Temperature-sensitive DNA Synthesis in a Mutant of *Bacillus subtilis*

By G. W. BAZILL* AND YVONNE RETIEF

*Medical Research Council, Radiobiological Research Unit, Harwell,
Didcot, Berkshire*

(Accepted for publication 3 December 1968)

SUMMARY

The properties of a temperature-sensitive mutant of *Bacillus subtilis* in which DNA synthesis is specifically and reversibly inhibited above 40° are described. When incubated at 45° there was no detectable breakdown of DNA in the mutant and the synthesis of deoxynucleoside triphosphates was normal. The temperature-sensitive element affects DNA synthesis *in vivo* but not *in vitro* and (unlike an initiator) it appears to be needed for DNA synthesis throughout the replication cycle.

INTRODUCTION

In the hope that biochemical studies of mutants with abnormal DNA synthesis might reveal new features of the replication mechanism, some temperature-sensitive mutants of *Bacillus subtilis* were isolated and examined. This report describes the properties of one mutant in which DNA synthesis, but not other macromolecular synthesis, ceases at 45°.

METHODS

Chemicals. Nucleosides, nucleotides, nucleoside triphosphates and bromodeoxyuridine were from Sigma Chemicals Ltd., London. Tritiated thymidine (methyl-T, 5 Ci/m-mole), [¹⁴C]thymine 58 mCi/m-mole, [¹⁴C]thymidine 36 mCi/m-mole, [^{5-³H}]uridine 3.5 Ci/m-mole, [¹⁴C]leucine 34 mCi/m-mole and carrier free H₃³²PO₄ were from the Radiochemical Centre, Amersham, Bucks., England. Schwartz BioResearch Inc., New York, supplied [³H]TTP, 4.8 Ci/m-mole. Amino acids were from British Drug Houses Ltd., chloramphenicol from Allen and Hanbury, London.

Bacteria. Mutant strains of *Bacillus subtilis* were obtained by courtesy of the following: 168 *ind⁻ thy⁻* (J. Farmer and F. Rothman); 168 *ind⁻* (C. Anagnostopoulos).

Spores were grown at 30° on Schaeffer agar plates and were prepared and purified as described by Yoshikawa (1965).

Media. Difco Antibiotic Medium no. 3 (16 g./l.) was used for routine culture and plating and is referred to throughout as broth. Salts medium was Spizizen minimal medium (Anagnostopoulos & Spizizen, 1961) supplemented when necessary with thymidine 5 or 10 µg./ml., L-tryptophan 50 µg./ml. and casein hydrolysate (Oxoid) 200 µg./ml.

* Present address: M.R.C. Microbial Genetics Unit, Department of Molecular Biology, The University, West Mains Road, Edinburgh 9.

For some experiments a rich defined medium (A.S. medium) was used which consisted of minimal salts medium supplemented with amino acids as in the spore germination medium of Donnellan, Nags & Levinson (1964). It was made as follows: 2 ml. concentrated minimal salts medium (Vogel & Bonner, 1956) plus 5 ml. 10 % (w/v) glucose was diluted to 100 ml. with a solution containing L-valine 0.2 g., L-arginine HCl 0.5 g., L-leucine 1.0 g., L-threonine, 0.5 g., L-serine 0.63 g. L-glutamine 4.7 g., L-alanine 1.25 g., L-asparagine 1.5 g./l. The concentrated salts, the glucose and amino acid mixture were autoclaved separately. Thymidine (5 μ g./ml.) and tryptophan (50 μ g./ml.) were added to the medium when required.

For experiments on ^{32}P -labelled nucleotide pools, bacteria were grown in 1 % Difco peptone. A sample of the batch of peptone used was analysed for total phosphate after digestion with perchloric acid. The 1 % (w/v) peptone medium contained 0.77 μ -mole phosphate per ml. Thymine-requiring strains grew well in this peptone; additional thymine was not needed.

Assays of DNA, RNA and protein synthesis. The incorporation of TCA-insoluble [^3H] or [^{14}C]thymidine, [^3H]uridine and [^{14}C]leucine was taken to be a measure of the synthesis of DNA, RNA and protein respectively. Duplicate 0.2 ml. samples were taken from the labelled culture at intervals. The samples were frozen immediately in acetone + solid CO_2 and stored at -20° . At a convenient time (generally the following day) they were allowed to melt and 3 ml. cold 6 % (w/v) TCA was added. After standing at 4° for not less than 20 min. the samples were filtered on 2 cm. Oxoid membrane filters, washed five times with cold TCA, twice with methylated spirit, placed in counting vials and allowed to dry at room temperature. The radioactivity was measured in a Beckman scintillation counter.

Cell extracts for in vitro assays. About 1 g. (wet weight) of bacteria growing exponentially at 30° in 2 l. of broth was collected by centrifugation. The packed organisms were washed once with 0.02 M-phosphate buffer (pH 7) suspended in 20 ml. of the same buffer and incubated for 1 hr. at 30° with 1 mg. lysozyme. The lysate was then centrifuged at 10° for 1 hr at 38,000 g. The supernatant fluid was decanted and frozen in CO_2 + acetone and stored at -20° .

Assay of DNA polymerase in vitro. Cell extract (0.1 ml. 5 to 8 mg. protein/ml.) was added to 0.9 ml. of 0.01 M-tris HCl buffer (pH 7.2) containing 1.6 μ -mole Na ATP, 2.5 μ -mole MgSO_4 , 85 μ m-mole dAMP, dCMP and dGMP, 7.0 μ m-mole [^3H]TTP (0.5 Ci/m-mole) and 80 μ g native *Bacillus subtilis* DNA, prepared by the method of Marmur (1961) from *B. subtilis* strain 168 ind.-. Incubation was in stoppered tubes at 30° or 45° . Samples of 0.025 ml. were removed at intervals on to filter-paper discs (Bollum, 1959) washed five times with 6 % TCA, twice with methylated spirit, dried and the radioactivity counted.

Analysis of nucleoside triphosphate pool. (1) [^3H]thymidine triphosphate: cultures of ts 230 (2×10^7 cells/ml.) in 10 ml. broth were labelled for 10 min. at 30° or 45° with [^3H]thymidine (2 μ Ci/ml.) after growth at 30° or after one hr at 45° . The organisms were filtered on 2 cm. membrane filters and washed rapidly with 0.5 ml. broth to remove extracellular [^3H]thymidine. The filters were covered with 0.8 ml. cold 6 % (w/v) TCA and the organisms were suspended by stirring with a fine glass rod. After 30 min. at 4° , the suspension was centrifuged and the supernatant fluid extracted six times with about 3 ml. diethyl ether to remove TCA. Remaining acidity was neutralized with one drop of 0.1 M-trisodium citrate solution. Carrier TMP and TTP

(0.5 mg. of each) was added and 0.1 ml. of this solution was applied to paper strips (Whatman 3 MM) for electrophoresis according to Smith (1955) in 0.05 M-citrate buffer (pH 3.75). After 2 hr. at 24 V./cm. the strips were dried and the u.v.-absorbing regions marked. The strips were then cut into 0.5 cm. sections and their radioactivities determined.

(2) ^{32}P nucleoside triphosphates: the techniques used were basically those of Randerath & Randerath (1964) and Neuhaard & Munch-Petersen (1966). Two volumes of 10 ml. were taken from an exponentially growing culture of ts 230 in 1 % peptone at a density of 3×10^7 bacteria/ml. One volume was incubated at 30° and the other at 45° for 30 min., then 0.1 mc $\text{H}_3^{32}\text{PO}_4$ (carrier-free) was added to each culture, to give a final specific activity of 13 $\mu\text{Ci}/\mu\text{-mole}$ phosphate. Incubation was continued at 30° and 45° for 15 min., then the organisms were filtered on 2 cm. membrane filters (Millipore, 0.45 μ) and washed rapidly with 1 ml. of peptone to remove extracellular $^{32}\text{PO}_4$. The membrane filters were placed in flat-bottomed tubes containing 1 ml. distilled water, frozen immediately in a CO_2 +acetone bath and stored at -40°.

To extract the ^{32}P -labelled nucleotides, 3 ml. cold 6 % (w/v) TCA was added to the frozen filtered bacteria, followed by 0.01 ml. of a mixture containing 3 $\mu\text{-mole/ml.}$ of ATP, UTP, GTP, CTP, dATP, TTP, dGTP, dCTP. Then 0.01 ml. M-potassium phosphate buffer, pH 7.0, was added, the mixture was allowed to thaw and kept at 4° for 20 min. before it was passed through a millipore filter (pore size 0.45 μ) and the filtrate was extracted six times with 3 ml. diethyl ether. The extract was made neutral with 0.05 ml. M- NH_4OH and incubated for a few minutes with shaking at 45° to remove traces of ether. It was then freeze-dried. The residue was dissolved in 0.2 ml. distilled water and stored at -40°.

Thin-layer plates (20 $\times$ 20 cm.) of polyethylene-imine cellulose were prepared according to Randerath & Randerath (1964). The ^{32}P -labelled extracts (0.005 ml.) were spotted on to the plates together with 5 $\mu\text{l.}$ of a mixture containing 3 $\mu\text{-mole/ml.}$ of each ribo- and deoxyribo-nucleoside triphosphate. Plates were developed in the solvent system described by Neuhaard & Munch-Petersen (1966): in the first dimension, M-acetic acid + M-lithium chloride; in the second dimension, 3 M-ammonium acetate + 5 % (w/v) boric acid, the pH value of the mixture being adjusted to 6.5 with ammonia. After development in the first solvent the plates were dried in a current of cold air and washed for 15 min. in methanol to remove lithium chloride. After the second development, the plates were dried in warm air and examined by u.v. radiation in a dark room. The position of the u.v. absorbing spots was marked and traced on to thin white paper. The plates were then taped to X-ray film (Kodak 'Kodirex' E.P. 6.5 $\times$ 8.5) and exposed for 24 hr. Sometimes a longer exposure was needed. If the autoradiograph was satisfactory, the spots previously located by u.v. absorption were excised from the thin-layer plate and their radioactivity counted. The excision was done with a scalpel, after first moistening the spot (ringed in pencil) with a drop of amyl alcohol. Correspondence between the carrier (u.v.-absorbing) and radioactive spots was exact.

Pulse labelling of DNA. The retention of tritiated thymidine in bacteria pulse-labelled at 30° then shifted to 45° was measured as follows: a suspension of strain ts 230 growing in 10 ml. Spizizen salts medium with thymidine 10 $\mu\text{g./ml.}$ and tryptophan 50 $\mu\text{g./ml.}$ was filtered at a density of 3×10^7 bacteria/ml. and suspended in 5 ml. medium without thymidine.

After 3 min. aeration at 30°, 10 μ Ci thymidine (18.6 Ci/m-mole) were added and aeration continued for 2 min. The bacteria were then collected on a membrane filter, washed and suspended in 16 ml. salts medium. The suspension was divided into four and incubated with and without thymidine (50 μ g./ml.) at 30° and 45°. Samples of 0.2 ml. were taken at intervals and the TCA-soluble radioactivity determined.

Isolation of temperature sensitive mutants. *Bacillus subtilis ind⁻ thy⁻*, grown to a density of 10⁷ bacteria/ml. in 100 ml. salts medium, was treated for 2 hr at room temperature with *N*-nitro-*N*¹-nitroso-guanidine 20 μ g./ml. A plate count at this stage showed approximately 10% survival. The mutagen was removed by membrane filtration and the bacteria were suspended in broth and kept at 4° overnight. Next morning the culture was revived in broth at 22°, then transferred to 10 ml. salts medium with 50 μ g./ml. tryptophan, 0.05% (w/v) casein hydrolysate and 20 μ g./ml. bromodeoxyuridine. It was then incubated for 1½ hr at 45° (many cells lysed at this stage). The remaining bacteria were collected on a membrane filter, suspended in 10 ml. broth and aerated overnight at 4°. Suitable dilutions were plated to determine the viable count, which was 10⁵ bacteria/ml. Seventy-five broth plates were spread, to give about 70 colonies per plate. After 2 days at 22°, replica plates were made (75 plates at 45°, 75 at 22°) and colonies growing at 22° but not at 45° were picked, streaked, then tested again for temperature sensitivity. Of 5000 colonies examined, twenty proved to be temperature sensitive mutants. Six of these showed restricted DNA synthesis at 45°. The most marked effect was in the strain numbered ts 230 and this was chosen for detailed study.

RESULTS

Temperature dependence of DNA synthesis. Figure 1 shows the uptake of [³H]thymidine at 37°, 45° and 49° by the temperature sensitive mutant (a) and the parent strain (b). The bacteria were prelabelled before the temperature shift by growth for more than ten generations in [³H]thymidine-A.S. medium.

RNA and protein synthesis. The uptake of [¹⁴C]leucine/[³H]thymidine (Fig. 2) and [5-³H]uridine/[¹⁴C]thymine (Fig. 3) was followed in simultaneous labelling experiments. RNA and protein synthesis continued in the absence of DNA synthesis for about 2 hr after the temperature was raised to 45°. In agreement with this observation, bacterial mass, as judged by turbidity, increased about sixfold during this time.

Morphology, viability and cell division. No unusual change in morphology was seen during the first 30 min. after raising the temperature of a log.-phase broth culture to 45°. Between 30 min. and 1 hr the bacteria began to lose motility and to grow in long chains, each organism becoming elongated. After 2 hr they had a contorted appearance and swollen regions could be seen, generally at one end of a bacterium but occasionally at both ends, or in the middle. On further incubation (3 to 5 hr) lysis occurred.

Although the total count increased eightfold during incubation at 45° in broth (Fig. 4) the viable count started to decrease after about half an hour (Fig. 5). In minimal salts medium the behaviour of the mutant was similar to that in broth, but there was less division, elongation and swelling.

Although there was rapid death of vegetative organisms at 45°, there was no loss of viability of ts 230 spores when kept at 45° in distilled water for 3 hr.

The utilization of exogenous thymidine. The contorted filaments formed by ts 230

Fig. 1. DNA synthesis in *ts 230* (a) and *168 ind- thy-* (b). Log.-phase bacteria growing in A.S. medium at 30° were diluted and transferred to 37°, 45° or 49° when the density was 5×10^6 bacteria/ml. 37°, ●●; 45°, ▲▲; 49°, ○○.

Fig. 2

Fig. 3

Fig. 2. Protein and DNA synthesis in *ts 230*. Cultures prelabelled by growth for many generations in A.S. medium containing ^{14}C leucine and ^3H thymidine were transferred from 30° to 45° and the simultaneous uptake of leucine and thymidine was measured. The medium contained $0.1 \mu\text{Ci/ml}$. ^{14}C leucine and $1.0 \mu\text{Ci/ml}$. ^3H thymidine with $5 \mu\text{g./ml}$. carrier thymidine. Bacterial density at temperature shift $5 \times 10^6/\text{ml}$. ^{14}C Leucine 32°, ●●; ^{14}C leucine 45°, ○○; ^3H TdR 32°, ▲▲; ^3H TdR 45°, △△.

Fig. 3. RNA and DNA synthesis in *ts 230*. Bacteria grown for many generations in A.S. medium containing ^3H uridine and ^{14}C thymine were shifted from 30° to 45° and the subsequent uptake of ^3H and ^{14}C measured. The medium contained $0.1 \mu\text{Ci/ml}$. ^{14}C thymine plus $5 \mu\text{g./ml}$. carrier thymine and $1.0 \mu\text{Ci/ml}$. ^3H uridine. Bacterial density at temperature shift was $5 \times 10^6/\text{ml}$. ^{14}C Thymine 30°, ●●; ^{14}C thymine 45°, ○○; ^3H uridine 30°, ▲▲; ^3H uridine 45°, △△.

at 45° appeared to be similar to those produced by the parent strain of *Bacillus subtilis* when incubated in a rich but thymineless medium (e.g. spore germination medium, Donnellan *et al.* (1964)). This suggested that the temperature sensitive lesion in ts 230 might be one which caused failure either to take up thymidine or to convert it to thymidine triphosphate. To test this possibility, ts 230 was grown in broth for 1 hr at 45°, then tritiated thymidine (2 µC/ml.) was added for 10 min. The bacteria were rapidly collected on membrane filters and the TCA-soluble extract was examined

Fig. 4. Increase of bacterial number and turbidity of ts 230 incubated in broth at 45°. Samples were removed at intervals, shaken with glass beads to disperse aggregates and counted in a Petroff Hauser chamber. Optical density 660 mµ, ○ ○; bacterial count, ● ●.

Fig. 5. Survival of ts 230 incubated in broth at 45°. Suitable dilutions were made and 0.1 ml. of cultures incubated at 26° and 45° were spread on broth plates. Colonies were counted after 2 days at room temperature. Viability at 26°, ● ●; viability at 45°, ○ ○.

by paper electrophoresis to check for the conversion of [³H]thymidine to [³H]thymidine triphosphate. About 70 % of the radioactivity on the strip was located in the TTP band (Fig. 6).

Synthesis of nucleoside triphosphates. Cultures of ts 230 and the non-temperature-sensitive parent strain 168 *thy*⁻ *ind*⁻ growing exponentially at 30°, were labelled with ³²P as follows: (a) ts 230 at 30°, shifted to 45° for 30 min., labelled with H₃³²PO₄ for 15 min. (b) ts 230 at 30° labelled with H₃³²PO₄ for 15 min. at 30°, (c) 168 *thy*⁻ *ind*⁻ at 30° shifted to 45° for 30 min., labelled with H₃³²PO₄ for 15 min. at 45°. After labelling, the bacteria were rapidly collected on membrane filters. Acid-soluble extracts were

prepared and analysed by thin-layer chromatography (see Methods). The radioactive regions located on the chromatographs by autoradiography corresponded exactly to the u.v.-absorbing regions of the carrier (non-radioactive) nucleoside triphosphates. The distribution of radioactivity among the triphosphates is given in Table 1.

It is evident that the pool of nucleoside triphosphates in the mutant held at 45° did not differ significantly from that of the controls (the wild-type at 45° or the mutant at 30°). None of the nucleoside triphosphates present in the controls was absent from the 45° extract; and there were no additional spots, indicative of abnormal nucleotides, on the 45° autoradiograph.

Table 1. *Distribution of radioactivity on chromatograms of nucleoside triphosphates in extracts of ts 230 (c.p.m. ³²P).*

	ATP	UTP	GTP	CTP	dATP	TTP	dGTP	dCTP
Ts 230 30°	8477	6504	3656	2764	673	537	379	878
Ts 230 45°	15031	5086	2370	5758	1247	881	702	726
168 ind ⁻ thy ⁻ 30°	10436	3256	1778	1370	427	1146	439	480
168 ind ⁻ thy ⁻ 45°	13455	9068	2597	1815	1360	1247	807	571

Fig. 6

Fig. 6. Conversion of [³H]thymidine to TMP, TDP, and TTP by *ts 230*. Bacteria were incubated 1 hr at 45° then labelled for 10 min. at 45° with [³H]thymidine. Distribution of the radioactivity on an electrophoretogram of the TCA-soluble extract is shown. Lines (—) show the position of u.v. absorbing bands of non-radioactive TMP, TDP and TTP added to the extract.

Fig. 7

Fig. 7. DNA polymerase and deoxynucleotide kinase activity in extracts of *ts 230* and *168 ind⁻ thy⁻*. The reaction mixtures contained 560 µg./ml. *ts 230* protein or 825 µg./ml. *168 ind⁻ thy⁻* protein, 7 µm-mole/ml. ³HTTP (82,000 c.p.m./µm-mole) and other components as given in Methods. The ordinate shows the TCA-soluble radioactivity of 0.025 ml. samples. *ts 230* extract incubated at 30°, △ △; *ts 230* extract incubated at 45°, ○ ○; *168 ind⁻ thy⁻* extract incubated at 30°, ▲ ▲; *168 ind⁻ thy⁻* extract incubated at 45°, ● ●; *168 ind⁻ thy⁻* extract incubated at 45°; no ATP, ■ ■.

DNA polymerase tested in vitro. Figure 7 shows the incorporation of [³H]TTP into DNA when cell-free extracts of *ts 230* and of the parent strain were supplied with [³H]TTP plus deoxynucleotides and ATP. The DNA polymerase was not inactivated by prolonged incubation at 45°. Similar results (not shown) were obtained when the

enzymes were extracted from organisms which had already been incubated for 1 hr at 45°.

Stability of *ts 230* DNA at 45°. No loss of TCA-soluble label was observed either from generally labelled or pulse-labelled organisms: samples of 2.5×10^6 bacteria labelled throughout their DNA remained at 5590 ± 130 c.p.m. over 180 min. at 45°. Bacteria pulse-labelled with tritiated thymidine of high specific activity (see Methods) also retained their TCA-insoluble label when incubated at 45°: samples of 4×10^5 bacteria taken between 15 and 60 min. stayed at $12,435 \pm 330$ c.p.m.; when incubation at 45° was in medium without thymidine, the count rate stayed at $13,030 \pm 360$ c.p.m.

Fig. 8

Fig. 9

Fig. 8. Resumption of DNA synthesis at 30° after inhibition at 45°. Log-phase *ts 230* bacteria at a density of 5×10^8 /ml. were incubated in A.S. medium (5 µg./ml. TdR) with $2.0 \mu\text{Ci } ^3\text{H-TdR}$ /ml. for 30 min. at 45°, then divided into two and transferred to 30°. To one half was added 50 µg./ml. chloramphenicol. No chloramphenicol, ▲▲; with chloramphenicol, △△; 30 min. at 45°, ●●.

Fig. 9. Resumption of DNA synthesis after thymine starvation at 45°. Log-phase cultures (10 ml.) of *ts 230* and 168 ind⁻ thy⁻ were grown in broth at 30° until the optical density at 600 mµ was 0.73. The bacteria were collected on membrane filters, washed, and suspended at a density of 5×10^7 /ml. in Spizizen salts medium supplemented with 20 µg/ml. tryptophan and 0.04 % casein hydrolysate; they were then aerated at 45° for 15 min. Tubes containing 5 ml. broth and 0.1 µCi/ml. [¹⁴C]thymidine were inoculated with 0.5 ml. of the thymine-starved *ts 230* or 168 ind⁻ thy⁻ suspension and were incubated as follows: (a) 168 ind⁻ thy⁻ at 45°, △△; (b) *ts 230* at 45°, ○○; (c) *ts 230*, ▲▲. Duplicate 0.25 ml. samples were withdrawn at intervals to determine the incorporation of [¹⁴C] thymidine.

Reversibility of the block in DNA synthesis. When a log-phase culture of ts 230 is shifted from 30° to 45° DNA synthesis is stopped within 30 min. If the temperature is then lowered to 30°, DNA synthesis starts again after a short lag. That the resumption of DNA synthesis does not require the synthesis of new protein is shown by the response in the presence of chloramphenicol (Fig. 8).

Thymine starvation at 45°. The inhibition, at temperatures above 40°, of DNA synthesis in ts 230 could be the result of progressive inactivation of an essential enzyme during the first 15 min. of incubation. If so, 15 min. at 45° in the absence of thymidine would inactivate the enzyme, and subsequent restoration of thymine at 45° would not produce any further DNA synthesis. On the other hand, if the limited amount of DNA synthesis at 45° was the result of the organism's inability to initiate new cycles of replication at that temperature, DNA synthesis should be observed after thymine was restored and it should continue until all cycles, interrupted at the time of thymine starvation, were completed.

An experiment to distinguish between these possibilities is shown in Fig. 9. DNA synthesis after thymine starvation for 15 min. at 45° was zero if thymine was restored at 45°. Control experiments in which thymine was restored to the temperature-sensitive mutant at 30° or to the parent strain at 45°, showed that the 15 min. period of thymine starvation at 45° did not itself abolish the capacity for DNA synthesis when thymine was restored.

DISCUSSION

Bacterial mass and number increase and RNA and protein synthesis continue when ts 230 is incubated at 45°. A non-specific lesion, for example, a change of membrane permeability or a defect in energy metabolism, can therefore be ruled out as a possible cause of the block in DNA synthesis. Of the many possible ways in which a specific block in DNA synthesis could occur, some can be eliminated on the evidence given, while others can be rated as more probable or less probable.

The morphological changes and the loss of viability after a lag period when ts 230 was incubated at 45° were reminiscent of thymineless death in the parent strain. However, the ts mutant could form TTP from externally supplied thymidine even after 1 hr at 45° (Fig. 6). Thin layer chromatography of the acid-soluble extracts of ³²P-labelled organisms confirmed that the ts mutant was still able to synthesize TTP after 45 min. at 45°, and further showed that the synthesis of dATP, dGTP and dCTP and the ribonucleoside triphosphates was normal at 45°. The temperature-sensitive step in ts 230 is therefore not in the biosynthetic pathways leading to nucleoside triphosphates. The continued activity, at 45°, of the deoxynucleotide kinases in the DNA polymerase assay (Fig. 7) supports this conclusion.

Since the DNA polymerase appeared to function normally at 45° *in vitro*, the mutant is not defective in the ability either to produce DNA substrates or to polymerize them. The defect may be due to inability of the organism's DNA to direct the synthesis of new DNA. Such a situation might be caused by double-strand breaks (Cairns & Davern, 1966) or it might arise if the initiation of new cycles of replication was prevented. These possibilities have been explored in the experiments on the stability of ts 230 DNA at 45° and on the effects of thymine starvation at 45°.

There was no appreciable release of acid soluble nucleotides from bacteria pre-labelled for several generations with tritiated thymidine when they were incubated at

45°. Bacteria pulse-labelled at the replicating fork similarly showed no evidence of breakdown of the DNA after a temperature shift. This shows that there was no gross breakdown (e.g. by an uncontrolled deoxyribonuclease) at this temperature. But the production of a small number of single-strand breaks would not have been detected. It is unlikely that double-strand breaks occur in view of the reversibility of the block in DNA synthesis (Fig. 8). There is evidence from studies on the effects of X-rays that *Escherichia coli* cannot repair double-strand breaks in its DNA (Kaplan, 1966). In *Bacillus subtilis*, which is not much more resistant to X-irradiation than *E. coli*, the effect of double-strand breaks would similarly be expected to be irreversible.

If single-strand breaks are the cause of inhibition of DNA synthesis, two possibilities may be considered: (1) single-strand breaks do not occur in the course of normal DNA function, but in ts 230 there is an aberrant enzyme which produces breaks at temperatures above 40°; (2) single-strand breaks do occur and are necessary for normal DNA functions; in ts 230 these breaks are repaired efficiently at 30° but inefficiently or not at all above 40°.

Neither of these possibilities can be discounted at present and to rule them out completely would require very refined experiments. However, it is by no means certain that the presence of single-strand breaks in DNA would necessarily prevent its replication. Indeed, unless the entire bacterial chromosome rotates when the DNA is replicated (and this seems mechanically difficult) a small number of single-strand breaks would be a prerequisite for replication. One might also expect that if breaks were not repaired they would be extended by exonuclease action. Prolonged exonuclease action should lead to appreciable loss of labelled thymine from the DNA but this did not take place.

The increase of DNA after a temperature shift to 45° in A.S. medium (50% in Fig. 1) is close to that expected (40%) for the completion of a cycle of replication and failure to initiate a new cycle. But if ts 230 had a temperature-sensitive initiator, replication cycles which could be completed at 45° should also be completed at 47° or 49°, the same amount of DNA being synthesized at each temperature. This was not found: the amounts of DNA synthesized decreased with increasing temperature (Fig. 1a). Further evidence against the theory that ts 230 is an initiator mutant comes from the experiment illustrated in Fig. 9: the bacteria were held for 15 min. at 45° in conditions (absence of thymine) where DNA synthesis was impossible. Synthesis of DNA did resume when thymine was restored at 30°, but not when it was restored at 45°. If the temperature-sensitive lesion was one which allowed DNA synthesis to continue to the end of a cycle, those replication cycles already in progress at the time of the temperature shift should have been completed when thymine was restored. But it is clear that they were not completed. Thus the evidence does not support the hypothesis of a temperature-sensitive initiator; rather, it suggests that some element which is continuously needed for DNA synthesis throughout the replication cycle becomes inactivated above 40° at a rate which increases with temperature.

In the experiments described, all the known components of the DNA replication system have been tested for possible temperature sensitivity, but none of them has proved to be abnormal. The results of the *in vitro* assay of ts 230 DNA polymerase must be interpreted with caution in view of the evidence of de Waard, Paul & Lehman (1965), that not all of the ts mutations mapping in the locus for T4 DNA polymerase

gave enzymes that were inactive *in vitro*. Thus the possibility remains that DNA polymerase may be abnormal in ts 230, present methods of assay being inadequate to detect the abnormality. It now seems very probable that the polymerase responsible for replicating the bacterial chromosome acts at a site on the cell membrane and forms part of a replicative complex through which the DNA duplex passes as it is unwound and copied. Such a complex would include a number of elements, any one of which could be temperature-sensitive *in vivo* without giving any sign of abnormal sensitivity *in vitro*. Our present hypothesis is that ts 230 is a representative of one such class of mutants.

One of us (G.B.) is indebted to Drs J. D. Gross and D. Karamata for helpful discussions and for communicating results prior to publication. The very competent assistance of Mrs J. Brogden in the later stages of the work is also gratefully acknowledged.

REFERENCES

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *B. subtilis*. *J. Bact.* **81**, 741.
- BOLLUM, F. J. (1959). Thermal conversion of non-priming DNA to primer. *J. biol. Chem.* **234**, 2733.
- CAIRNS, J. & DAVERN, C. I. (1966). Effect of ^{32}P decay upon DNA synthesis by a radiation-sensitive strain of *E. coli*. *J. molec. Biol.* **17**, 418.
- DONNELLAN, J. E., NAGS, E. H. & LEVINSON, H. S. (1964). Synthetic media for *B. subtilis* spores. *J. Bact.* **87**, 332.
- KAPLAN, H. S. (1966). DNA-strand scission and loss of viability after X-irradiation of normal and sensitized bacterial cells. *Proc. natn. Acad. Sci. U.S.A.* **55**, 1442.
- MARMUR, J. (1961). A procedure for the isolation of DNA from micro-organisms. *J. molec. Biol.* **3**, 208.
- NEUHARD, J. & MUNCH-PETERSEN, A. (1966). Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *E. coli* during thymine starvation. *Biochim. biophys. Acta* **114**, 61.
- RANDERATH, K. & RANDERATH, E. (1964). Ion exchange chromatography of nucleotides on polyethyleneimine cellulose thin layers. *J. Chromatog.* **16**, 111.
- SMITH, J. D. (1955). The electrophoretic separation of nucleic acid components. In *The Nucleic Acids*. Ed. by E. Chargaff & J. N. Davidson, vol. 1. New York: Academic Press Inc.
- VOGEL, H. J. & BONNER, D. M. (1956). Acetylornithinase of *E. coli*: partial purification and some properties. *J. biol. Chem.* **218**, 97.
- DE WARD, A., PAUL, A. V. & LEHMAN, I. R. (1965). The structural gene for DNA polymerase in bacteriophages T4 and T5. *Proc. natn. Acad. Sci. U.S.A.* **54**, 1241.
- YOSHIKAWA, H. (1965). DNA synthesis during germination of *B. subtilis*. *Proc. natn. Acad. Sci. U.S.A.* **53**, 1476.