Effect of Thymidine Auxotrophy, Thymidine Starvation and Nalidixic Acid Inhibition on the Properties of DNA Labelled by a Pulse of [^3H]Thymidine in *Staphylococcus aureus*

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(Received 26 February 1980)

The labelling of DNA by pulse/chase experiments in *Staphylococcus aureus* has been investigated, analysing the products by alkaline sucrose velocity centrifugation. In *S. aureus* NCTC 8325 a short (60 s) pulse of [^3H]thymidine labels both small (10 to 20 S) fragments and DNA that co-sediments with long-term label. In a thymidine-requiring derivative, 8325*thy*, most pulse label is incorporated into small fragments. In both bacterial strains small fragments can be chased into high molecular weight DNA. Thymidine starvation of 8325*thy* prior to pulse labelling results in smaller fragments (4 to 10S) being labelled. In a subsequent chase with unlabelled thymidine this label is incorporated into high molecular weight DNA, although more slowly than in the absence of thymidine starvation. The fact that nalidixic acid, an antibiotic which specifically inhibits DNA replication in *S. aureus*, does not inhibit the [^3H]thymidine incorporation immediately after thymidine starvation and that nalidixic acid slows down the increase in size of pulse-labelled fragments through inhibition of DNA synthesis suggests that thymidine starvation results in changes at the replication fork. The possible nature of these changes is discussed. It is proposed that one of the results of thymidine starvation is to cause a long-lived gap between DNA synthesized before starvation and DNA synthesized after starvation.

INTRODUCTION

Investigation of the biochemistry of DNA replication in *Staphylococcus aureus* has been confined mainly to the study of plasmid DNA replication (Novick *et al.*, 1975; Sheehy & Novick, 1975). To complement the knowledge gained in this area we have attempted to study various aspects of both the genetics and biochemistry of bacterial chromosome replication in *S. aureus* (Thomas & Dyke, 1978*a, b*). An interesting aspect of the biochemistry of bacterial chromosome replication is the nature of nascent DNA synthesized at the replication origin. One way of looking at this DNA and its conversion into normal chromosomal-sized fragments is through experiments in which DNA is pulse-labelled with radioactive DNA precursors. In *Escherichia coli* and *Bacillus subtilis*, the early demonstrations of 'Okazaki type' nascent DNA fragments have been partly reinterpreted in the light of recent results which indicate that excision repair of uracil (Tye *et al.*, 1977; Tamanoi & Okazaki, 1978) may accentuate the apparent discontinuity of replication. The exact balance between these processes in producing 'Okazaki fragments' in both *E. coli* and *B. subtilis* varies with experimental conditions and is not fully resolved at present (Tamanoi &

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Okazaki, 1978; Anderson, 1978; Kwoh *et al.*, 1979). In seeking optimum conditions for labelling nascent DNA with [^3H]thymidine in *S. aureus*, we found qualitative differences between the sedimentation characteristics of pulse-labelled DNA in a thymidine-independent and a thymidine-dependent strain. The latter gave almost exclusive labelling of small DNA fragments especially after thymidine starvation as recommended in early experimental protocols for investigating nascent DNA (Okazaki, 1974). However, these conditions are the very ones most likely to cause artefacts due to uracil incorporation and excision repair. We have therefore investigated the result of thymidine starvation on subsequent [^3H]thymidine incorporation.

Exploratory experiments with nalidixic acid indicated that this antibiotic is a specific inhibitor of DNA replication in *S. aureus*. We have therefore used it to investigate changes in [^3H]thymidine incorporation brought about by thymidine starvation. Recent work with *E. coli* has demonstrated that nalidixic acid acts as an inhibitor of the enzyme DNA gyrase (Sugino *et al.*, 1977; Gellert *et al.*, 1977). It is likely, therefore, that inhibition of DNA replication by nalidixic acid is the result of a block in the unwinding of the DNA helix in front of the replication complex. On the other hand, different types of experiments had led to the hypotheses that nalidixic acid blocked the process of joining of nascent DNA fragments (Diaz *et al.*, 1975; Crumplin & Smith, 1976) or the initiation of synthesis of these fragments (Brewin, 1977). Results described here show that in *S. aureus* nalidixic acid does not inhibit joining of nascent DNA fragments but appears to exert its action directly on synthesis of DNA and therefore possibly on the forward movement of the replication fork. After thymidine starvation there is a burst of incorporation that is resistant to nalidixic acid. The fact that the chase of this material into high molecular weight DNA is inhibited by nalidixic acid suggests that a gap is formed between DNA synthesized before and after starvation.

METHODS

Bacterial strains, media and cultural conditions. The bacterial strains used in this study were *Staphylococcus aureus* NCTC 8325 and a thymidine-requiring derivative of this strain obtained by the method of Stacey & Simson (1965). Either CY medium (Novick, 1963) or synthetic broth AOAC (Difco) were used for liquid or solid media. Stock cultures were grown on AOAC agar at 30 °C and stored at 4 °C. For the experiments described, inocula were grown for 12 h at 30 °C and then diluted into fresh, pre-warmed medium to about 3×10^7 colony-forming units (c.f.u.) ml^{-1} and grown to the required bacterial density. Cell densities were determined by measuring the absorbance at 675 nm (A_{675}). For normal growth of 8325*thy*, media were supplemented with thymidine ($10 \mu\text{g ml}^{-1}$).

Incorporation of radioactive precursors of DNA into whole bacteria. For the determination of incorporation of [$6\text{-}^3\text{H}$]thymidine or [$2\text{-}^{14}\text{C}$]thymidine into bacteria, 0.5 ml samples were mixed with 0.5 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) and after 30 to 180 min at 0 °C samples were filtered on to 2.1 cm diam. Whatman GF/C filters, washed with 3×5 ml ice-cold 5% (w/v) TCA and 4×5 ml ethanol and then dried at 80 °C *in vacuo*.

For labelling of cultures not starved of thymidine, the bacterial culture, grown to about 5×10^8 c.f.u. ml^{-1} ($400 \mu\text{g}$ bacterial dry wt ml^{-1}) at 30 °C in AOAC medium, was centrifuged, the pellet was washed with AOAC medium containing $100 \text{ ng thymidine ml}^{-1}$ and after resuspension at 30 or 20 °C in AOAC ($+100 \text{ ng thymidine ml}^{-1}$) an equal volume of AOAC containing [$6\text{-}^3\text{H}$]thymidine was added to give the desired specific activity of thymidine. For thymidine starvation, a similar bacterial culture was centrifuged, washed with thymidine-free AOAC, resuspended in the same medium at 20 °C and incubated at this temperature for up to 60 min to deplete the internal thymidine pool. Labelling was then either by dilution with an equal volume of AOAC containing [$6\text{-}^3\text{H}$]thymidine [$2 \mu\text{Ci ml}^{-1}$ (74 kBq ml^{-1}), 20 ng ml^{-1}] or by the addition of a small quantity of [$6\text{-}^3\text{H}$]thymidine solution to give $1 \mu\text{Ci ml}^{-1}$ (37 kBq ml^{-1}) and 10 ng ml^{-1} . For long-term labelling with [$2\text{-}^{14}\text{C}$]thymidine, this nucleotide was added to bacteria growing in AOAC ($+10 \mu\text{g thymidine ml}^{-1}$) to give a concentration of $1 \mu\text{Ci ml}^{-1}$ (37 kBq ml^{-1}).

The shift from 30 to 20 °C for [^3H]thymidine labelling was to provide internal consistency in all experiments since this temperature was used for investigating 'discontinuous' synthesis of DNA.

Analysis of nascent DNA by alkaline sucrose sedimentation. To halt DNA metabolism rapidly so that [$6\text{-}^3\text{H}$]thymidine incorporated into nascent DNA could be investigated, samples were withdrawn into an

equal volume of ice-cold solution containing 75% (v/v) ethanol, 2% (w/v) phenol, 21 mM-sodium acetate (pH 5.3) and 2 mM-EDTA (Okazaki, 1974).

After washing with a solution containing 0.15 M-NaCl and 17 mM- NaN_3 , the bacteria were resuspended at a density of 5×10^8 to 10^{10} c.f.u. ml⁻¹. The bacterial suspension (0.15 ml) was mixed with 0.15 ml lysostaphin solution (about 300 $\mu\text{g ml}^{-1}$ in 0.1 M-NaCl, 50 mM-EDTA, pH 7.0) and incubated at 18 °C for 15 min. A solution (0.2 ml) of 1% (w/v) Brij 58 and 0.4% (w/v) sodium dodecyl sulphate in 0.2 M-NaOH was added and the final volume of 0.5 ml was treated at 70 °C for 10 min. After cooling on ice for 2 min, 0.2 ml samples were placed on the top of 4.9 ml gradients which consisted of 4.4 ml of a 5 to 20% (w/v) linear sucrose gradient and, at the bottom, 0.6 g CsCl in 0.5 ml 50% (w/v) sucrose. The whole gradient also contained 0.7 M-NaCl, 0.3 M-NaOH and 1 mM-EDTA. The CsCl solution at the bottom was present to float non-lysed cells; denatured DNA sedimented through this solution. Gradients were centrifuged in polyallomer tubes in a Spinco SW50.1 rotor at 18 °C for 3 h 25 min at 35000 rev. min⁻¹ and then sampled either by collecting drops through a needle puncturing the bottom of the tube or by sucking 0.1 ml fractions from the top of the gradient with a syringe. Fractions were collected on to strips of Whatman GF/B paper, precipitated by immersion in ice-cold TCA and then washed with ice-cold TCA and ethanol and dried at 80 °C *in vacuo*.

The sedimentation of long-term labelled DNA under the same conditions was determined for a culture that had been grown for at least two generations in the presence of radioactive thymidine at 30 °C in AOAC medium. In some cases the culture that was later given a short-term label was used but alternatively the long-term label was in a parallel culture which was then mixed with the pulse-labelled bacteria prior to lysis.

To provide a calibration system for determining approximate sedimentation values, ³²P-labelled bacteriophage T5 DNA was denatured and sedimented under identical conditions (Abelson & Thomas, 1966).

To test whether the material precipitated by ice-cold 5% (w/v) TCA and labelled with [6-³H]thymidine was DNA, pooled fractions from alkaline sucrose gradients were neutralized with HCl. Samples (0.1 ml) were subjected to different treatments: alkali (0.3 M-NaOH; 37 °C; 60 min); RNAase (pancreatic RNAase I and RNAase T₁, each 100 $\mu\text{g ml}^{-1}$ in 0.1 M-Tris, pH 7.5, and 0.1 M-EDTA; 37 °C; 60 min); pronase (1 mg ml⁻¹; then as for RNAase); DNAase (50 $\mu\text{g ml}^{-1}$ in 0.15 M-NaCl, 0.1 M-Tris, pH 7.0, and 40 mM-MgSO₄; 37 °C; 60 min); hot TCA (5%, w/v; 70 °C; 30 min). The effect of these treatments on the total radioactivity precipitated by ice-cold 5% (w/v) TCA was determined.

Measurement of radioactivity. Samples of ice-cold TCA precipitated material, on glass fibre paper, washed and dried as above, were immersed in 2.5 ml of 5% (w/v) 2-(4'-*tert*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole (butyl-PBD) in toluene and counted in a liquid scintillation counter.

Chemicals and enzymes. [2-¹⁴C]Thymidine and [6-³H]thymidine were from The Radiochemical Centre, Amersham. Lysostaphin was from Beckton Dickinson; RNAase, DNAase (Grade I) and Brij 58 were from Sigma. Pronase and nalidixic acid were from Calbiochem. All other chemicals were reagent grade from standard sources. ³²P-labelled bacteriophage T5 was a gift from Mr R. D. Everett.

RESULTS

Labelling of nascent DNA with [³H]thymidine in S. aureus 8325 and 8325thy

To pulse label DNA, cultures of *S. aureus* 8325 and 8325*thy* that had been grown at 30 °C in the presence of thymidine were centrifuged and the pellets were resuspended in medium containing high specific activity [6-³H]thymidine. Under the conditions used the incorporation of [³H]thymidine by 8325*thy* was similar or up to 30% lower than for 8325. Between 5 and 15% of added [³H]thymidine, depending on the cell density, was incorporated during the pulse labelling period. When the pulse was not chased, incorporation was generally linear until approximately 60% of the [³H]thymidine was incorporated and then it continued at a slightly reduced rate to a plateau of 80 to 90% of total label. After 60 s, an excess of non-radioactive thymidine was added. Approximately 2×10^4 c.p.m. of [³H]-thymidine were incorporated per 8×10^8 bacteria during the pulse and TCA-precipitable radioactivity increased two- to threefold during the 10 min chase period. Bacterial lysates were analysed on alkaline sucrose velocity gradients to determine the sedimentation profile of TCA-precipitable radioactivity (Fig. 1*a, b*). More than 90% of the TCA-precipitated radioactivity was DNA, based on its sensitivity to DNAase and hot TCA but resistance to RNAase, pronase and alkali. With strain 8325, the 60 s pulse label was approximately equally distributed between fast-sedimenting material (> 40S) and slower-sedimenting material (< 40S) with a peak at 10 to 55S. Approximately 25% of the incorporated label

Fig. 1. Sedimentation analysis of pulse-labelled DNA prepared from *S. aureus* 8325 (a) or 8325thy (b). Bacteria were grown in AOAC medium (+10 μg thymidine ml^{-1}) at 30 °C, pelleted and labelled for 60 s with [^3H]thymidine (1 $\mu\text{Ci ml}^{-1}$, 10 ng ml^{-1}) after resuspension in thymidine-free medium. Pulse labelling was terminated by addition of non-radioactive thymidine to a final concentration of 1 mg ml^{-1} . Bacteria in samples taken at 60 s ($\circ$) and 10 min ($\bullet$) were lysed and analysed on alkaline sucrose gradients as described in Methods. In parallel gradients using bacteriophage T5 DNA, 15S material was found in fraction 39/40 and 40S material in fraction 25.

was at the bottom of the tube where long-term labelled DNA is typically found. With strain 8325thy, more than 80% of the pulse was in material smaller than 40S with a broad peak at 10 to 15S. The two strains therefore appear to differ in that the thymidine auxotroph did not show immediate incorporation into material co-sedimenting with long-term labelled DNA. With both strains, the chase with excess thymidine resulted in all radioactive material having a sedimentation coefficient greater than 60S. While it is likely that the slowly-sedimenting pulse-labelled DNA had been converted to high molecular weight material this was not certain because the small pieces could have been degraded and large molecules synthesized from the intracellular labelled nucleoside pool.

Effect of thymidine starvation

To improve the characteristics of [^3H]thymidine incorporation during pulse labelling and the subsequent chase period we investigated the effect of thymidine starvation to deplete the thymidine pool (Okazaki, 1974). In addition, to slow down the rate of nascent DNA processing, pool depletion and labelling were carried out at 20 °C. Initially a culture of strain 8325thy, grown at 30 °C, was starved of thymidine for 60 min at 20 °C and then pulse-labelled for 90 s followed by addition of excess thymidine. The rate of [^3H]thymidine incorporation by such starved bacteria was approximately threefold higher than for non-starved bacteria and again was linear for incorporation of about 60% of added label reaching a plateau of about 80 to 90% incorporation of total added [^3H]thymidine. At the cell densities used for pulse labelling prior to DNA analysis approximately 50% of total [^3H]thymidine was incorporated during the 60 or 90 s pulse. After addition of excess thymidine the rate of incorporation fell off rapidly and incorporation of [^3H]thymidine increased by only 10 to 20% over the subsequent 30 min. Sedimentation analysis indicated

Fig. 2. Sedimentation analysis of DNA of *S. aureus* 8325thy pulse-labelled after thymidine starvation. Bacteria were grown at either 30 °C (a) or 20 °C (b) in AOAC medium with 10 μ g thymidine ml⁻¹ and then resuspended in AOAC medium and incubated at 20 °C for 60 min to exhaust any remaining thymidine. [³H]Thymidine (1 μ Ci ml⁻¹, 10 ng ml⁻¹) was then added, followed 90 s later by an excess of non-radioactive thymidine. Samples taken at 90 s (○) and 10 min (●) were analysed as in Fig. 1.

that most of the pulse-labelled DNA was found in small pieces of approximately 5 to 10S while a 10 min chase resulted in an increase in this size to a distribution of from 20 to 60S (Fig. 2a). When 8325thy was grown throughout at 20 °C, thymidine starvation for 60 min had the same effect, although the reduction in size of the pulse-labelled fragments was less marked and these were more rapidly converted to higher molecular weight during the chase period (Fig. 2b). We concluded that preferential labelling of smaller fragments and retardation of their conversion to high molecular weight was the result of thymidine starvation. In subsequent experiments this effect was observed for thymidine starvation periods as short as 10 min at 20 °C.

Since the thymidine level has recently been reported to have effects on nascent DNA metabolism in *E. coli* due to relationships with dUTP levels and uracil incorporation into DNA and its subsequent excision repair (Tye *et al.*, 1977), it was decided to investigate further this effect of thymidine starvation. Thymidine starvation for up to 60 min did not result in a change in sedimentation characteristics or recovery of [¹⁴C]thymidine label that had been incorporated over two generations prior to thymidine starvation (data not shown). However, evidence with *B. subtilis* (Ramareddy & Reiter, 1969) indicates that under conditions where fork movement is halted, preferential degradation of newly synthesized material may occur at the replication fork. For this reason we labelled a culture of 8325thy with [³H]thymidine for 2 min just prior to thymidine starvation. Over a subsequent 60 min period of thymidine starvation less than 5% of the incorporated radioactivity was degraded to acid-soluble material. Analysis on alkaline sucrose gradients indicated that this radioactively labelled DNA sediments mostly as high molecular weight material both before and after thymidine starvation and that no breakdown to smaller pieces occurred during this period (Fig. 3).

To determine whether the rate of thymidine incorporation is affected by thymidine starvation, a culture of 8325thy was monitored before and after starvation; it showed no

Fig. 3

Fig. 4

Fig. 3. Stability of DNA of *S. aureus* 8325thy during thymidine starvation. Bacteria were grown at 30 °C in AOAC medium with 10 µg thymidine ml⁻¹ and then harvested and resuspended in AOAC medium containing 100 ng thymidine ml⁻¹ and 1 µCi [³H]thymidine ml⁻¹ at 20 °C. After 2 min the suspension was centrifuged and the pellet was resuspended in AOAC medium without thymidine. A sample was immediately removed and the bacteria were lysed and analysed on an alkaline sucrose gradient (○). The remaining resuspension was incubated at 20 °C for 60 min and then a sample was analysed as above (●).

Fig. 4. Effect of nalidixic acid on the incorporation of [³H]thymidine into thymidine-starved *S. aureus* 8325thy. A thymidine-starved culture at 20 °C was divided into three and nalidixic acid was added to give a final concentration of 0 µg ml⁻¹ (○), 20 µg ml⁻¹ (●) or 100 µg ml⁻¹ (△). After 10 min [³H]thymidine was added (1 µCi ml⁻¹, 110 ng ml⁻¹). Samples (0.5 ml) were withdrawn periodically to determine incorporation into ice-cold TCA-precipitable material.

decrease in the rate of incorporation of [³H]thymidine. These determinations were carried out at low concentrations of thymidine (100 ng ml⁻¹) to minimize the time required for the internal nucleotide pool to attain the same specific activity as exogenous thymidine. In control experiments it was found that [³H]thymidine was incorporated into 8325thy at approximately the same rate in terms of moles of thymidine in the concentration range of 100 ng ml⁻¹ to 10 µg ml⁻¹. Therefore, it was concluded that thymidine starvation does not have a gross effect on DNA structure or DNA replication.

Effect of nalidixic acid

Before using nalidixic acid as a specific inhibitor of DNA replication its effects were characterized. The minimum inhibitory concentration of nalidixic acid for *S. aureus* 8325 and 8325thy growing in CY medium at 30 °C was 100 µg ml⁻¹, at which concentration the *A*₈₇₅ increased about 20-fold and then stopped. Over the range 20 to 100 µg nalidixic acid ml⁻¹, DNA synthesis, estimated by chemical determination of DNA content (Burton, 1956), was inhibited more than the *A*₆₇₅. This indicates that, as in *E. coli* (Goss *et al.*, 1965) and *B. subtilis* (Cook *et al.*, 1966; Ramareddy & Reiter, 1969), the target of nalidixic acid in

Fig. 5. Incorporation of small quantities of thymidine into thymidine-starved *S. aureus* 8325thy. A culture was starved for thymidine at 20°C for 60 min and then either no nalidixic acid (a) or $50 \mu\text{g}$ nalidixic acid ml^{-1} (b) was added. After 10 min, $[{}^3\text{H}]\text{thymidine}$ (33 Ci mmol^{-1}) was added to give $13 \text{ pmol per } 10^8 \text{ bacteria}$ ($\circ$) or $25 \text{ pmol per } 10^8 \text{ bacteria}$ ($\bullet$). Incorporation into TCA-precipitable material was determined in $50 \mu\text{l}$ samples.

Fig. 6. Analysis in alkaline sucrose gradients of newly synthesized DNA of *S. aureus* 8325thy. Samples were removed from cultures starved of thymidine at 20°C , preincubated for 10 min without nalidixic acid (a) or with $100 \mu\text{g}$ nalidixic acid ml^{-1} and then $[{}^3\text{H}]\text{thymidine}$ ($1 \mu\text{Ci ml}^{-1}$, 10 ng ml^{-1}) was added. After 60 s an excess of non-radioactive thymidine was added. Samples were withdrawn at 3 min ($\circ$), 10 min ($\bullet$) and 30 min ($\blacktriangle$).

S. aureus may be DNA synthesis. In AOAC medium, nalidixic acid had a similar effect except that the A_{675} for strain 8325thy did not increase as much, possibly due to a lower DNA content of this strain when grown in defined medium.

The effect of various concentrations of nalidixic acid on $[{}^3\text{H}]\text{thymidine}$ incorporation correlated with the effect on total DNA synthesis as determined chemically. When the effect of nalidixic acid on $[{}^3\text{H}]\text{thymidine}$ incorporation was measured before and after

Fig. 7. Analysis on alkaline sucrose gradients of newly synthesized DNA of *S. aureus* 8325thy synthesized in the absence (○) or presence (●) of nalidixic acid ($50 \mu\text{g ml}^{-1}$) after addition of [^3H]-thymidine to give 13 pmol per 10^8 bacteria (a, b) or 25 pmol 10^8 bacteria (c, d). The bacteria, grown at 30°C , had previously been starved of thymidine at 20°C . Nalidixic acid was added 10 min prior to labelling. Samples were withdrawn at 3 min (a, c) and 20 min (b, d) after addition of the [^3H]-thymidine and analysed on alkaline sucrose gradients.

thymidine starvation, it was found that after thymidine starvation there was a period of very little inhibition followed by a decreased rate of incorporation equivalent to the reduced rate observed for nalidixic acid inhibition prior to starvation (Fig. 4). Further investigation of the period of incorporation immediately after thymidine starvation showed that at very limiting amounts of thymidine (about 13 pmol thymidine per 10^8 bacteria) there was no inhibition by nalidixic acid and only at higher amounts of thymidine (e.g. about 25 pmol thymidine per 10^8 bacteria) was inhibition observed (Fig. 5). The lack of inhibition by nalidixic acid in this initial period thus appears to reflect the fact that there was a certain

small potential for nalidixic acid-resistant incorporation after thymidine starvation. With higher concentrations of thymidine, the initial rate of incorporation increased slightly and a small degree of inhibition by nalidixic acid was observed (Figs 4 and 5), suggesting that, after thymidine starvation, at maximum incorporation rates the initial incorporation was probably composed of a nalidixic acid-sensitive and a nalidixic acid-resistant component.

Investigation of the effect of nalidixic acid on the sedimentation velocity of DNA labelled by a pulse of [^3H]thymidine after thymidine starvation indicated that during a chase period with excess thymidine, nalidixic acid caused a retardation of the increase in size of DNA (Fig. 6). When the concentration of nalidixic acid was increased, the retardation effect increased in the range 20 to 300 $\mu\text{g ml}^{-1}$, the maximum retardation being found at 100 and 300 $\mu\text{g ml}^{-1}$ correlating with the maximum inhibition of DNA synthesis. Attempts to demonstrate a retardation of increase in size of fragments labelled in cultures of 8325 and 8325*thy* that had not been starved for thymidine were unsuccessful (data not shown). It was possible, therefore, that the effect of nalidixic acid on maturation of labelled fragments was not the primary effect. Under conditions where limiting thymidine was added to thymidine-starved bacteria, it was found that when no inhibition of incorporation was observed (13 pmol thymidine per 10^8 bacteria, Fig. 5) no effect on the size of fragments was observed (Fig. 7*a, b*). At slightly higher amounts of thymidine, where some inhibition by nalidixic acid was observed (25 pmol thymidine per 10^8 bacteria, Fig. 5), still no effect on the fragment size increase was observed except that incorporation into the smallest fragments was inhibited by nalidixic acid at later times (Fig. 7*c, d*). Our hypothesis is, therefore, that nalidixic acid inhibits the synthesis of DNA and that this inhibition may explain why the growth of fragments labelled by a pulse of [^3H]thymidine after thymidine starvation was retarded.

DISCUSSION

When a culture of *S. aureus* 8325 is pulse-labelled in exponential phase with [^3H]thymidine radioactivity is found in material that co-sediments with [^{14}C]thymidine-long-term labelled DNA and in small fragments of DNA tentatively proposed to be 'Okazaki' fragments. During a chase with non-radioactive thymidine these fragments disappear and it is proposed that they are incorporated into high molecular weight DNA. With this strain, however, we were unable to obtain conditions where incorporation of pulse label was halted completely during the chase period so that from our data it is not possible to conclude that the low molecular weight pulse-labelled DNA is indeed converted to high molecular weight species rather than being degraded. With the derivative 8325*thy* it was possible to obtain better pulse/chase labelling, but the *thy* mutation appears to result in a change in the properties of pulse-labelled material such that even prior to thymidine starvation very little incorporation into fast-sedimenting material was observed during the 60 or 90 s pulse, although the pulse-labelled material was all converted to fast-sedimenting material during a chase period of 10 min. To explain this effect of thymidine auxotrophy further experiments are necessary, although from what is known with *E. coli* (Tye *et al.*, 1977) and *B. subtilis* (Tamanoi & Okazaki, 1978) we hypothesize that the *thy* mutation results in reduced TTP pool size and therefore a higher dUTP/TTP ratio. The higher dUMP incorporation would therefore result in most newly synthesized DNA passing through a small-fragment stage due to excision repair of uracil.

In order to achieve good incorporation during a [^3H]thymidine pulse that could be rapidly reduced by addition of non-radioactive thymidine it was necessary to pre-starve the bacteria for thymidine. The result of this starvation was to increase incorporation of [^3H]thymidine into small fragments and, in bacteria grown at 30 °C, to cause a reduction in the average size of small labelled fragments from 10 to 20S, to 4 to 10S. In addition, the fragments labelled after thymidine starvation were converted to high molecular weight

species more slowly than before starvation. Thymidine starvation therefore results in changes in DNA metabolism.

Indeed, after thymidine starvation there is the potential for a certain amount of nalidixic acid-resistant thymidine incorporation. Calculations show that this amounts to about 2.6×10^4 nucleotide bases incorporated per bacterium. A similar phenomenon has been observed in *E. coli* (Brewin, 1977). From experiments we have carried out with nalidixic acid it appears that nalidixic acid has no effect on the joining of DNA fragments but does inhibit their synthesis. This is consistent with the demonstrated effect of nalidixic acid on DNA gyrase in *E. coli*. The fact that nalidixic acid slows down the increase in molecular weight of labelled DNA fragments in a pulse/chase experiment after thymidine starvation, but not before starvation, suggests that after thymidine starvation increase in molecular weight is due to joining of DNA synthesized during or after the pulse period rather than joining to the high molecular weight DNA already present in the cell. Our hypothesis is that thymidine starvation results in an alteration of the DNA, probably at, or near, the replication fork such that when thymidine is added after starvation discontinuities are left between DNA synthesized before and after starvation.

It should be stressed that while we feel that this is the most likely explanation, the data in no way prove where the incorporation is taking place, either at, or near, the replication fork or distributed around the chromosome. Since, however, there is no evidence of chromosome breakdown and the DNA synthesized adjacent to labelled fragments during the chase period must be very large (10 to 20% of the chromosome), we think it is more likely to be at or near the replication fork. If nalidixic acid acts on a DNA gyrase in *S. aureus* as it does in *E. coli* then resistance to nalidixic acid probably means that the incorporation after thymidine starvation takes place at a previously unwound region of DNA. The incorporation could be repair of uracil-rich DNA or replicative elongation of fragments whose initiation may be stimulated by thymidine starvation (see Brewin & Cairns, 1977). A third possibility is that re-initiation of chromosome replication takes place. Further experiments are required to distinguish between these possibilities.

In conclusion, the results described in this paper suggest that DNA replication in *S. aureus* involves a partly discontinuous process and that attempts to study this process using a thymidine-requiring strain alter the process in ways similar to that found in *E. coli* and *B. subtilis*. Nalidixic acid appears to inhibit synthesis of DNA in a manner consistent with its being an inhibitor of fork movement.

C. M. T. was the holder of an MRC Studentship for training in research methods during the course of this research.

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