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The Occurrence of Two Types of Synthesis of Deoxyribonucleic Acid during Normal Growth in *Bacillus subtilis*

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A study of the relative utilization of thymine and thymidine as precursors for DNA synthesis during normal growth in *Bacillus subtilis* showed that thymine serves preferentially as a precursor for 'repair' synthesis, whereas thymidine is used preferentially for 'replicative' synthesis. Further, evidence was obtained which suggests that during normal growth both 'replicative' and 'repair' DNA syntheses occur simultaneously. 'Repair' synthesis is distinguished not only on the basis of its preferential utilization of thymine but also by its selective inhibition by caffeine. 'Replicative' synthesis, however, is selectively inhibited by 6-(*p*-hydroxyphenylazo)-uracil. 'Repair' synthesis would seem to be a 'pre-fork' phenomenon and its inhibition is highly lethal to the cell.

Recently, it has become clear that all cells, both prokaryotic and eukaryotic, possess several enzymes which are capable of catalysing the synthesis of DNA and are all present during normal growth of cells. *Escherichia coli*, for example, possesses at least three DNA polymerase species (EC 2.7.7.7), pol I, pol II and pol III (for review, see Pato, 1972), and *Bacillus subtilis* also possesses three (Ganesan *et al.*, 1973). It appears that not all of these polymerases are essential for DNA replication; indeed mutants essentially deficient in the most abundant form of DNA polymerase, pol I, grow as normally as wild-type cells (DeLucia & Cairns, 1969; Laipis & Ganesan, 1972). The characteristic property of these mutants is increased sensitivity to irradiation with u.v. light or X-rays, which implies a role of pol I in the repair of lesions thus introduced into the DNA. During normal growth, however, it is unlikely that cells accumulate sufficient lesions to account for the occurrence of significant amounts of pol I or for the presence of multiple molecular forms of DNA polymerase. Rather, the possibility is raised of there being more than one type of DNA synthesis during normal growth. Preliminary investigations have lent some support to this concept and have suggested that two types of DNA synthesis are essential for normal cell growth and viability. Possible roles for such DNA syntheses have been discussed by Harris (1973).

Two types of DNA synthesis are known to occur *in vivo*. One type, which is termed 'semi-conservative' or 'replicative', results in daughter molecules that contain one completely intact strand of parental DNA and one of daughter DNA. The second type of synthesis, which is observed after cells have been exposed to agents that introduce lesions into DNA, involves excision and replacement of some parental DNA and is referred to as 'repair' or 'non-conservative' synthesis. Results are described in the present paper which are interpreted as evidence that during normal growth in *B. subtilis* at least two types of DNA synthesis occur simultaneously: a 'replicative-type' and a synthesis that more closely resembles 'repair-type'. A preliminary report of some of these findings has appeared (Fraser *et al.*, 1972).

Experimental

Materials

[6-^{3}H]Thymidine (approx. 15Ci/mmol), [6-^{3}H]-thymine (approx. 20Ci/mmol) and [2-^{14}C]thymidine (50Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; $^{15}NH_4Cl$ (95% enrichment) was from C.Z. Scientific Instruments Ltd., London W1A 2AR, U.K.; Deuterated Algal Whole Hydrolysate was from Merck, Sharp and Dohme Ltd., Quebec, Ont., Canada; nitrocellulose filters (Sartorius; 2.5cm diameter, 0.45μm pore size) were from V. A. Howe Ltd., London SW6 3EP, U.K.; and Whatman GF/C glass-fibre discs were from H. Reeve Angel and Co., London EC4 6AY, U.K. Lysozyme and bovine pancreatic ribonuclease A, type 1-A, were the products of Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Trichloroacetic acid, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were AnalaR grade where possible. HOPhNNUra* was generously given by Dr. B. W.

* Abbreviation: HOPhNNUra, 6-(*p*-hydroxyphenylazo)uracil.

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Methods

Growth of cells. The bacterial strain used throughout this work was *Bacillus subtilis* 168T (*try*C_2, *thy*), obtained from Professor C. Anagnostopoulos, Gif-sur-Yvette, France. Cells were usually grown overnight on Tryptose Blood Agar Base (Oxoid Ltd., London E.C.4, U.K.) and inoculated into liquid growth medium to an initial cell concentration of 10^8 cells/ml. This growth medium, described by Kelly & Pritchard (1965), was supplemented with casein hydrolysate to 0.05% (w/v), 50μg of L-tryptophan/ml, and 5μg of thymidine/ml. Cells were grown in 5ml batches in 22mm×150mm test tubes at 37°C with vigorous shaking. Under these conditions cells were in mid-exponential phase after 2–3h and entered stationary phase after 4–5h. Viable cell concentrations were determined after dilution into minimal salts medium by plating appropriate dilutions on Tryptose Blood Agar.

Incorporation of radioactive precursors into cells and measurement of DNA synthesis. CsCl-density-gradient-centrifugation experiments were all performed by using a single batch of stock cells, which were labelled uniformly with ^{15}N, ^{2}H and [2-^{14}C]-thymidine by the following procedure. Minimal salt solution was prepared containing 18.3g of K_2HPO_4, 6g of KH_2PO_4, 1g of sodium citrate and 2g of $^{15}NH_4Cl$ in 1 litre of water. A loopful of cells was inoculated into 5ml of the above salts medium and 0.05ml of Deuterated Algal Whole Hydrolysate was added. After growth overnight the cells were sedimented by centrifugation and resuspended in 20ml of this medium containing [2-^{14}C]thymidine (0.1μCi/ml). After growth for a further 6h the cells were washed four times with $^{14}NH_4Cl$-containing minimal salts medium and finally resuspended in 40ml of $^{14}NH_4Cl$-containing medium. Glycerol was then added to 15% (v/v) and the cells were stored at −70°C in 2ml portions. For subsequent experimentation 2ml samples were thawed as required, sedimented by centrifugation and resuspended in 10ml of growth medium. This gave an initial cell concentration of approx. 10^8 cells/ml.

In experiments measuring the radioactivity incorporated during short pulses, cells (5ml) were grown to mid-exponential phase (2–3h) and duplicate 0.5ml portions were labelled with either [6-^{3}H]-thymine (2.5μCi/ml) or [6-^{3}H]thymidine (0.5μCi/ml) in the absence or presence of caffeine at 4mg/ml or HOPhNNUra at 10μg/ml. Radioactive labelling of cells was terminated by the addition of 0.1vol. of ice-cold saline–EDTA–azide (0.15M-NaCl, 0.1M-EDTA, 0.1M-NaN_3, pH8.0) and the cells were sedimented by centrifugation in the MSE 4L Mistral centrifuge (10min at 4°C, 2700*g*, $r_{av.}$ 18cm). They were then washed four times with saline–EDTA–azide and finally resuspended in 0.5ml of saline–citrate (0.15M-NaCl, 0.015M-sodium citrate, pH7.0). Lysozyme (400μg/ml final concn.) and ribonuclease (50μg/ml final concn.) were then added and the cells incubated at 37°C for 30min. Trichloroacetic acid was then added to 5% (w/v) and the mixture left in ice for 10min; the precipitate was then collected on a nitrocellulose filter that was washed twice with 10ml of 5% (w/v) trichloroacetic acid and twice with 10ml of 0.5M-HCl before being dried at 70°C. Ribonuclease was prepared as a solution of 2mg/ml in 0.15M-NaCl, pH5.0, and heated at 80°C for 10min before use. This procedure is subsequently referred to as the 'standard assay procedure'.

In experiments where DNA was extracted for CsCl-density-gradient analysis, the cells were digested with lysozyme and ribonuclease as described above and then 4-aminosalicylate was added to 6% (w/v) along with an equal volume of a phenol–hydroxyquinoline solution (90g of phenol, 10ml of water and 0.1g of 8-hydroxyquinoline). To ensure uniform shearing of the DNA, phenol extracts were mixed for 3min on a Vortex mixer before separation of the aqueous and phenol phases by centrifugation at 7°C, 2700*g*, for 20min in the MSE 4L Mistral centrifuge ($r_{av.}$ 18cm). The upper aqueous phase was added directly to CsCl.

Preparative ultracentrifugation procedures. Density-gradient equilibrium-sedimentation studies were performed as previously described (Harris & Barr, 1969). Stock CsCl solution (4ml, 1.6g/ml) in 0.04M-potassium phosphate buffer, pH8.0, was mixed with 0.5ml of the DNA sample and the refractive index adjusted to 1.4010 at room temperature in an Abbé refractometer. The sample was placed in a 10ml polypropylene tube, then overlaid with 3ml of liquid paraffin and centrifuged in the MSE Superspeed 65 ultracentrifuge in the MSE 10×10ml fixed-angle rotor (rotor no. 59113) for 66h at 15°C and 100000*g* ($r_{av.}$ 6.0cm). Fractions (eight drops each) were collected by upward displacement of the CsCl gradient, directly on to glass-fibre filter discs, which were then immersed for 10min in baths of ice-cold 5% (w/v) trichloroacetic acid, 0.05M-HCl and ethanol. They were then dried and measured for radioactivity; 40–45 fractions were usually obtained from each gradient.

Zone centrifugation through sucrose was performed by preparing 5–20% (w/v) linear sucrose gradients at pH12.0 as previously described (Harris & Barr, 1969), centrifuging in the MSE Superspeed 65 ultracentrifuge in the 3×5ml SW rotor no. 59589 at 100000*g* for 90min at 5°C ($r_{av.}$ 6.4cm). Fractions were collected and analysed as described for CsCl-density-gradient centrifugation.

Radioactivity measurements. Dual-labelled samples were immersed in 2ml of scintillation fluid, which contained 5g of 2,5-dimethyloxazole and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene, and counted for radioactivity in the Intertechnique liquid-scintillation spectrometer model SL 30. There was 18% overlap of ^{14}C into the ^{3}H channel.

Results

Initial studies focussed attention on two aspects of DNA synthesis in *B. subtilis*. First, the apparent amount of DNA synthesis occurring during pulse-labelling of cells *in vivo* was found to vary according to the procedure used for measurement of DNA synthesis, and secondly, thymine and thymidine behaved differently as precursors for DNA synthesis.

Measurement of DNA synthesis

The effect of pretreatment of cell extracts with ribonuclease on the radioactivity incorporated from thymine or thymidine into DNA is described by Table 1. In both cases, radioactivity is incorporated into ribonuclease-sensitive material though to a much more pronounced degree with thymine as

Table 1. *Effect of ribonuclease on the incorporation of radioactivity into DNA*

Cell suspension (10ml) was grown to mid-exponential phase (2–3h), then divided into 0.5ml batches. Duplicate 0.5ml samples were then grown for 4min with either [6-^{3}H]thymidine (0.5μCi/ml) or [6-^{3}H]-thymine (2.5μCi/ml) and assayed as follows. Extraction procedure (i): trichloroacetic acid was added to the cell suspension to 6% (w/v), then the cells were left in ice for 30min. The resulting precipitate was collected on a nitrocellulose filter, which was then washed twice with 5% (w/v) trichloroacetic acid and twice with 10ml of 0.5M-HCl. The filter was dried and counted for radioactivity. Extraction procedure (ii): cells were analysed by the standard procedure described in the Experimental section. The table describes three separate experiments with cells at different stages of exponential growth, results being expressed as the mean of duplicate samples. The same batch of cells was used for Expt. (1) thymine and Expt. (1) thymidine.

Precursor	Expt. no.	Incorporation (c.p.m.)		Ribonuclease-sensitive incorporation (%)
Procedure	...	(i)	(ii)	
Thymidine	1	5897	5421	8
	2	9906	9074	8
	3	24065	20490	15
Thymine	1	2092	718	66
	2	3040	2146	28
	3	621	136	78

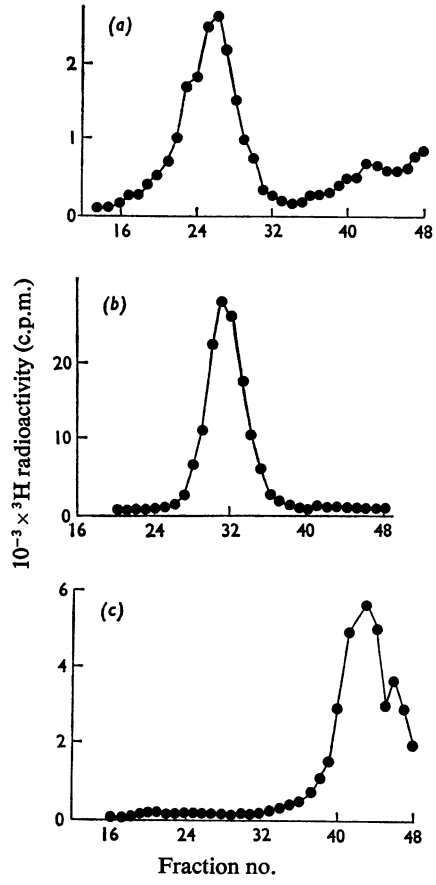


Fig. 1. *CsCl-density-gradient analysis of DNA and RNA radioactively labelled with [6-^{3}H]thymine, [6-^{3}H]thymidine and [5,6-^{3}H]uridine*

Cell culture (5ml) was grown to mid-exponential phase and 1ml batches were grown for 10min in the presence of (*a*) [6-^{3}H]thymine (2.5μCi/ml), (*b*) [6-^{3}H]thymidine (0.5μCi/ml) and (*c*) [5,6-^{3}H]uridine (0.5μCi/ml). DNA was extracted from the cells for CsCl-density-gradient analysis as described in the Experimental section except that treatment with ribonuclease was omitted. Centrifugation was performed for 66h at 15°C and 100000*g* ($r_{av.}$ 6.0cm), and fractions were collected as described in the Experimental section. Fraction 1 represents the top of the gradient. All of the radioactivity collected from the gradient was precipitable with trichloroacetic acid.

precursor. A similar phenomenon was found with alkali-sensitive trichloroacetic acid-insoluble material, but this gave poor reproducibility with duplicate samples. Because of this, digestion of cells with lysozyme and ribonuclease was adopted as standard for measurement of DNA synthesis.

Evidence that the ribonuclease-sensitive material is indeed RNA is presented in Fig. 1. With thymine as precursor, radioactivity is clearly recovered from within the CsCl gradients at the positions expected for both DNA and RNA (Fig. 1*a*, and compare with Fig. 1*c*), whereas the radioactivity incorporated from thymidine is almost exclusively within the DNA. The absolute amount of radioactivity recovered as RNA was highly variable and depended on the state of the growth of the cells. Nevertheless, there seems to be an efficient mechanism for the incorporation of thymine into RNA, presumably as uracil. In contrast, Barlati (1970) showed that in *B. subtilis* uridine can be recovered as cytosine in DNA. Clearly then, care must be exercised in the interpretation of results of experiments that use thymine and uridine as specific markers for DNA and RNA synthesis respectively.

Utilization of thymine and thymidine as precursors for DNA synthesis

There are reports of variation in the utilization of thymine and thymidine as precursors for DNA

Table 2. *Relative utilization of thymine and thymidine as precursors for synthesis of DNA*

Cell suspension (10ml) was grown to mid-exponential phase and duplicate 0.5ml samples were grown with either [6-^{3}H]thymine (2.5μCi/ml) or [6-^{3}H]thymidine (0.5μCi/ml) for 2min, before and after irradiation of the cells with u.v. light. Radioactivity incorporated was measured by the standard procedure described in the Experimental section. The dose of u.v. light was such that there was a 20% survival of cells. The table describes two separate experiments with different batches of cells, results being expressed as the mean of duplicate samples.

		Incorporation in 2min (c.p.m.)	
Precursor	Expt. no.	Before irradiation	After irradiation
Thymine	1	1444	730
	2	5094	2959
Thymidine	1	3997	536
	2	4607	900

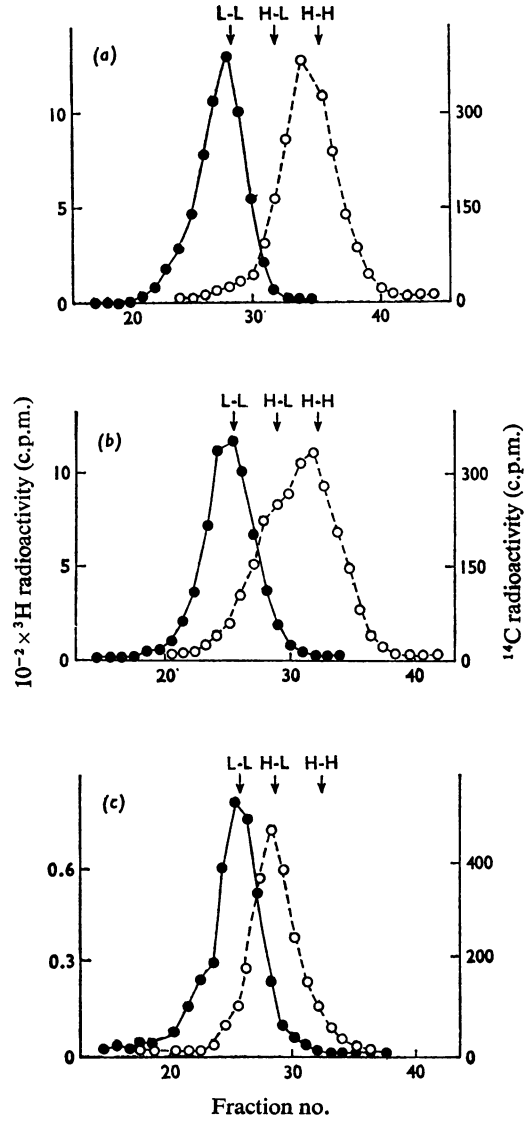
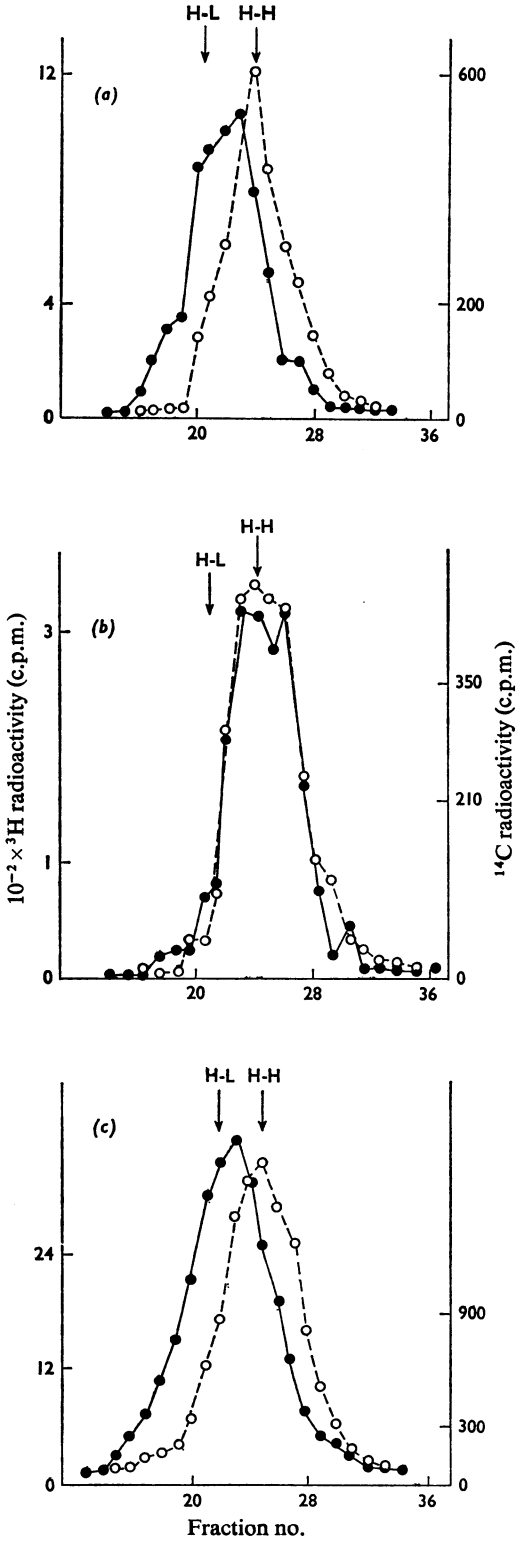


Fig. 2. *Kinetics of transfer of DNA from the dense to the hybrid position in CsCl-density-gradient analysis*

DNA was extracted from cells labelled with [2-^{14}C]-thymidine, ^{15}N and ^{2}H as detailed in the Experimental section. The density of this DNA (○) compared with added marker DNA containing [6-^{3}H]thymidine, ^{14}N and ^{1}H (●) is represented in (*a*). The profile resulting after subsequent growth of cells for 1h (*b*) or 3h (*c*) is compared also with added marker ^{3}H-labelled DNA; 40–45 fractions were obtained from each gradient, all other conditions being as detailed for Fig. 1. The position characteristic of the dense species is referred to as H-H, that of the hybrid as H-L and the added marker DNA as L-L.



synthesis in *E. coli* (Werner, 1971; Rosenbaum-Oliver & Zamenhof, 1972), and Billen *et al.* (1971) have shown that in *B. subtilis* thymine is preferentially incorporated into the DNA of cells that have been frozen and thawed after u.v. irradiation, but thymidine is used preferentially before irradiation.

Two experiments measuring the incorporation of thymine or thymidine into the DNA of cells before and after u.v. irradiation (Table 2) demonstrate this phenomenon *in vivo*. These results show that the u.v. irradiation decreases the incorporation of thymine into DNA by 40–50%, whereas incorporation of thymidine is decreased by 80–90% by the same dose of radiation. If it is assumed that the same amount of DNA synthesis is actually occurring irrespective of the radioactive precursor, then it must be concluded that thymine and thymidine are used differentially as precursors for this DNA synthesis. Further, if we consider the DNA synthesis before irradiation to be 'replicative-type' and the synthesis after irradiation to be 'repair-type', then thymine seems to be used as a preferential precursor for 'repair' synthesis and thymidine a precursor for 'replicative' synthesis. This is in agreement with the conclusion of Billen *et al.* (1971).

Occurrence of 'replicative' and 'repair' DNA synthesis during normal growth of cells

To distinguish between 'replicative' and 'repair' DNA synthesis, cells of *B. subtilis* were prepared in which their DNA was uniformly labelled with [2-^{14}C]-thymidine, ^{15}N and ^{2}H. The density of this DNA is approx. 1.710g/ml, compared with 1.703g/ml for normal DNA of *B. subtilis* (Fig. 2*a*). This dense DNA which possesses ^{15}N and ^{2}H in both strands, is referred to as H-H DNA and the control marker

Fig. 3. *CsCl-density-gradient analysis of cells pulse-labelled with* [6-^{3}*H*]*thymidine*

A batch (2ml) of frozen cells, previously labelled with [2-^{14}C]thymidine, ^{15}N and ^{2}H as described in the Experimental section, was grown in 10ml of growth medium for 60min; then 1ml samples were grown with [6-^{3}H]thymidine (0.5μCi/ml) for 10min (*a*) before u.v. irradiation, (*b*) after u.v. irradiation and (*c*) without u.v. irradiation but in the presence of caffeine (4mg/ml final concn.). The arrows mark the position expected for dense (H-H) and hybrid (H-L) DNA respectively. ○, ^{14}C radioactivity. ●, ^{3}H radioactivity. All other conditions were as detailed for Fig. 1.

DNA as L-L (^{14}N and ^{1}H in both strands). On subsequent growth of these density-labelled cells in ^{14}N- and ^{1}H-containing medium, the DNA was gradually transferred to a less-dense position when analysed by CsCl-density-gradient centrifugation (Figs. 2*b* and 2*c*). After 1 h of growth (Fig. 2*b*) 10–30% of the DNA was recovered in a position of intermediate density between the H-H DNA and the control marker L-L DNA. This is due to the replication of the dense DNA giving rise to daughter molecules possessing one strand of parental DNA (containing ^{15}N and ^{2}H) and one strand of newly replicated DNA (containing ^{14}N and ^{1}H). The resulting DNA molecules are hybrid, designated as H-L. These profiles are characteristic of semiconservative 'repli-

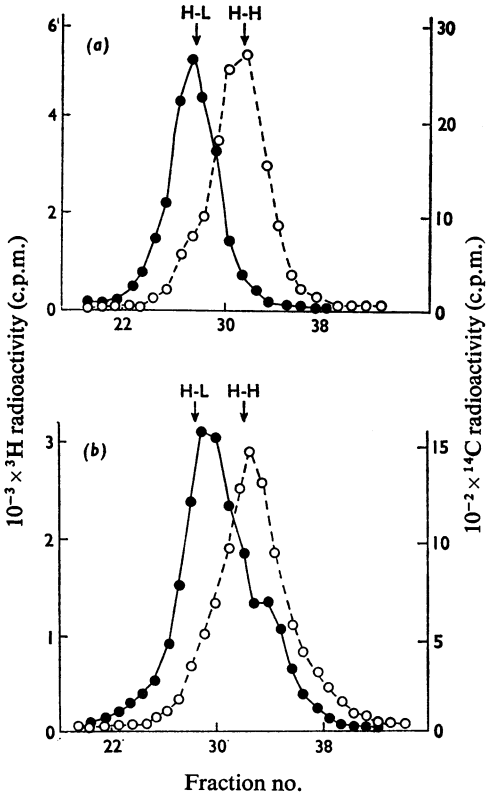
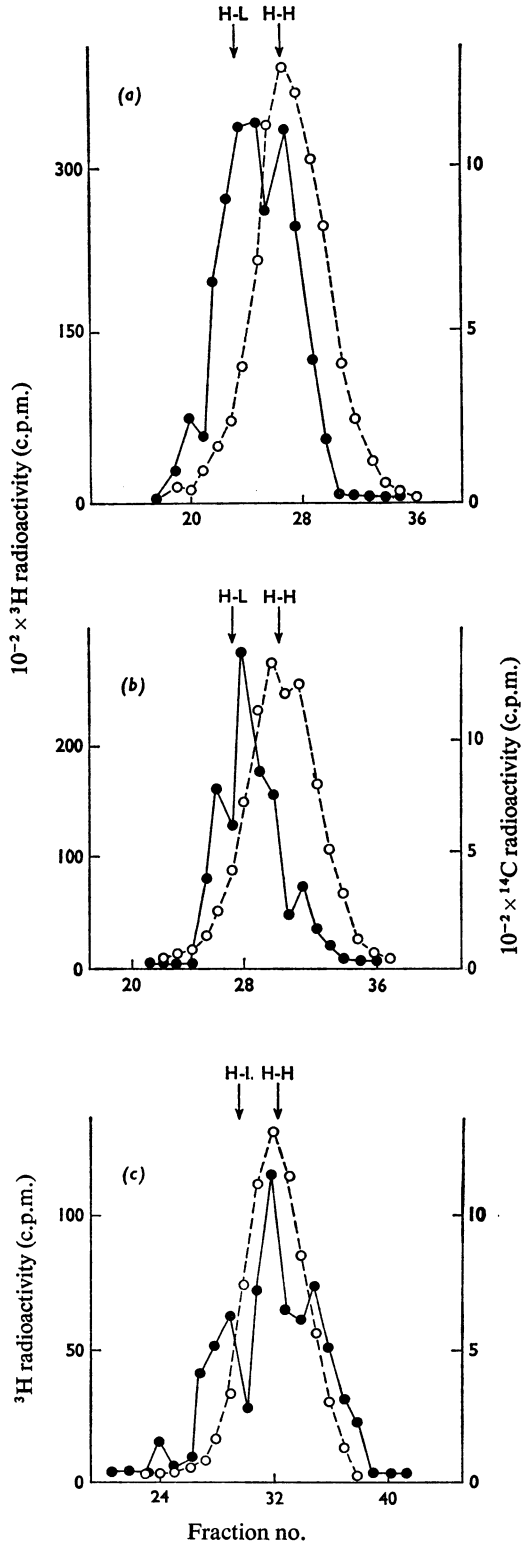


Fig. 4. *CsCl-density-gradient analysis of DNA extracted from cells radioactively labelled with [6-^{3}H]thymine and [6-^{3}H]thymidine*

Stock cells, grown for 60 min as described for Fig. 3, were divided into 1 ml samples and grown for 10 min with (*a*) [6-^{3}H]thymidine (0.5 μCi/ml) or (*b*) [6-^{3}H]-thymine (2.5 μCi/ml). The arrows mark the positions expected for H-H and H-L DNA species respectively. ○, ^{14}C radioactivity. ●, ^{3}H radioactivity.



cative' DNA synthesis. After 3h of growth all the DNA has been replicated.

To distinguish between 'repair' and 'replicative' synthesis the cells were grown for 60–90min so that 10–30% of the DNA was recovered within the hybrid (H-L) region that is replicated. In this time it is reasonable to assume that most of the cells had initiated DNA replication. These cells were then pulse-labelled with either thymine or thymidine and the distribution of the incorporated radioactivity in the DNA was analysed in CsCl density gradients. That this technique distinguishes between the conventional semiconservative 'replicative' synthesis on the one hand and non-conservative 'repair' synthesis on the other is demonstrated by Fig. 3. All the radioactivity incorporated before irradiation of the cells was recovered in the hybrid (H-L) position (Fig. 3*a*), whereas after irradiation the radioactivity was located extensively in the dense position (Fig. 3*b*).

A comparison of the distribution of pulses of thymine and thymidine within CsCl density gradients is presented in Fig. 4. Although the radioactivity incorporated from thymidine was exclusively within the hybrid (H-L) position (Fig. 4*a*), the radioactivity incorporated from thymine (Fig. 4*b*) was located in the positions characteristic of both 'replicative' (H-L) and 'repair' synthesis (H-H). During normal growth there seems to be simultaneous 'replicative' and 'repair' synthesis, though the latter is detectable only if thymine is used as precursor. Although cells are usually grown in the presence of thymidine (5μg/ml), at the time of addition of radioactive precursors essentially all of this material has been converted into thymine.

It should be stressed that these cells were not exposed to any agents likely to introduce artificial lesions into the DNA and hence one would not expect significant 'repair' synthesis owing to the excision–repair of lesions in the DNA. As discussed below, this 'repair' synthesis may not be related to the excision–repair of lesions, but since this type of synthesis exhibits a behaviour analogous to that observed during the excision–repair of lesions it is here referred to as 'repair' synthesis.

Fig. 5. *CsCl-density-gradient analysis of cells labelled with [6-^{3}H]thymine in the presence of caffeine or HOPhNNUra*

Stock cells were grown for 60min as described for Fig. 3 and then 1ml samples were grown for 4min with [6-^{3}H]thymine (50μCi/ml) (*a*) in the absence of inhibitor, (*b*) in the presence of caffeine (4mg/ml final concn.) and (*c*) in the presence of HOPhNNUra (10μg/ml final concn.). The arrows mark the positions expected of H-H and H-L DNA species respectively. ○, ^{14}C radioactivity. ●, ^{3}H radioactivity.

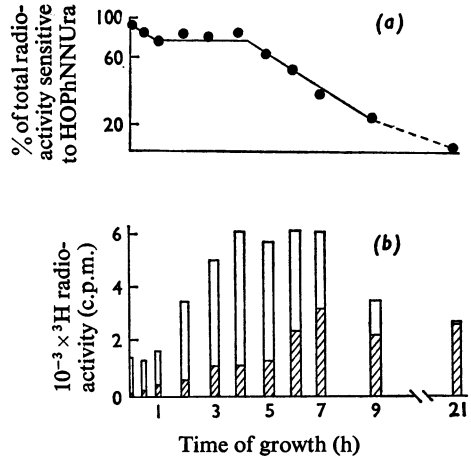


Fig. 6. *Effect of HOPhNNUra on pulse-labelling of DNA throughout the growth cycle*

Cell suspension (50ml) was grown at 37°C with shaking in a 250ml Ehrlenmeyer flask. Under these growth conditions cells were in mid-exponential phase in 2–3h and entered stationary phase at about 5h. At 1h intervals throughout growth 2ml samples of cells were removed and duplicate 0.5ml portions were grown for 2min in the presence of [6-^{3}H]-thymidine (2.5μCi/ml) with HOPhNNUra at a concentration of 10μg/ml. Duplicate portions were similarly labelled in the absence of HOPhNNUra. Results are expressed as the mean of duplicate samples. Gross radioactivity incorporated into trichloroacetic acid-insoluble DNA is shown in (*b*), and the percentages of each pulse sensitive to HOPhNNUra are represented in (*a*). □, ^{3}H in control cell DNA; ■, ^{3}H in DNA of cells treated with HOPhNNUra.

Effect of caffeine and HOPhNNUra on 'replicative' and 'repair' DNA synthesis

If there are two distinguishable types of DNA synthesis, it should be possible specifically to inhibit either type. Caffeine has been described as an inhibitor of repair reactions within the DNA of both *E. coli* (Lieb, 1961) and of mammalian cells (Cleaver, 1969). The effect of caffeine on the CsCl-density-gradient profile of a short pulse of [6-^{3}H]-thymidine (Fig. 3*c*) is minor, possibly eliminating a small amount of 'repair' synthesis detected at fractions 27 and 28 in Fig. 3(*a*). However, caffeine has a profound effect on the sedimentation profile resulting from a 4min pulse of thymine; for example, Fig. 5(*b*) demonstrates that caffeine selectively eliminates the 'repair' DNA synthesis.

Brown (1971) presented convincing evidence that in *B. subtilis* HOPhNNUra is a specific inhibitor of DNA replication. The effect of this compound on the CsCl-density-gradient profile after pulse-labelling with [6-^{3}H]thymine shows (Fig. 5*c*) that although incorporation is low HOPhNNUra does seem to eliminate the 'replicative' DNA synthesis preferentially. Neither caffeine nor HOPhNNUra exert a substantial effect on the uptake of thymine or thymidine into the cells.

Measurement of the amount of 'replicative' and 'repair' DNA synthesis occurring throughout the growth cycle

On the basis of the apparent specificity of inhibition of 'replicative' and 'repair' DNA synthesis by HOPhNNUra and caffeine respectively, the effectiveness of HOPhNNUra throughout the growth cycle was examined (Fig. 6). Two points are worthy of note: first, HOPhNNUra is ineffective against the residual DNA synthesis occurring in stationary-phase cells; further, throughout the growth cycle there seems to be a significant amount of HOPhNNUra-resistant (i.e. 'repair') synthesis occurring, the absolute amount increasing somewhat in the stationary phase.

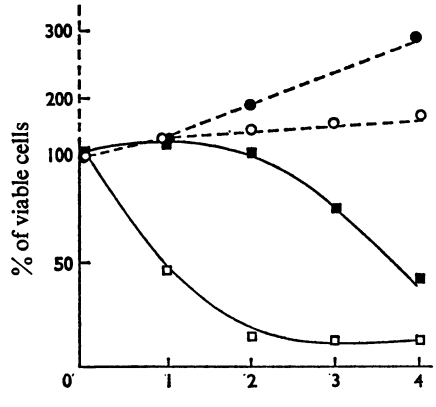


Fig. 7. *Effect of caffeine and HOPhNNUra upon cell viability*

Cell suspension (30ml) was grown to mid-exponential phase as described for Fig. 6 and then divided into 5ml batches. Growth was continued in the presence of caffeine at 2mg/ml (○), caffeine at 4mg/ml (□), HOPhNNUra at 1μg/ml (●), HOPhNNUra at 10μg/ml (■). At 1h intervals, the cells were serially diluted on tryptose blood agar and incubated. Results are expressed as the mean of triplicate plate counts.

Effect of caffeine and HOPhNNUra on cell viability

Prolonged contact of *B. subtilis* with caffeine and HOPhNNUra at concentrations at which they exert their selective effects on DNA synthesis

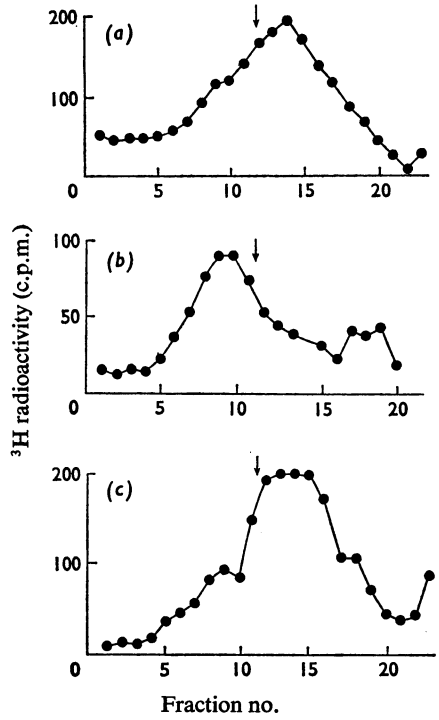


Fig. 8. *Alkaline sucrose density gradients of DNA extracted from cells grown in the presence of caffeine or HOPhNNUra*

Cell suspension (20ml) was grown for 2h at 37°C in the presence of [6-^{3}H]thymidine (0.1μCi/ml). The cells were then sedimented by centrifugation and washed twice with minimal salts medium before resuspension in 20ml of fresh growth medium. Samples (5ml) of cells were then grown for 3h (*a*) in the absence of inhibitor, (*b*) in the presence of caffeine (4mg/ml) and (*c*) in the presence of HOPhNNUra (10μg/ml). The cells were then collected by centrifugation and a lysate was prepared as described in the Experimental section. The resulting lysate was then dialysed overnight at 4°C against standard saline–citrate, and then carefully layered on a 5ml 5–20% (w/v) sucrose density gradient, pH12.0. Then 0.1vol. of 0.5M-NaOH was added to the top of the gradient, and after 15min at room temperature, tubes were centrifuged as described in the Experimental section. Fractions (20–23) were collected, fraction 1 representing the top of the tube. The arrow marks the position of added ^{14}C-labelled *E. coli* DNA.

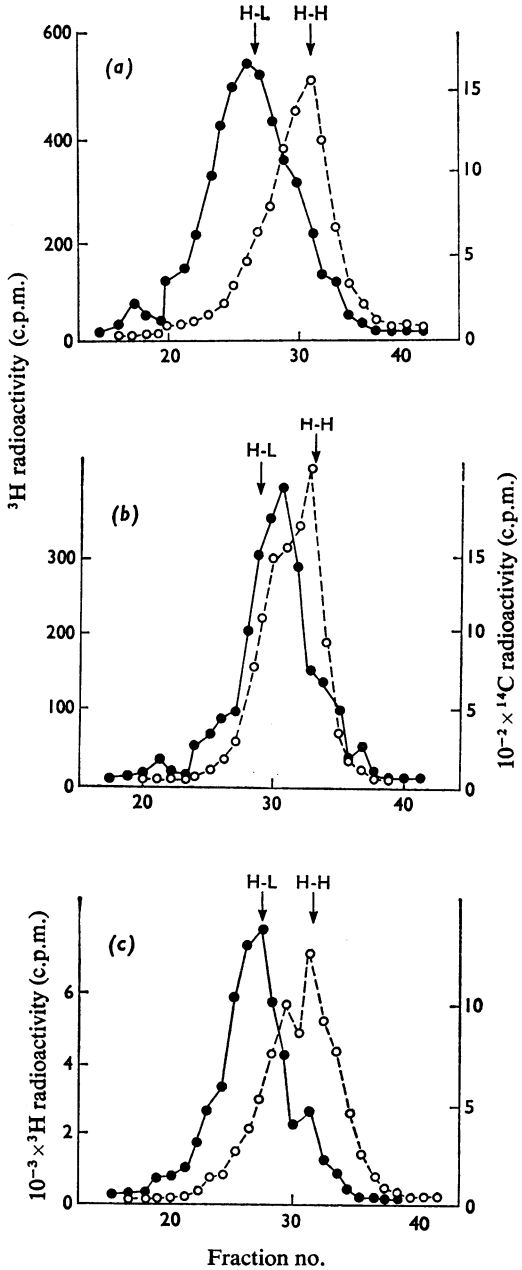


Fig. 9. *CsCl-density-gradient analysis of 'repair' DNA synthesis during continued growth of the cells*

Stock cells were grown for 60min as described for Fig. 3 and then 1ml samples were grown with [6-^{3}H]thymine (50μCi/ml) as follows. (*a*) Cells were pulse-labelled for 10min in the presence of HOPhNNUra (10μg/ml), and then both radioactivity and inhibitor removed from the cells by washing on a nitrocellulose filter four times with minimal salts medium. The cells were then resuspended in fresh medium and growth was continued for a further 10min before extraction of the DNA. (*b*) Cells were labelled for 10min in the presence of HOPhNNUra and the DNA was extracted immediately. (*c*) Cells were pulse-labelled for 10min in the absence of inhibitor. The arrows mark the positions expected for H-H and H-L DNA respectively. ○, ^{14}C radioactivity; ●, ^{3}H radioactivity.

Table 3. *Effect of caffeine and HOPhNNUra on DNA synthesis before and after irradiation of cells with u.v. light*

Cell suspension (10ml) was grown to mid-exponential phase and duplicate 0.5ml samples were removed and grown with [6-^{3}H]thymine (2.5μCi/ml) for 4min. Radioactivity incorporated into DNA was measured as described in the Experimental section. The amount of radioactivity (%) compared with control samples was measured in the presence of either caffeine (4mg/ml final concn.) or HOPhNNUra (10μg/ml final concn.). The table describes two separate experiments, the radioactivity incorporated being of the same order as in Table 2.

	Inhibition (%)			
	Before irradiation		After irradiation	
Expt. no.	Caffeine	HOPhNNUra	Caffeine	HOPhNNUra
1	61.4	89.5	49.3	45.8
2	55.0	67.0	59.0	40.2

shows that caffeine is highly bacteriocidal, whereas HOPhNNUra is initially bacteriostatic (Fig. 7). Moreover incubation of cells with caffeine causes the accumulation of single-strand breaks within the DNA, whereas HOPhNNUra has no such effect (Fig. 8).

Relationship between 'replicative' and 'repair' DNA synthesis during normal growth of cells

The two inhibitory effects of caffeine and HOPhNNUra on DNA synthesis within the same culture of cells before irradiation are not simply additive (Table 3), implying that either the two inhibitors do not have a specific effect or that the 'replicative' and 'repair' DNA synthesis are interrelated. The latter seems more likely, since if the cells were previously exposed to u.v. light the effects of the two inhibitors were additive.

A further indication of the interdependence of

'replicative' and 'repair' DNA synthesis is provided by examination of the fate of the 'repair' synthesis on continued growth of the cells (Fig. 9). When cells were given a 10min pulse with [6-^{3}H]thymine the majority of the radioactivity is within the hybrid (H-L) position, indicative of 'replicative' synthesis (Fig. 9*c*), with a small component in the dense (H-H) position. If the cells were given a 10min pulse in the presence of HOPhNNUra (Fig. 9*b*) the incorporated radioactivity was more significantly within the dense position. Fig. 9(*a*) is the profile obtained if cells were pulse-labelled for 10min in the presence of HOPhNNUra, which was then washed away and growth of the cells continued for a further 10min in the presence of non-radioactive thymidine. Unfortunately, in such experiments it was not found possible to deplete completely the nucleotide pool and hence approx. 30% more radioactivity was incorporated during the period of the chase. Nevertheless, it seems reasonable to conclude that a substantial proportion of the 'repair' synthesis is subsequently rapidly recovered within the replicated fraction.

Discussion

Evidence is presented which suggests that in *B. subtilis* thymine and thymidine behave differently as precursors for DNA synthesis, thymine serving as a preferential precursor for 'repair' DNA synthesis and thymidine for 'replicative' synthesis. Further, these two types of DNA synthesis seem to occur simultaneously during normal growth. In the present context, 'replicative' and 'repair' DNA synthesis are defined by the observed profiles within CsCl density gradients and are not synonymous with the overall process of DNA replication and excision–repair of artificial lesions (see the text).

In contrast, Werner (1971) concluded that in *E. coli* thymine is an immediate precursor for DNA replication and thymidine is the preferred precursor for excision–repair reactions. The preferential utilization of precursors in *B. subtilis* is detectable within normally growing cells only during short pulses of synthesis; over a longer time-scale (greater than 20min) no substantial difference in profiles in CsCl density gradients is observed. Clearly then, thymine and thymidine are interconvertible. These observations raise the possibility of different intermediates in the pathways of 'replicative' and 'repair' DNA synthesis, and indeed Werner (1971) suggested the existence of a compound dTMP-X exclusive to DNA replication. Preliminary investigations showed that in *B. subtilis* thymine is more easily incorporated into dTTP than is thymidine, although attempts to detect consistent differential labelling of nucleotide pools have been unsuccessful (Fraser *et al.*, 1972). This tends to suggest that the explanation for differential utilization of precursors stems from other factors, such as compartmentalization of precursors (Billen *et al.*, 1971) or the direct absorption of thymidine into a multienzyme complex. This differential utilization would presumably extend also to differential utilization of bromouracil and bromodeoxyuridine, compounds often used as density labels in the study of DNA replication.

Several findings suggest that two types of DNA synthesis can be distinguished. Cells which have replicated some 20% of their DNA still incorporate radioactivity into the dense (H-H) species (Fig. 4). If only 'replicative' synthesis were occurring, such radioactivity should have represented elongation of the previously synthesized 20% of the DNA and should have been exclusively within the hybrid position. The 'repair' synthesis can further be distinguished from 'replicative' by its preferential utilization of thymine as precursor and its inhibition by caffeine (Fig. 5). Also, this 'repair' synthesis occurs at a relatively constant rate throughout the growth cycle and increases during the stationary phase.

Two observations suggest a relationship between the 'replicative' and 'repair' synthesis. First, Table 3 indicates that although the effects of caffeine and HOPhNNUra are additive after cells have been irradiated, no such additivity occurs before irradiation. Secondly, the CsCl-density-gradient profiles of Fig. 9 suggest that a proportion of the 'repair' synthesis is rapidly transferred to the 'replicative' position. Because of this interrelationship it is not possible to assess 'repair' synthesis which could be occurring within the DNA that has already been replicated. Nevertheless, it seems reasonable to conclude that most of the 'repair' synthesis, or more accurately the 'repair' synthesis occurring in exponential phase cells, is within DNA that has yet to be replicated, that is 'repair' synthesis is a 'pre-fork' synthesis.

Several possible roles for 'repair' synthesis are worthy of consideration. It may represent a true repair synthesis, being responsible for the repair of defects introduced by the previous round of replication. This seems unlikely, since it is possible to increase the overall amount of 'repair' synthesis under physiological conditions which would not introduce base-pair lesions into DNA, that is during growth to competence (Harris & Barr, 1971) and after thymine deprivation (Pauling & Hanawalt, 1965).

Thus it is unlikely that 'repair' synthesis occurring in exponential phase cells is due to the repair of base-pair lesions, though such an explanation may be feasible for 'repair' synthesis in stationary phase. Alternative roles have previously been suggested (Harris, 1973) and it is noteworthy that the behaviour of 'repair' synthesis in relation to the replication fork is in good agreement with the 'pre-fork' model for

DNA replication suggested by Haskell & Davern (1969).

Finally, consideration should be given to the effects of caffeine and HOPhNNUra on cell viability. At concentrations at which they exert their specific effects caffeine is highly lethal whereas HOPhNNUra is initially bacteriostatic. This can be reconciled with the finding that 'repair' synthesis is a 'pre-fork' synthesis, since failure to effect this synthesis could be considered to shear the DNA double helix on replication, a lethal event. This is supported to some extent by the finding that caffeine introduces single-strand breaks into DNA (Fig. 9). A model invoking single-strand breaks has been proposed by Grigg (1968) to explain caffeine-induced death in *E. coli*.

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