. Bishop and Sueoka (1972) isolated BU-tolerant, thymine-requiring strains which exhibited a *decreased* preferential incorporation into DNA of thymine over BU, and an increased rate of DNA synthesis in the presence of the analogue. This suggests that the mechanisms of BU-tolerance exhibited by their strains differed from that operating for BUdR-tolerance in *but-32*.

The results of incorporation experiments and density

gradient analysis also suggested that the rate of DNA replication of the BUdR-sensitive parent strain was decreased to a much greater extent than the rate in the But strain when the cells were grown in medium containing BUdR. In addition, the parent strain incorporated more BUdR into DNA than the tolerant strain. Marker-frequency analysis of DNA replication also suggested that BUdR had a much greater inhibitory effect on the rate of chromosome replication in the parent strain than in but-32. The inhibitory effect of BUdR on growth may have been a direct consequence of the degree of incorporation into DNA, although the level of incorporation is presumably dependent on the level of uptake into the whole cell, as the tolerant strain excluded more BUdR from the cell than the parent, resulting in less incorporation of the analogue into DNA.

The possibility could still not be ruled out that, for the parent strain, a greater intracellular BUdR concentration was exerting some metabolic effect resulting in unbalanced growth. A high intracellular BUdR concentration in these cells could also have induced a low intracellular TdR concentration, leading to "thymineless" death, which occurs when thymine-requiring cells continue to grow in the absence of balanced DNA synthesis, until they lose the power to multiply (Cohen and Barner, 1956). Although viability measurements were not made during growth of both strains in this work, BUdR had little effect on cell viability and division

of either strain during sporulation (Tables 12 and 15).

Since BUdR did not appear to inhibit ribonucleotide reductase, and it was unlikely that thymidylate synthetase and thymidine kinase were the target enzymes for its action (see Introduction), inhibition of pyrimidine metabolism did not appear to be the basis for the inhibitory action on growth. It is possible that incorporation of the analogue into both strands of DNA may have had a more direct effect on some chromosome-associated function, such as the normal operation of the chromosome-membrane complex during DNA replication and cell division. Nagley and Wake (1969) observed that thymine-requiring germinating spores of B. subtilis only started to lose viability in BU medium after the time of initiation of a second round of DNA replication, when DNA was labelled in both strands with BU.

The tolerance phenotype appeared to be a result of increased preferential uptake of TdR over BUdR, resulting in less incorporation of the analogue into DNA than was found with the parent strain. This could have arisen as a result of alterations in the membrane or surface properties of the cell. A comparison of membrane proteins by two-dimensional polyacrylamide gel electrophoresis, or membrane lipids and fatty acids by thin-layer and gas-liquid chromatography, using preparations from cells of the tolerant and sensitive strains, may reveal differences in the membrane properties of these strains. If these

exist BUdR-tolerance presumably arose by a mutation in a gene (or genes) coding for a membrane component, perhaps involved in transport across the cell membrane. In *B. subtilis* TdR is phosphorylated by thymidine kinase and incorporated into DNA (Bodmer and Grether, 1965; Rinehart and Copeland, 1973). Therefore, the basis for BUdR-tolerance may be an increased affinity of thymidine kinase for TdR over BUdR compared to that exhibited by the enzyme in the sensitive parent strain, resulting in increased preferential uptake of TdR over BUdR in the tolerant strain.

II. DNA replication and inhibition of sporulation by BUdR

The inhibitory effect of BUdR on sporulation of strain *but-32*, which could grow at an almost normal rate in medium containing BUdR, occured without affecting cell viability, at least during the first 6h of sporulation (Tables 12 & 15). Inhibition of sporulation by BUdR was shown not to be due to a limiting TdR concentration, as the sporulation capacity of *but-32* was normal in SM containing TdR at 1 $\mu g ml^{-1}$. BUdR did not appear to inhibit transcription in general during sporulation, as the rate of gross RNA synthesis was not affected by the analogue (Fig. 26), and the expression of the inducible enzyme histidase was not altered (Coote, 1977). The rate of gross protein synthesis also did not appear to be affected by BUdR during the first hour of sporulation, although after this time no further incorporation

of $[^3H]$-methionine occured in this medium, whereas incorporation continued in the control culture with excess TdR (Fig. 24). However, the culture with TdR at 1 $\mu g ml^{-1}$ also did not incorporate $[^3H]$- methionine after the first hour. Due to the rapid turnover of proteins during sporulation, the experiment could have been improved by measuring the incorporation of radioactivity by cell samples pulse-labelled for a specific time, rather than by continuous measurement of incorporated radioactivity by the whole culture.

Davidson and Kaufman (1977) proposed that during eukaryotic differentiation BUdR starves cells for dCyd residues by inhibiting ribonucleotide reductase. However, BUdR did not apparently inhibit this enzyme during sporulation of B. subtilis but-32, as addition of equimolar or excess dCyd to SM containing BUdR did not relieve the inhibitory action of the analogue. Coote (1977) found that BUdR allowed normal, or slightly increased, production of two early marker events of sporulation, exoprotease and alkaline phosphatase, but the bulk of the cells became blocked before spore septum formation, and did not produce dipicolinic acid, a later event. This suggested that BUdR interfered with an early event during sporogenesis, and since BUdR appeared to be incorporated into DNA of the tolerant strain during both growth and sporulation (Fig.'s 10. 11, 14, 21, and 25) it was thought that inhibition of sporulation by BUdR might be associated with the requirement for DNA replication at the onset of sporulation.

A. The association between DNA replication and inhibition of sporulation by BUdR

The use of HPUra, a specific inhibitor of DNA synthesis, has indicated that successful sporulation depends on the completion of chromosome replication at the onset of the process (Dunn et al, 1978). In these experiments the wild-type strain began to escape the inhibitory effect of HPUra on sporulation about 35 min after initiation, and this correlated with termination of DNA replication, measured by marker-frequency analysis. Therefore, the number of cells which escape from HPUra inhibition of sporulation can be regarded as an index of termination of replication required for sporulation.

Unlike the wild-type, cells of but-32 began to escape the inhibitory effect of HPUra at a later time, about 60-75 min after initiation (Fig's 27, 28, 30, 31 and 32). This correlated with termination of DNA replication, monitored by marker-frequency analysis (Fig. 38). The spore incidence with strain but-32 continued to rise until about 6h after initiation (Fig. 28), unlike the wild-type, where escape was essentially complete 2h after initiation (Dunn et al, 1978). Therefore, DNA replication required for sporulation of the TdR-requiring strain occured over a longer period of time at the onset of sporulation than replication in the wild-type. The chromosome replication time during growth in CH medium plus excess TdR of strain but-32 was estimated

as 50 and 82 min in two experiments (Tables 9 and 10), compared to value of 55 min for the wild-type (Ephrati-Elizur and Borenstein, 1971). The rate of DNA replication is little affected by transferring cells from the rich to the poor medium (Young and Jeffs, 1978), so similar values can be assumed during sporulation. Other work has also indicated that the rate of chromosome replication is slower in TdR-requiring strains (Ephrati-Elizur and Borenstein, 1971; Gillin and Ganesan, 1975), but slowing the rate to increase the chromosome replication time from 55 min to 82 min could still not account for the whole of the prolonged escape of but-32 from HPUra inhibition of sporulation. This disparity may be related to the possibility that replication essential for sporogenesis, although proceeding at the same rate as in the wild-type, is initiated over a wider time span under starvation conditions in TdR-requiring strains (Sargent, 1980 b).

The pattern of escape from BUdR inhibition of sporulation closely paralleled that of HPUra and consistently preceded escape from HPUra (Fig.'s 27, 28, 30, 31 and 32). The effect of BUdR on sporulation therefore appeared to be limited to the period of DNA replication. Supportive evidence for an association between DNA replication and inhibition of sporulation by BUdR was obtained when cells grown and resuspended in a subinhibitory concentration of HPUra, which slows down the rate of DNA synthesis (Fig. 22), exhibited delayed escape from inhibition of sporulation

by both BUdR and HPUra, at the fully inhibitory concentration. It was of interest that a subinhibitory HPUra concentration, which did not affect the growth rate, also inhibited sporulation to some extent, presumably by slowing down the rate of DNA synthesis. This conflicted with the results of other work, using the wild-type strain (Young and Jeffs, 1978). Since cells grown and resuspended with BUdR also sporulated poorly and experienced delayed escape of sporulation from inhibition by the higher HPUra concentration (Fig.'s 31 and 32), it was thought that the basis for inhibition by the analogue may have been a delay in termination of replication, while other changes necessary for sporulation continued normally. This was supported by the observation that incorporation of radiolabelled TdR into acid-insoluble material continued for much longer in sporulating cells exposed to BUdR (Fig.'s 37 and 56).

Therefore, it seemed possible that an imbalance between DNA replication and other sporulation events, due to exposure of sporulating cells to BUdR, may have resulted in inhibition of the process. The fact that escape from BUdR inhibition of sporulation consistently preceded that from HPUra suggested that an event earlier than termination of replication, such as initiation of replication, was affected by BUdR. It seemed possible that continued reinitiation of DNA replication by BUdR could bring about an imbalance between DNA replication and other events occuring during sporulation, such that termination of

chromosome replication, a prerequisite for successful sporulation, was prevented.

B. Recovery from BUdR inhibition of sporulation

Before further examining the effect of BUdR on the known requirement for termination of DNA replication, it was important to determine that BUdR had no adverse irreversible effects on DNA replication. This seemed unlikely, as recovery from inhibition of sporulation could be obtained if the analogue was removed from the sporulation medium and replaced by TdR (Coote, 1977; Table 19). Continued DNA replication was required for recovery, as this was not obtained if DNA synthesis was prevented by TdR starvation or addition of HPUra (Table 19). In this experiment the cells only began to regain the capacity to sporulate after about 60 min, roughly equivalent to a complete chromosome replication time. Continued DNA synthesis was required for recovery since, if HPUra was added before 60 min, DNA replication stopped and sporulation was inhibited.

Further analysis of the capacity for cells to recover from BUdR inhibition indicated that cells could recover sporulation capacity after removal of BUdR at any time up to t_8 (Table 20). However, the degree of recovery tended to decline the longer the cells were exposed to the analogue, and this correlated with a decrease in the rate of DNA replication on

transfer to TdR medium. This raised the possibility that BUdR might be preventing termination of replication in the majority of cells, thereby preventing sporulation. However, replication appeared to proceed normally in cells exposed to BUdR at various times after initiation of sporulation except that the rate of incorporation of radiolabelled TdR continued up to t_7 in cells given BUdR from t_{zero} (Fig.'s 37 and 56), whereas in general replication was terminated by around t_4-t_5 in cells given excess TdR, or TdR at 1 $\mu g ml^{-1}$, from t_{zero} (Fig.'s 36 and 56). The day-to-day variation observed for the time of termination in TdR alone may have been caused by differences in the small amount of rich CH medium carried over into SM at the time of initiation of sporulation, resulting in differences in the times of induction of sporulation between experiments. The prolonged period of DNA replication in cells exposed to BUdR agreed with the later escape time from HPUra inhibition of sporulation exhibited by BUdR-treated cells (Fig.'s 31 and 32). However, when cells were exposed to BUdR after 6h with TdR, when replication had terminated, radioactive TdR was incorporated at a linear rate for a further 2h (Fig.37). This suggested that BUdR might be promoting initiation of DNA synthesis during sporulation rather than delaying termination of existing rounds of replication. Marker-frequency analysis of these cells might have confirmed that the DNA synthesis on transfer to BUdR medium after 6h in TdR medium was due to reinitiation from the origin of replication.

C. Marker-frequency analysis of sporulating cells

This technique was used in an attempt to determine the effect of BUdR on termination of DNA replication during sporulation. The *pur*$^+$/ *met*$^+$ ratio from cells given TdR fell over an initial 90 min period (Fig. 38), and thereafter did not alter significantly up to $t_{7.5}$ (Fig. 39). For BUdR-treated cells the ratio only levelled off about 180 min after initiation, and also remained relatively constant up to $t_{7.5}$. This suggested that in BUdR medium replication was terminated in most of the cells, but termination was delayed. When DNA replication was synchronised during growth of *Ts134*, a TdR-requiring strain ts for initiation of DNA replication, and the cells initiated to sporulate at the permissive temperature, the *pur*$^+$/ *met*$^+$ ratio for cells in BUdR medium fell rapidly, but again not as quickly as the ratio for cells in TdR alone (Fig.'s 44 and 45). In addition, the ratio for BUdR-treated cells did not decrease to the level of the control values. An explanation for this could be seen when DNA synthesis was followed by incorporation of radiolabelled TdR into acid-insoluble material (Fig. 46). Whereas DNA replication had apparently terminated in TdR medium by $t_{1½}$, in BUdR medium DNA synthesis continued at an almost linear rate until at least t_6, and sporulation was inhibited. It therefore seemed possible that BUdR allowed more rounds of replication to occur in SM than occured in TdR alone. When the rate of DNA synthesis was decreased using a subinhibitory HPUra

concentration, sporulation was also inhibited, but not to the same extent as in BUdR medium. The fall in the *pur*$^+$/ *met*$^+$ marker ratio during sporulation was also delayed, but DNA synthesis appeared to stop by about t_3 under these conditions. It therefore seemed that BUdR was not mimicking the action on DNA synthesis of a subinhibitory HPUra concentration.

A direct test of the effect of BUdR on completion of replication at the chromosome terminus during sporulation was unsuccessful, for the reasons given in the results section. When this strain was initiated to sporulate at 47^{o}C, when further initiation of DNA replication was prevented, sporulation was inhibited, presumably by an effect on cell viability (Table 21). An inhibitor of initiation of chromosome replication, such as chloramphenicol, could have been used instead of a ts mutant, but it was not clear what other effects this drug might have had, since it inhibits bacterial protein synthesis generally. There was a negligible effect of BUdR on termination of synchronised chromosome replication during growth of both *Ts134* (Fig. 47) and a BUdR-tolerant derivative of this strain (Fig. 49) at the restrictive temperature. Therefore, as initiation of DNA replication was prevented under these conditions, and termination apparently proceeded normally, it seemed possible that the continued replication in the presence of BUdR during sporulation was due to reinitiation of DNA replication which did not normally occur.

D. Analysis of DNA replication and BUdR inhibition of sporulation, using BUdR as a density-label

Much experimental work has been reported indicating the requirement for termination of DNA replication for sporulation (Mandelstam et al, 1971; Dunn et al, 1978; Young and Jeffs, 1978). In addition, the work of Mandelstam and Higgs (1974), Keynan et al (1976) and Dunn et al (1978) has suggested that there is a critical time during the DNA replication cycle for induction of sporulation. The consensus of opinion has been that sporulation could be induced in cells under starvation conditions when the chromosome replication fork was passing through a particular segment of the chromosome some 20 min after replication began at the origin. Therefore, those cells with a replication fork situated after this critical point were presumed to terminate that particular round and reinitiate another complete round, whereas those cells with a replication fork situated before the critical point at the time of initiation of sporulation were thought to terminate that round, with no further initiation, so that sporogenesis could continue.

However, Sargent (1980 b) recently suggested that all chromosomes destined to be packaged into spores may be replicated in full under starvation conditions and that, regardless of the time at which replication of the spore chromosome is completed, the template strand is always of vegetative origin. It was hoped,

therefore, that by using BUdR as a density label to study DNA replication during sporulation of a BUdR-tolerant strain, it might be possible to determine firstly, how much chromosome replication is required for a spore to be made, and secondly, how much incorporation of BUdR into DNA occurs during sporulation.

The fate of DNA replicated during sporulation was examined by density-gradient analysis using BUdR as the density label. By t_4 during sporulation in medium containing TdR (1 μgml^{-1}) + BUdR (15 μgml^{-1}), after growth in TdR alone, most of the cellular DNA (66%) contained BUdR incorporated into one strand, with 17% labelled in both strands, and 17% unsubstituted (Fig. 50). By t_7 in this medium there was an equal amount of DNA with BUdR in either both strands (heavy-heavy) or in one strand (heavy-light)(each 40% of the total DNA), with 20% unsubstituted (Fig.53). Unsubstituted DNA was presumably unreplicated DNA, as radiolabelled TdR was present during both growth and sporulation. The appearance of heavy-heavy (HH) DNA during sporulation in BUdR medium indicated that at least one initiation of DNA replication had occured under starvation conditions, since BUdR had been absent during growth. However, in both experiments no HH DNA was detected in the few spores formed in BUdR medium, the DNA being entirely of intermediate, or heavy-light (HL) density (Fig.'s 51 and 54). Therefore, either continued reinitiation of DNA replication, promoted by BUdR, was the basis of inhibition of sporulation, or the presence of BUdR in both strands of

DNA precluded sporulation by somehow interfering with the normal packaging of a chromosome into a spore. Also, the absence of light-light (LL) density DNA in these spores indicated that one full round of DNA replication has to be initiated in a SM before the chromosome can be incorporated into a spore.

Supportive evidence for this latter conclusion came from the result of the converse experiment, in which cells were grown in medium containing TdR (1 $\mu g ml^{-1}$) + BUdR (15 $\mu g ml^{-1}$) until DNA was substituted in both strands with the analogue (Fig. 57), then initiated to sporulate in medium containing only added TdR. (Note that HH DNA can only be obtained using a But strain). The fate of HH DNA during sporulation in TdR was monitored by the same density approach. Under these conditions the sporulation level was similar to that obtained from cells grown and initiated to sporulate in TdR alone. The DNA from spores formed in this medium appeared mainly of LL density, corresponding to 70% of the total spore DNA, with a smaller peak of HL density, representing 30% of the total spore DNA (Fig. 59). However, no HH fraction was detected in the spores. If those cells in which a replication fork lies before a critical point for induction of sporulation some distance from the origin merely had to terminate that particular round of replication for sporulation to continue, some HH DNA woule be expected in the spores formed in this experiment, and some LL DNA in the spores formed in the converse

experiment (Fig.'s 51 and 54). However, neither were detected, suggesting that a full round of DNA replication must be initiated in a SM for a chromosome to be packaged into a spore.

It was possible that no HH DNA was detected in the latter experiment simply because it cannot be incorporated into a spore. However, if this was the case, the level of sporulation ought to have been markedly reduced, unless more than one initiation of replication had occured in SM. This seemed unlikely, as the cells terminated DNA synthesis during sporulation in TdR medium at the same time, whether grown in BUdR or TdR medium (Fig. 56). However, there may have only been a minimal amount of HH DNA present in those cells used to obtain the data in Fig. 56 as they were grown for only approximately four generations in BUdR medium.

The distribution of spore DNA into 70% LL form and 30% HL form, instead of mostly HL density, after growth in BUdR and sporulation in TdR (Fig. 59), may have been the result of a complete round of chromosome replication in SM in addition to, and coming after, completion of all rounds of replication which had already been initiated during growth, and which subsequently terminated in SM. Dichotomous replication might also explain the relatively long period of time during sporulation over which but-32 escaped HPUra inhibition of sporulation. Sargent (1980 b)

also noted that termination of chromosome replication necessary for sporulation of a TdR-requiring strain was distributed over a lengthy period of time.

However, even this amount of replication may not be enough to account for the high percentage of LL DNA in the spores. If the pur$^+$/ met$^+$ marker ratio of but-32 DNA from cells grown in BUdR is taken as 4 (see Table 9), the average number of replication forks per cell is 2. Therefore, cells initiated to sporulate in TdR medium after growth in BUdR probably contained either 1 or 3 replication forks at t_{zero}. If these rounds had to terminate under starvation conditions and were then followed by a full round of chromosome replication necessary for sporulation, on the basis of the model presented (Fig. 60), most of the DNA in spores would be expected to be in the HL form, with a smaller peak of LL density.

Fig. 60 illustrates replication of DNA (conveniently linearized) under starvation conditions if cells terminate rounds of DNA replication already initiated during growth in BUdR medium and then further initiate a full round in SM. It is assumed that 50% of the cells in this medium contained a chromosome with one replication fork, positioned approximately halfway along the chromosome, and 50% of the cells contained a chromosome with three replication forks, one positioned close to the terminus (approximately 7/8 th of the total length of the chromosome),

Figure 60. Diagrammatic representation of possible patterns of chromosome replication during sporulation of but-32.

For the purposes of the model it is assumed that strain but-32 was grown to mid-exponential phase in medium containing TdR + BUdR until both strands of DNA were substituted with BUdR, and then initiated to sporulate in SM + TdR (see legend to Fig.'s 57-59). At the point of resuspension it was assumed that 50% of cells contained one replication fork (A), and 50% contained three forks (B). Substituted DNA is represented by a continuous line, unsubstituted DNA by a broken line. The symbol * denotes the chromosome entering the spore if only one round of replication occured in SM, and if it is assumed that the chromosome with the least amount of vegetative DNA is incorporated into the forespore compartment. If two rounds of chromosome replication occured in SM, an extra round of replication and division are shown. O and T refer to the origin and terminus of replication.

Fig. 60(A)

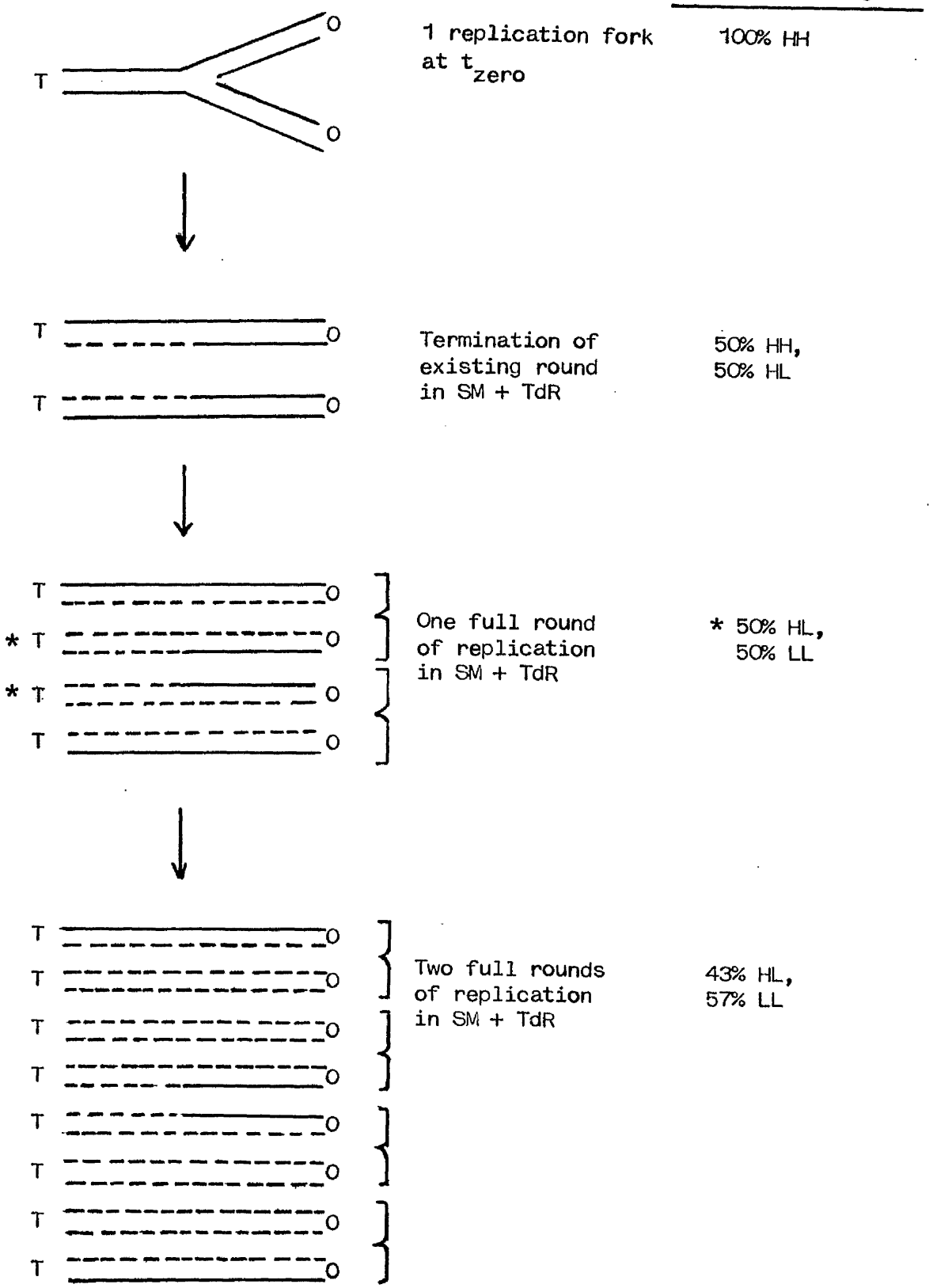
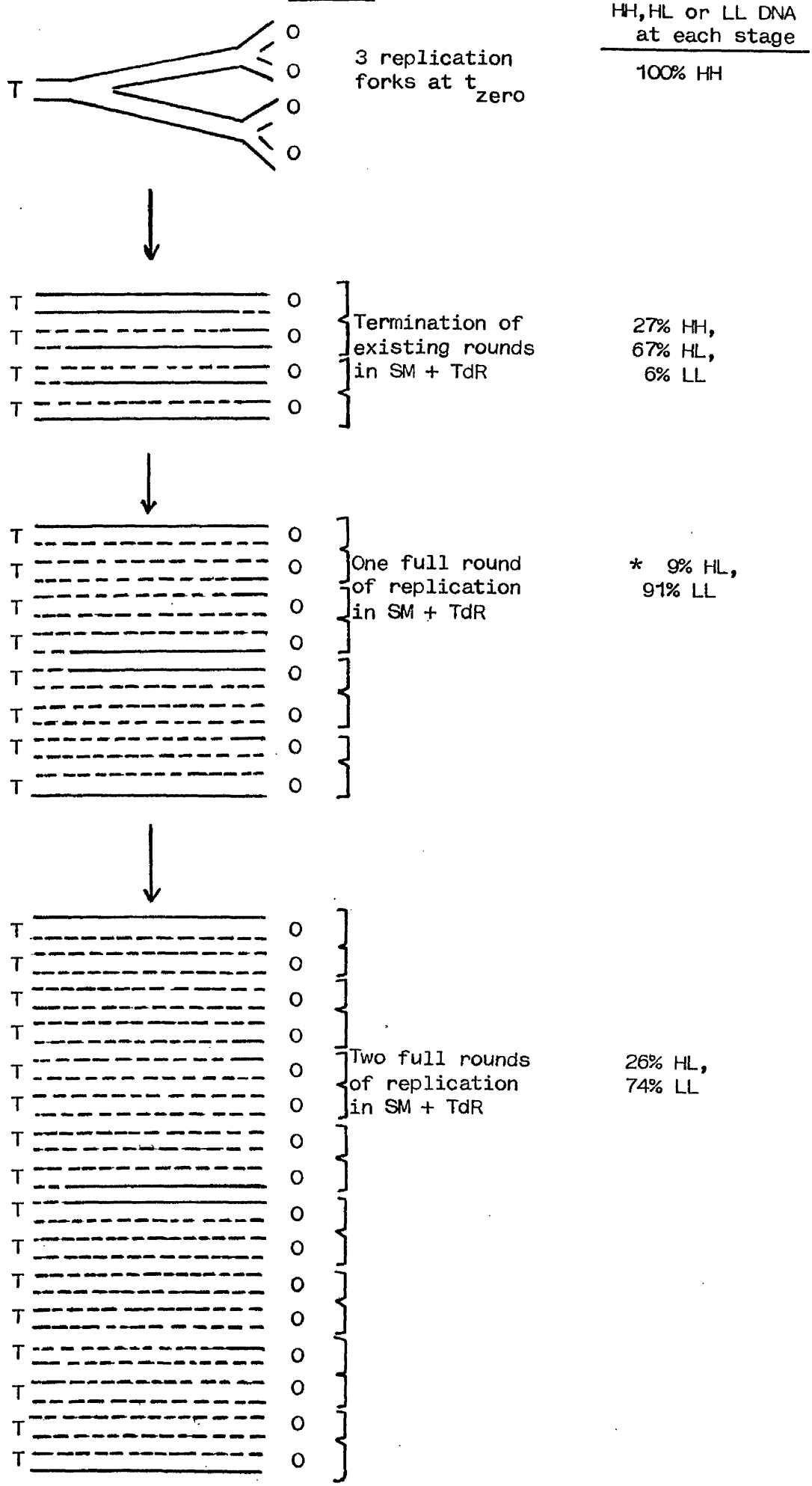

Percentage HH, HL, LL DNA at each stage
T
O
1 replication fork at t_{zero}
100% HH
Termination of existing round in SM + TdR
50% HH, 50% HL
One full round of replication in SM + TdR
* 50% HL, 50% LL
Two full rounds of replication in SM + TdR
43% HL, 57% LL

Fig. 60(B)


Percentage HH, HL or LL DNA at each stage
3 replication forks at t_{zero}
100% HH
Termination of existing rounds in SM + TdR
27% HH,
67% HL,
6% LL
One full round of replication in SM + TdR
* 9% HL,
91% LL
Two full rounds of replication in SM + TdR
26% HL,
74% LL

and two forks close to the origin (approximately 1/8 th of the length of the chromosome from the origin). Substituted DNA is represented as a continuous line, and unsubstituted DNA as a broken line. After completion of the chromosomes, and division of the cells, it was then assumed that there was an equal probability of either of the two chromosomes entering the forespore compartment, and that there were twice the number of spores eventually formed from those cells which contained 3 replication forks at t_{zero} than the number formed from those cells which had only one fork. The expected composition of double-stranded spore DNA in a density gradient was 70% HL and 30% LL density forms. However, the actual composition of the spore DNA in this experiment was 30% HL and 70% LL. Therefore, this result suggested that either the chromosome containing the least amount of vegetative DNA was preferentially incorporated into the spore, or that two full rounds of DNA replication, plus an extra division of cells, occured under these conditions, in order to reduce the amount of BUdR present in the chromosome "destined" for the forespore compartment. When a similar analysis to the one above was carried out, but assuming that the chromosome with the least amount of vegetative DNA was incorporated into the spore, 77% of the DNA would be expected in the LL form, and 23% in the HL form. If two full rounds of replication occured in sporulation medium, 68% of spore DNA would be expected in the LL form, and 32% in the HL form.

Although the experimental results tend to favour the hypothesis that two full rounds of replication occured under starvation conditions, it is possible that either model could fit the results. Sargent (1980 b) described an experiment to determine how much vegetative DNA appears in the completed spore. It involved radiolabelling the DNA of cells during growth with $[^{3}H]$ -TdR, halving the culture, resuspending half the cells in SM with label present and half with label absent. A comparision of the level of radioactivity per 10^{8} spores formed in both half cultures indicated that almost exactly half the level of radioactivity was obtained for cells sporulated in medium lacking radioactive TdR, compared to the level of radioactivity in cells grown and sporulated in medium containing radiolabel. This suggested that one strand of spore DNA was of vegetative origin. However, such an experiment would not indicate which of the two theories above was the basis for the composition of spore DNA found when cells were initiated to sporulate in TdR medium after growth in BUdR medium, as they both predict similar low percentages of vegetative DNA in the spore. However, the theory predicting two full rounds of DNA replication also predicts an extra division of cells. Therefore, direct counting and measurement of cell viability during sporulation under these conditions might indicate that twice as many cells would appear during sporulation in a culture of cells grown in BUdR medium until DNA was substituted to a maximum level, and initiated to sporulate in medium with TdR,

compared to the number in a culture of cells grown and sporulated in TdR alone, if this theory is correct. If the cells grown in BUdR and initiated to sporulate in TdR did not undergo a second round of replication and an extra division, as predicted by the other model, a similar number of cells would be expected under these conditions to the number in a control culture grown and resuspended in TdR alone.

It is clear, however, that the actual composition of the spore DNA in this experiment was not what would be expected if one strand was of vegetative origin. Perhaps the normal level of sporulation achieved by cells grown in BUdR medium but resuspended in TdR medium was obtained via abnormal behaviour of the replicating chromosomes, in such a way as to effectively minimise the amount of BUdR appearing in DNA of the mature spore.

In summary, therefore, BUdR inhibition of sporulation was limited to the period of DNA replication which occurs at the onset of sporulation, and BUdR appeared to delay termination of DNA replication required for sporulation. Prolonged DNA synthesis in sporulation medium with BUdR was confirmed by, a) direct measurement of the rate of incorporation of radiolabelled TdR into acid-insoluble material, b) density-gradient analysis and c) by marker-frequency analysis of DNA replication during sporulation. The results of density-gradient analysis of DNA replication suggested either that incorporation of BUdR into both

strands of DNA prevented sporulation, or that continued initiation of DNA replication, induced by BUdR, was the basis of inhibition of sporulation. The same analysis also indicated that a full round of chromosome replication must be initiated from the origin in a sporulation medium before sporulation can continue. The conclusion is therefore not consistent with the hypothesis that for sporulation to be induced, the cell, under starvation conditions, must contain a replication fork passing through a segment of the chromosome some distance from the origin (Mandelstam and Higgs, 1974). Sargent (1980 b) tentatively suggested that possibly all chromosomes destined to be packaged into spores are initiated in the sporulation medium. The results of this work are consistent with this suggestion, but also indicate that initiation of chromosome replication in starvation medium is a requirement for sporulation in B. subtilis.

Finally, the association between DNA replication and inhibition of sporulation by BUdR is generally consistent with the action of the analogue on differentiation of eukaryotic cells. However, major differences exist between chromosome replication of prokaryotes and eukaryotes. Unlike prokaryotes, eukaryotes do not replicate DNA from one unique origin, and replication occurs at numerous points along the chromosome. Also, segregation of replicated chromosomes in eukaryotes is achieved via mitosis, a process which does not occur in prokaryotes. The use

of BUdR may clarify the role of chromosome replication during differentiation of particular organisms. However, because of the major differences between chromosome replication in prokaryotes and eukaryotes, and because of the different possible sites of action of BUdR during differentiation, discussed earlier, it seems unlikely that its use would enable a close analogy to be drawn between prokaryotic and eukaryotic differentiating systems.

ADDENDUM - DNA REPAIR SYNTHESIS

The structure of DNA is susceptible to different kinds of damage, all of which may be lethal or mutational events. Those which include changes to the structure which make its replication difficult or impossible e.g. pyrimidine dimer formation, would normally be lethal lesions, but DNA repair mechanisms can eliminate them (Moseley, B.E.B. & Williams, E : 1977, Advances in Microbial Physiology 16, 89-156). Also, more subtle modifications to DNA bases which do not impede DNA replication, but which can mispair more often than do normal bases, can enhance mutation frequencies unless removed. The excision mechanism of DNA repair is a multienzyme process involving single-strand incision of DNA by an endonuclease adjacent to the region of damage, the excision of a short stretch of DNA containing the damage site, resynthesis of the region by DNA polymerase I, using the complementary strand as a template, and, finally, ligation of adjacent oligonucleotide sequences. An excision repair mechanism exists for the repair of potentially mutagenic bases (Demple, B. & Linn, S: 1980, Nature 287, 203-208). Glycosylases remove individual bases by hydrolysis of the bond between the base and the sugar moiety so that an apurine or apyrimidinic (AP) site is created, leaving intact the sugar-phosphate backbone. This AP site may be recognised by a specific endonuclease, culminating in excision repair of DNA.

It has been shown that the introduction of 5-bromouracil into B. subtilis 168 (trp, thy) DNA allows ultraviolet and visible light to produce single-strand breaks in fully

BUdR- substituted DNA at a 500-fold greater rate than that found in DNA containing no bromouracil (Hutchinson, F. & Hales, H.B: 1970, Journal of Molecular Biology 50, 59-69). Introduction of 5-bromouracil into DNA during replication of strain but-32, during sporulation in the presence of BUdR, resulted in approximately 20% substitution of TdR residues by BUdR (see Results). If this level of substitution induced, for example a significant degree of single-strand breakage in DNA, it is possible that the prolonged period of DNA synthesis during sporulation in the presence of BUdR (Fig.'s 37 & 56) was due more to excision repair of the sites of damage, rather than to normal semi-conservative replication. Since HPUra is a selective inhibitor of semi-conservative replication in B. subtilis, and does not inhibit repair synthesis (Brown, N: 1971, Journal of Molecular Biology 59, 1-16), the effect of the addition of this drug during DNA replication under starvation conditions in the presence of BUdR would indicate whether semi-conservative DNA replication or repair synthesis was the basis for the prolonged incorporation of radiolabelled TdR into acid-insoluble material. However, there is evidence from this work which might indicate that little repair synthesis was, in fact, being undertaken by cells containing BUdR- substituted DNA. When cells were shifted from BUdR to TdR medium at t_3 during sporulation (Fig. 33), by which time HH and HL DNA would have formed, incorporation of $[^{14}C]$- TdR into acid-insoluble material was immediately blocked on addition of HPUra to these cells. In addition, the symmetrical nature of the HL and LL density peaks of DNA isolated from spores formed in TdR medium, after previous growth in BUdR medium, might not have been expected if extensive

repair of HH and HL DNA occurred during sporulation in TdR medium. If a significant degree of repair synthesis of HL DNA occurred under these conditions, after shearing of DNA during the extraction procedure, the appearance of short pieces of radioactively-labelled double-stranded DNA containing HL and LL DNA (HLL) would have been expected. However, the density gradient method, as used in these experiments, might not have been sensitive enough to detect this DNA.

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APPENDIX

Media

Liquid Growth Media

I. Casein Hydrolysate (CH) Medium:

The basal medium contained 10 g of casein hydrolysate (Oxoid), 3.68 g L-glutamic acid, 1.25 g L-alanine, 1.39 g L-asparagine, 1.36g KH_2PO_4, 0.107 g Na_2SO_4, 0.001 g $FeCl_3.6H_2O$, 0.096 g NH_4NO_3 and 0.54 g NH_4Cl in 1 litre of distilled water. The pH was adjusted to 7.0 using 40% NaOH and the medium sterilised by autoclaving at 15 $lb(in^2)^{-1}$ for 15 min.

Additions to the CH medium

CH_3 : This contained 2.0 g $MgSO_4.7H_2O$ plus 0.4 g $CaCl_2.2H_2O$ per litre of distilled water.

CH_4 : This contained 1.1 g $MnSO_4.4H_2O$ per 100 ml distilled water.

Both CH_3 and CH_4 additions were sterilised by autoclaving at 15 $lb(in^2)^{-1}$ for 15 min.

Complete CH Medium

This medium is similar to that described by Sterlini and Mandelstam (1969).

To each 100 ml of basal medium was added 5.0 ml CH_3 plus 0.2 ml CH_4. 1.0 ml 0.2% (w/v) L-tryptophan was included for growth of tryptophan-requiring strains. This medium is referred to in the text as CH medium.

Also, an appropriate volume of 0.2% (w/v) thymidine was included, depending on the concentration required in an experiment, for growth of TdR-requiring strains.

Tryptophan and TdR solutions were sterilised by filtration through a cellulose acetate membrane filter (Oxoid, pore size 0.45 μm)

II. Resuspension Medium (SM) for Initiation of Sporulation

The following stock solutions were used in the preparation of the resuspension medium.

Solution A.

$FeCl_3.6H_2O$, 0.1 g; $MgCl_2.6H_2O$, 0.83 g; and $MnCl_2.4H_2O$, 1.98 g; made up to 100 ml with distilled water and stored at 4 °C.

Solution B.

NH_4Cl, 53.5 g; Na_2SO_4, 10.6 g; KH_2PO_4, 6.8 g, and NH_4NO_3, 9.7 g; made up to 1 litre with distilled water and stored at 4 °C.

Basal Salts Solution

To 900 ml distilled water was added 1.0 ml stock solution A and 10 ml stock solution B. The pH was adjusted to 7.0 using 40% NaOH, and the medium sterilised by autoclaving at 15 $lb(in^2)^{-1}$ for 15 min.

Additions to the Basal Salts Medium

The following solutions were prepared separately, and sterilised by autoclaving as above.

1) 1.5% (w/v) L-glutamate, brought to pH 7.0 using 40% NaOH.

2) 0.1 M $CaCl_2.2H_2O$ solution.

3) M $MgSO_4.7H_2O$ solution

Resuspension Medium (SM) for Induction of Sporulation

This is the replacement medium described by Sterlini and Mandelstam (1969). To each 100 ml basal salts medium was added 10 ml L-glutamate solution, 1.0 ml $CaCl_2.2H_2O$ solution and 4.0 ml $MgSO_4.7H_2O$ solution. 1.0 ml 0.2% (w/v) L-tryptophan was included for tryptophan-requiring strains, and an appropriate volume of 0.2% (w/v) TdR for TdR-requiring strains. This medium contains an adequate nitrogen source in the form of ammonium but only a poor carbon source in the form of L-glutamate.

III. Minimal Growth Medium (MM)

This defined medium was used for growth and sporulation of B. subtilis by the nutrient exhaustion method. (see Materials and Methods, Section IV).

To 100 ml basal salts medium (see above) was added:- 10 ml L-glutamate solution, 2 ml $CaCl_2.2H_2O$ solution, 5 ml 10% (w/v) D-glucose (sterilised by filtration), 1.0 ml 2.5% (w/v)

$MgSO_4.7H_2O$ solution, 1 ml 0.05% (w/v) $FeSO_4.7H_2O$ solution, and 1.0 ml 0.05% $MnCl_2 4H_2O$ solution. (these 3 solutions were sterilised by autoclaving as above). 1 ml 0.2% (w/v) L-tryptophan was added, plus an appropriate volume of 0.2% (w/v) TdR solution for tryptophan- and TdR- requiring strains.

Solid Growth Media

I. Nutrient agar (NA)

28 g Oxoid nutrient agar was added to 1 litre distilled water and autoclaved at 15 $lb(in^2)^{-1}$ for 15 min.

II. Minimal agar (MA)

15 g purified agar (Oxoid) was added to 1 litre solution containing:

900 ml basal salts medium

90 ml 1.5% (w/v) L-glutamate

10 ml 0.1 M $CaCl_2.2H_2O$

This was sterilised by autoclaving as above. Before pouring molten agar at 60°C into sterile plates, 5 ml 40% (w/v) D-glucose was added. (Auxotrophic requirements were added either at this point, or were spread onto plates of solid agar).

Transformation Media

When method 1 was used the following were prepared:-

a) Stock amino-acid solutions

20 mg of each of the following were made up to 100 ml distilled water at pH 7.0 :-

Glycine, L-alanine, L-phenylalanine, L-methionine, L-threonine, L-aspartate, L-valine, L-lysine, L-leucine, L-isoleucine, L-proline, L-cysteine, L-cystine, L- tyrosine, L-serine, L-glutamine, L-arginine, L-histidine, L-asparagine and L-trytophan. Like all amino acid solutions used in this work, this was filter sterilised. For the final stage of the method a solution omitting an amino-acid was prepared, where this particular amino-acid was missing in the selective medium.

b) Spizizen's Minimal Medium (SMM)

This medium was based on that used by Spizizen (1958). The following salts were dissolved in distilled water in the order shown;

$(NH_4)_2SO_4$	0.2% (w/v)
K_2HPO_4	1.4% (w/v)
KH_2PO_4	0.6% (w/v)
Sodium citrate. $2H_2O$	0.1% (w/v)
$MgSO_4.7H_2O$.	0.02% (w/v)

The solution was sterilised by autoclaving.

c) 40% (w/v) D-glucose, 10^{-5}M $MnSO_4.4H_2O$ and 0.8% (w/v) α,α'-dipyridyl solutions were made and sterilised by filtration.

d) PTM

For approximately 20 ml volumes, the following were used: 18 ml SMM. 0.3 ml 40% (w/v) D-glucose, 0.2 ml 10^{-5}M $MnSO_4$ and 2 ml stock amino-acid mixture. Other requirements were added separately.

g) TM

For 20 ml volumes, the following were used:- 18 ml SMM, 0.3 ml 40% (w/v) D-glucose, 1.0 ml α,α'-dipyridyl solution, plus 2 ml of an amino-acid mixture omitting one amino-acid. Other requirements were added separately.

When method 2 was followed the following media were prepared:-

I. Semi-defined medium (Bott and Wilson, 1967)

For approximately 20 ml of culture, using Mu8u5u16 as a recipient:-

18.0	ml	SMM		
0.4	ml	1%	(w/v)	casein hydrolysate (in distilled water)
0.25	ml	40%	(w/v)	D-glucose
0.4	ml	2.5%	(w/v)	$MgSO_4.7H_2O$
0.5	ml	0.2%	(w/v)	L-tryptophan
0.5	ml	0.2%	(w/v)	L-histidine

To this basic medium the auxotrophic requirements of this strain were added, i. e. 0.5 ml each of 0.2% (w/v) adenine, L-leucine and L-methionine.

II. Defined medium-1 (Wilson and Bott, 1968) For approximately 20 ml of culture, the following were used:-

18.0 ml	SMM	
0.25 ml	40% (w/v)	D-glucose
0.4 ml	2.5% (w/v)	$MgSO_4.7H_2O$
0.5 ml	A9 supplement	

A9 supplement is a mixture of nine amino-acids made up in a stock solution, each at 2 $mgml^{-1}$, sterilised by filtration, and stored at $4^{o}C$. The amino acids are L-tryptophan L-arginine, L-histidine, L-valine, L-lysine, L-threonine, glycine, L-aspartate and L-methionine. The final concentration of each in the medium was 50 μgml^{-1}.

III. Defined medium -2 (Bott and Wilson, 1968)

This medium was identical to the previous medium, except that the $(NH_4)_2SO_4$ concentration was increased from 0.2% to 0.6%, the K_2HPO_4 concentration reduced from 1.4% to 1.24%, the KH_2PO_4 concentration raised from 0.6% to 0.76%, and the pH raised to 6.7 using 40% (w/v) NaOH.

Selective media

Selection for growth of transformants was made on minimal agar (MA, see above). Using Mu8u5u16 as a recipient for transformation, when pur^+ transformants were selected, the MA also contained L-leucine (10 $\mu g ml^{-1}$, final concentration) and L-methionine (10 $\mu g ml^{-1}$, final concentration). When met^+ transformants were selected, MA contained L-leucine (10 $\mu g ml^{-1}$, final concentration) and adenine (10 $\mu g ml^{-1}$, final concentration).

Reagents for DNA extraction

I. Preparation of DNA from cells in CH or SM

A. Stock reagents

0.1 M sodium azide

0.15 M NaCl (pH 5.0)

0.15 M NaCl, 0.1 M EDTA buffer (pH 8.0)

Standard saline citrate (SSC):- 0.15 M NaCl containing 0.015 M trisodium citrate, pH 7.0.

0.1 X SSC: a one-tenth dilution of SSC using distilled water. These reagents were sterilised by filtration.

B. Freshly-prepared reagents

0.5% (w/v) lysozyme, made up in SSC

0.1% (w/v) ribonuclease, made up in 0.15 M NaCl (pH 5.0)

0.1% (w/v) protease, made up in NaCl/EDTA buffer (see above).

II. Preparation of spore DNA

A. Reagents for spore preparation

0.1 M KH_2PO_4, 0.01 M $MgCl_2.6H_2O$ (pH 7.8)

lysozyme

0.1% (w/v) sodium dodecyl sulphate (SDS)

sodium dodecyl sulphate (crystalline, Sigma)

45% Urografin (made by dilution of a 72% stock solution using 0.1% SDS)

B. Reagents for spore DNA preparation

Spore solution A. (freshly-prepared, pH 9.8 with 40% NaOH)

8 M Urea

1 M tris

1% (w/v) SDS

0.1 M Ammonium acetate

0.1 M Dithioerythritol (DTE).

Spore solution B

1 M NaCl

0.01 M DTE

Spore solution C.

0.1 M tris/HCl buffer, pH 7.3

0.25% (W/V) Tween 80 (Polyoxyethylene Sorbitan Mono-oleate)

0.01 M EDTA (Ethylene diaminetetracetic acid, disodium salt)

0.01 M DTE

Spore solution C + sucrose

0.5 M sucrose in solution C.

Buffer-saturated phenol

Per 500g bottle of phenol (Analar) :-

Phenol saturated with 0.1 M tris, 0.005 M EDTA (pH 8.0), plus 50 ml m-cresol;

0.5g 8-hydroxyquinoline

Buffer-saturated ether

Diethyl ether saturated with 0.1 M tris, 0.005 M EDTA(pH 8.0)

Other reagents

Lysozyme, sodium lauryl sarcosinate, protease, 3 M sodium acetate and 95% ethanol.