Studies on the Replication of Bacteriophage T5

By JANET M. CARRINGTON AND MARY R. LUNT

Microbiology Unit, Department of Biochemistry, South Parks Road, Oxford, England

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SUMMARY

Bacteria infected with bacteriophage T5 were disrupted with lysozyme and mild detergents and the intracellular phage-specific components resolved by sedimentation through neutral sucrose gradients. In pulse-chase experiments with [3H]-thymidine most of the radioactivity initially appeared in a fast-sedimenting form of DNA (fsf) which was very shear-sensitive and bound tightly to nitrocellulose. Label next appeared in a slower sedimenting form (ssf), then phage heads and finally virus particles. The ssf showed susceptibility to shear similar to that of DNA from intact virus, and sedimented with it on neutral gradients. The ssf DNA differed from virus DNA by binding to nitrocellulose and showing a different sedimentation profile on alkaline-sucrose gradients. The pulse-labelled replicating DNA was very heterogeneous in mol. wt. and appeared to contain many single-strand nicks which were extensively repaired while the DNA was still in the fast-sedimenting form. The conversion sequence fsf -> ssf -> heads -> phage was supported by the accumulation of components in non-permissive host bacteria infected with certain amber mutants of T5. One of these, T5.B1, could not form the T5 phage-induced 5' exonuclease and in these infections there was no conversion of replicating DNA to ssf, and net DNA synthesis stopped prematurely. It was concluded that maturation of T5 virus involved mature virus-size pieces of DNA of unusual structure as intermediates between replicating DNA and phage heads. The process appeared to require functional T5 induced exonuclease, but the role of this enzyme was unclear.

INTRODUCTION

The formation of mature virus particles requires that a constant amount of DNA must be incorporated into each virus particle at the expense of intracellular replicating DNA. We have investigated this process using bacteriophage T5.

Bacterial viruses which contain linear duplexes of DNA include two groups of organisms: those in which the DNA molecules contain sequences which are circular permutations of each other (e.g. phages T2, T4, P1, P22), and those in which the DNA molecules are identical, and unique for any one strain of virus (e.g. phages T1, T3, T5, T7 and λ). Terminal redundancy, or possession of the same sequence at both ends of a linear DNA duplex, occurs in both groups (Thomas, 1967; Thomas & MacHattie, 1967). The DNA of T5 possesses 10 % terminal redundancy (Rhoades & Rhoades, 1972), although the results of physiological experiments (Lanni, 1968) and genetic mapping (Hendrickson & McCorquodale, 1971) indicate that the same end of the phage DNA molecule (First Step Transfer
fragment) is always injected first into the host, and that the corresponding T5 genes must be expressed before the remaining virus DNA enters the cell. These results imply that the virus DNA must be uniquely orientated within the phage head. Phage T5 is also unusual in that its DNA possesses interruptions in one of the strands of the duplex, the position of these breaks being constant for any one phage strain (Abelson & Thomas, 1966; Bujard, 1969).

Unlike mature virus DNA, the replicating DNA of λ and T-even phage consists of high mol. wt. ‘concatemers’ which correspond in size to several mature phage lengths of DNA (Frankel, 1962, 1968; Smith & Burton, 1966; Young & Sinheimer, 1968); branch points have been detected in replicating T4 DNA (Werner, 1968; Broker & Lehmann, 1971). Little is known of the precise mechanisms whereby a length of DNA corresponding to one virus genome is selected from the replicating DNA and incorporated into a new virus particle. However, T4 phage deletion mutants have increased terminal redundancy in their DNA, and Streisinger has proposed that the DNA is packaged directly into the head structure, the final cutting of each strand at one ‘headful’ of nucleic acid automatically producing a collection of randomly permuted molecules (Streisinger, Emrich & Stahl, 1967). Direct evidence for this mechanism has recently been obtained by Luftig, Wood & Okinata (1971), and by Frankel, Batcheler & Clark (1970).

In contrast to T4, deletion mutants of phages T5, T7 and λ contain reduced amounts of virus DNA with no increase in terminal redundancy (Ritchie & Malcolm 1970; Parkinson & Huskey 1971). This suggests that the production of a collection of unique DNA molecules involves a mechanism whereby, within a concatemer, nucleotide sequences corresponding to the potential ends of mature virus DNA molecules are recognized and cut, possibly as proposed by Kelly & Thomas (1969). Such a mechanism predicts that mature virus-sized lengths of DNA are intermediates in phage production, and that virus-induced enzymes, including sequence-specific nucleases, are involved. We decided to test these possibilities with phage T5. We have avoided the use of strong detergents or phenol deproteinization and have chosen gentle methods of cell lysis in order to distinguish free intracellular DNA from components such as virus particles or phage heads. Preliminary experiments of this kind were described by Lunt & Kay (1968).

**METHODS**

*Bacteriophages.* Stocks and radioactive preparations of phages T2(H), λ, T5 and the T5 amber mutants B1, B2 and B3 were grown, purified and assayed by the methods of Fielding & Lunt (1970), who also describe the selection of T5 mutants after mutagenesis by bromodeoxyuridine; the amber mutant T5 N1 was isolated from a stock of T5 which had been mutagenized with nitrous acid (Bautz-Freese & Freese, 1961).

*Bacteria.* *Escherichia coli* strain B/2 was routinely used to grow and assay T5, and strain B/1·5 to check the purity of stocks. *E. coli* cr63 was used to grow amber mutants. *E. coli* c3000 thiamine was used for most of the metabolic experiments; it is non-permissive for amber mutants.

*Media.* Tryptone broth, glucose-salts medium and phage dilution medium were those used by Fielding & Lunt (1970). Low-phosphate minimal medium was that of Hershey (1955). Tris-EDTA buffer contained 0·01 M-tris-HCl, 0·001 M-EDTA, pH 8·1. SSC contained 0·15 M-NaCl, 0·015 M-sodium citrate, pH 7·0.

*DNA in bacterial cultures.* This was measured according to Burton (1956).

*Radioactive materials and their measurement.* Carrier free [32P]-phosphate, [35S]-sulphate,
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[6-3H]-thymidine (17 to 28 Ci/m-mol), [5-3H]-uracil (1 Ci/m-mol) and [2-14C]-thymidine (56-2 m-Ci/m-mol) were obtained from the Radiochemical Centre, Amersham, Bucks. Aqueous samples (0.2 ml) which contained radioactive material were counted after mixing with scintillation fluid which contained 7 g butyl PBD, 80 g naphthalene, 400 ml methoxy-ethanol and 600 ml toluene. Radioactivity was measured in a Beckman scintillation counter using the appropriate iso-sets, or in a Wallac scintillation counter. The counts were corrected for background and, in double labelling experiments, for overlap between the two channels.

Collection and counting of samples from sucrose density gradients. Normally a hole was punched in the bottom of the centrifuge tube and, when [H]-thymidine was the only label, fractions were collected directly on to strips of glass fibre paper (Whatman Chromedia GF/82). The strips were dried, washed with cold 7% TCA, then ethanol, and dried. The strips were cut into sections corresponding to each fraction and each section placed in a vial with 2 ml of scintillation fluid (0.7% butyl PBD in toluene). When DNA was labelled with [32P], or with [3H] and [32P], the gradients were drop-collected into tubes and samples incubated with 0.3 N NaOH to hydrolyse RNA before acid precipitating the DNA on glass-fibre discs prior to counting (Lunt & Kay, 1968). A comparison of counts in complete acid hydrolysates with counts of acid-precipitated gradient samples showed that in the latter method the efficiency of counting of [3H] in free DNA was the same as in virus particles. The same result was obtained with [32P]-labelled components.

When it was necessary to avoid shearing DNA which was to be re-centrifuged, fractions were collected from the tops of the gradients. To do this a perspex adapter with a wide bore (2 mm) outlet was fitted over the top of the centrifuge tube. The gradient was then slowly displaced by sucrose solution (50% w/v) in 2 M NaCl, 0.05 M tris-HCl, pH 7.8 which was run in to the bottom of the centrifuge tube through a hollow needle connected to a reservoir.

Nitrocellulose binding test on DNA. Nitrocellulose powder was washed with 2 × SSC solution and tightly packed into sawn-off Harshaw Pasteur pipettes of 4 mm internal diameter (Harshaw Chemicals Ltd., Daventry, Northants.) to give columns of 2 cm height. Samples of DNA were diluted to contain up to 1.5 μg/ml in 2 × SSC and 0.1 ml layered on to the nitrocellulose. Several washings of 1 ml batches of 2 × SSC were collected and assayed for radioactivity. Each column was used only once. Duplicates were always performed and they gave consistent results. The amount of DNA bound to the nitrocellulose was calculated from the difference between that applied and the amount recovered in the column washings.

Reagents. Nitrocellulose powder was Nitrocel-S from Serva, Heidelberg, Germany. Chloramphenicol was obtained from Parke-Davis; rifampicin, B grade, from Calbiochem; Brij 58 (polyoxethyleneeetyl ether) from Honeywell and Stein, Carshalton, Surrey; sarkosyl NL 30, was sodium lauryl sarkosinate from Koch Light Ltd; sodium deoxycholate was from British Drug Houses Ltd; butyl PBD (5-(4-biphenylyl)-2-(4-tert-butyphenyl)-1-oxa-3:4 diazole) was from CIBA, Duxford, Cambridge.

Enzymes. Egg-white lysozyme, Grade 1, was obtained from Sigma Chemical Co.; crystalline DNase was from Sigma, and Pronase, Grade B from Calbiochem. Ltd.

Radioactive phage DNA preparations. These were obtained by phenol extraction of suspensions of T2 or T5 phages according to Mandell & Hershey (1960). The extracted DNA was dialysed for 24 h against three changes of 0.01 M tris-HCl, 0.001 M EDTA, pH 8.1.

Growth of infected cultures. Routinely an overnight culture of E. coli C3000 was diluted into fresh Hershey low-phosphate medium and grown at 37 °C with forced aeration for four to five generations until the bacteria were at a cell density of 5 × 10⁸/ml. The culture was made 1 mM with CaCl₂ and infected with T5 phage at an input multiplicity of 10 p.f.u./cell.
The addition of radioactive substances and other materials is described under the individual experiments. A two-necked flask was used to prevent interruption of aeration during sampling. With these conditions the latent period for phage T5 was 45 to 50 min.

**Preparation and analysis of bacterial lysates.** Samples (5 ml) of the infected cultures were poured on to frozen 0.1 M-NaCl which stopped further DNA synthesis, and the bacteria collected by centrifuging for 5 min at 3500 g. The bacteria were resuspended in 0.4 ml of 25% sucrose in 0.01 M-tris-HCl, pH 8.1 and converted to spheroplasts by mixing with 0.2 ml of a 1:1 mixture of lysozyme (0.85 mg/ml) in 0.25 M-tris-HCl, pH 8.1 and EDTA (2.7 mg/ml) according to Godson & Sinsheimer (1967). The mixture was kept on ice for 5 min before lysis by one of the methods given below.

**Brij lysis.** To 0.6 ml of spheroplast suspension was added 0.4 ml of a 1:1 mixture of Brij 58 and sodium deoxycholate prepared as described by Godson & Sinsheimer (1967), except that Mg\textsuperscript{2+} ions were absent. The lysis conditions do not affect the viability of T5 phage. The lysate samples (0.1 to 0.2 ml) were layered directly on to sucrose gradients. After centrifuging, the recovery of radioactivity in DNA was always better than 80%.

**Sarkosyl lysis** (Earhart et al. 1968). A spheroplast suspension (0.6 ml) prepared as above was gently mixed with 0.4 ml of 0.02 M-MgCl\textsubscript{2}. An 0.1 sample of this was then layered on to a sucrose gradient (see below) with 0.01 ml of 1% sarkosyl and the spheroplasts lysed by gently mixing with the tip of a pipette. White crystals of a Mg\textsuperscript{2+}-sarkosyl complex were formed, and after centrifuging for 15 min at 15000 rev/min in the Spinco SW39 rotor, the complex appeared as the visible ‘M-band’ half way down the tube. The M-band could be removed with a syringe, and contained pulse-labelled DNA. Exogenously added $[^{3}H]$-phage T5 or T5 DNA were found in the top-fraction above the M-band, from which they were absent.

**Sucrose density gradients.** Linear neutral gradients were prepared from sucrose 10 to 25% (w/v) made up in 0.05 M-tris-HCl, pH 7.8, with 1 M-NaCl. Alkaline gradients contained 5 to 20% (w/v) sucrose in 0.1 M-NaOH, 0.9 M-NaCl. For analysis of sarkosyl lysates, 2.5 ml of 15% (w/v) sucrose in 0.01 M-tris-HCl, pH 7.5, 0.1 M-magnesium acetate, 0.1 M-KCl (TMK) was layered on to 2.5 ml 45% (w/v) sucrose in TMK. Sedimentation was in the SW 39 or SW 50 rotor of a Spinco Model L centrifuge. Conditions are given in Fig. legends or in the text.

**RESULTS**

**Brij lysates of T5 infected bacteria: identification of intracellular components**

A culture of *E. coli* c3000 was grown with aeration in low phosphate medium to a cell density of $5 \times 10^8$/ml when T5 phage were added to give an input multiplicity of 10 p.f.u./cell. The culture was labelled with $[^{3}H]$-thymidine (0.5 $\mu$Ci/ml) from 30 to 32 min after infection and the bacteria then harvested and lysed by the lysozyme-Brij procedure. A sample of the lysate was centrifuged for 30 min at 30000 rev/min through a 10 to 25% sucrose density gradient, after which fractionation revealed the presence of the two rapidly sedimenting species previously identified as phage heads and intact virus particles (Lunt & Kay, 1968), with a large amount of slower sedimenting material. The latter was converted to acid-soluble material by treatment of the lysate with DNase (0.25 $\mu$g/ml) indicating that the slow-moving peak consisted of free intracellular DNA. When a similarly prepared lysate was centrifuged at 38000 rev/min for 105 min, the phage particles and heads formed a pellet above which the gradient contained two types of DNA. One heterogeneous species sedimented up to 2.5 times faster than $[^{32}P]$-labelled marker DNA from intact T5 phage. The slower sedimenting species coincided with the virus DNA marker. These two intracellular DNA
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Bottom to Top

(a) 10 20 30 Fraction number

(b) 6 6 4 4 2 2 0 6

(c) 6 6 4 4 2 2 0 6

Top to Bottom

(d) 10 20 30 Fraction number

(e) 6 6 4 4 2 2 0 6

(f) 6 6 4 4 2 2 0 6

Fig. 1. ‘Pulse-chase’ experiment. Distribution of radioactivity from Brij lysates of T5-infected bacteria after sedimentation through neutral sucrose gradients at 38000 rev/min for 105 min. Two-drop fractions were collected , [3H]; ———, [32P]. Continuous [32P]-phosphate label and [3H]-thymidine 20 s pulse at 30 min after infection. Times of samples during ‘chase’ period: (a) 30 min 20 s, (b) 30 min 40 s, (c) 31 min 21 s, (d) 32 min 40 s, (e) 35 min 20 s, (f) 38 min 20 s.

components were named fast sedimenting form (fsf) and slow sedimenting form (ssf) respectively.

Kinetics of labelling of intracellular DNA components

To investigate the sequence of formation of the different components, [32P]-phosphate was added to label all cellular DNA and the fate of a [3H]-thymidine pulse label was then followed by measuring the ratio [3H/32P] in the different fractions.

In one experiment, an exponentially growing culture of E. coli 3000 at 3 × 10⁸/ml was provided with [32P]-phosphate (3 μCi/ml). When the cell density reached 5 × 10⁸/ml, the bacteria were infected with T5. After 30 min of infection, [3H]-thymidine was added (2 μCi/ml) and 20 s later 0.5 mg of ‘cold’ thymidine to end the pulse. Five ml samples of the culture were removed at intervals for preparation of Brij lysates. Total DNA was also estimated at intervals to ensure that the infection was proceeding normally. Fig. 1 shows the distribution of [32P] and [3H] in the sucrose density gradient analyses of 0.1 ml samples of the Brij lysates made during the ‘chase’ period.

Using the procedures of Lunt & Kay (1968), an estimate of the total DNA in the different
components was determined after summing the $[^{32}P]$ counts in the fsf (10 fractions) and the ssf (5 fractions) of the different gradients. The $[^{32}P]$ distribution in Fig. 1f shows poor resolution of the fsf and ssf. Nevertheless, the results in Fig. 2 support the overall $[^{32}P]$ recoveries of Fig. 1 and indicate that the amounts of fsf and ssf showed little change during the chase period, although the total DNA content of the culture increased rapidly. The total $[^{3}H]$ content of the fsf and ssf decreased during the chase, as was to be expected for intermediates in phage formation. The $[^{3}H]/[^{32}P]$ ratios were measured after recounting the vials corresponding to the three peak fractions for both the fsf and ssf and averaging the values obtained. The specific activities of the fsf and ssf were calculated from the radioactivity ratios and are shown in Fig. 3. Although rapidly labelled DNA includes some material which sediments in the position of the ssf, initially the specific activity of the fsf was the greater, and while it dropped that of the ssf rose to a maximum before it too began to fall. This labelling pattern is consistent with the fsf being a precursor of the ssf.

In an exactly similar 'pulse-chase' experiment Brij lysates were sedimented through sucrose gradients (15 to 30%, w/v, in 0·05 M-tris-HCl, pH 7·8) at slow speed (30,000 rev/min for 30 min) so that all four components, namely heads, phage, fsf and ssf could be distinguished on one gradient. The resolution was sufficient to estimate the changes in specific activity and show that the ssf was a precursor of phage heads (Fig. 4). As found previously (Lunt & Kay, 1968), DNA estimations showed that the amounts of the heads remained constant while those of phage increased.

To determine at what time after infection the ssf was first made, a 1 min pulse of $[^{3}H]$-thymidine was given to a culture at 12 min after infection. Analysis of Brij lysates of samples taken at 13 to 24 min showed that although fsf was present at the beginning of the chase, the ssf did not appear until 15 min, while accumulation of radioactivity in the ssf at the expense of fsf was not apparent until between 20 and 24 min after infection. Similar results
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Fig. 3. Changes in specific activities of fsf and ssf DNA during the ‘pulse-chase’ experiment shown in Fig. 1. ▲—▲, fsf; ○—○, ssf.

Fig. 4. Changes in specific activities of ssf DNA and phage heads during a ‘pulse-chase’ experiment. ○—○, ssf; ●—●, phage heads. Specific activities were determined according to Lunt & Kay (1968).
were obtained in experiments where the pulse labels were administered at different times after infection.

**Characterization of the fast-sedimenting and slow-sedimenting forms of intracellular T5 DNA**

To prepare fsf and ssf DNA, Brij lysates were made from bacteria immediately after they had been labelled for 2 min with $[^3H]$-thymidine at 30 min after infection. The lysates were centrifuged through sucrose gradients and, to prevent shearing, the fractions were collected by displacing the fractions out of the tops of the centrifuge tubes. Alternate one-drop and three-drop fractions were collected and the peaks of radioactivity located by measuring the radioactivity in the one-drop fractions. The two three-drop fractions closest to the fsf and the ssf peaks were pooled separately.
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Fig. 6. Breakage of [3H] ssf by shear: conditions see Fig. 5. (a) unsheared; (b) gently sheared; (c) vigorously sheared. ––, [3H] counts; –––, [14C] counts of T5 DNA mixed with ssf before shearing.

Sedimentation rate and sensitivity to shear

The isolated fractions (0.5 ml) were diluted with tris-EDTA buffer mixed with 0.04 ml of [14C]-T5 DNA and 0.1 ml samples were re-run on sucrose density gradients with and without subjecting them to shear by blowing them through a 0.1 ml pipette. The ssf contained some material which sedimented in the position occupied by the fsf, but, apart from this, the sedimentation characteristics of both forms were retained on recentrifugation. The fsf was very shear-sensitive, even with gentle pipetting which hardly affected the sedimentation of the ssf, or [14C]-labelled T5 phage marker DNA (Figs. 5, 6).

Absence of protein in fsf and ssf DNA

Treatment with pronase (100 µg/ml) at 37 °C did not affect the sedimentation rates, nor did [35S]-labelled material in the lysates sediment with the free DNA species when [35S]-sulphate was added to the culture before or after infection.
Adsorption to nitrocellulose powder

Nitrocellulose powder adsorbs single-stranded, but not double-stranded DNA from concentrated salt solutions (Nygaard & Hall, 1963; Oishi, 1968). We found that ssf DNA was almost completely (96%) bound and at the end of a chase the ssf DNA was 86% bound to nitrocellulose powder. Under the same conditions, denatured T5 DNA was 98% adsorbed, while native T5 DNA, and native phage λ DNA which has single-stranded ends twelve nucleotides long (Wu & Taylor, 1971) were not retained by the nitrocellulose columns. These results suggested that the ssf DNA was not identical with native DNA from mature phage, although it possessed closely similar sedimentation properties. To test whether the ssf T5 DNA might have complementary single-stranded ends as occur in λ DNA, we heated preparations of the ssf at 65 °C for 40 min in SSC (Ritchie et al. 1967). After this treatment there was no change in the sedimentation rate showing that no circles or concatemers had been formed, and that complementary single-stranded ends were therefore probably absent.

Alkaline sucrose density gradient sedimentation

DNA from wild-type T5 phage shows three or more major peaks of radioactivity when sedimented through alkaline gradients. The size distribution of major single-strand molecules depends on the positions of single-strand nicks in the original native T5 DNA and is characteristic of the phage strain used (Abelson & Thomas, 1966; Hayward & Smith, 1972a, b). The pattern we obtained with our T5 strain is shown in Fig. 7a. It resembles that found by Abelson & Thomas (1966) and is consistent with the distribution of nicks proposed by Bujard (1969) and by Hayward & Smith (1972a, b).

To examine the size distribution of rapidly labelled DNA, a 2 min [3H]-thymidine pulse-labelled sample was isolated from a neutral gradient and then material from the leading edge and middle of the ssf, and material trailing into the ssf region were separately examined on alkaline sucrose gradients with [32P]-labelled T5 phage DNA as marker. The results in Fig. 7 show that all the pulse-labelled DNA yields material with a continuous size distribution, and not a series of radioactive peaks as would be expected for a collection of fragments with a few different unique sizes. About 20% of the fsf radioactivity sedimented faster than the longest single strands from mature virus DNA.

FsF DNA which had been continuously labelled with [32P]-phosphate was also isolated on neutral and then analysed on alkaline gradients (Fig. 8). It showed heterogeneity in mol. wt. distribution, but with two major peaks of radioactivity, unlike the symmetrical distribution found in the pulse-labelled ssf DNA.

The patterns of radioactivity observed suggest that the newly synthesized (i.e. pulse-labelled) DNA contains a large number of randomly spaced single-strand nicks, the repair of which produces the asymmetrical spread of radioactivity found on alkaline sedimentation of the continuously labelled ssf DNA.

Following a 1 min [3H]-thymidine pulse/7.5 min chase, ssf DNA from a neutral gradient was analysed on an alkaline gradient. The result (Fig. 9) showed that the single stranded fragments sedimented heterogeneously with two radioactive peaks roughly corresponding to the two faster-moving components observed with [32P]-labelled DNA from mature phage. There was a noticeable absence of [3H] counts from the region of the slower moving [32P]-labelled peak from mature T5 phage DNA.
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Fig. 7. Alkaline sucrose density gradients of 2 min [3H]-pulse-labelled DNA previously isolated from a neutral gradient. Sedimentation was at 39,000 rev/min for 140 min. ———, [3H] counts; ——, [32P] counts of marker T5-phage DNA. † indicates peak positions of [32P] counts of markers in gradients (b) and (c). (a) leading edge of pulse-labelled DNA; (b) middle region of pulse-labelled DNA; (c) pulse-labelled DNA from region of ssf and T5 phage DNA on neutral gradient.

Analysis of sarkosyl lysates of T5 phage-infected bacteria

An alternative method using mild lysis conditions with sarkosyl was investigated in case it could indicate whether the ssf DNA might only occur in Brij lysates as a breakdown product of a fragile precursor of phage heads.

Trial experiments showed that whereas 70% of [3H]-thymidine pulse-labelled DNA appeared in the M-band, [32P]-labelled T5 phage or mature phage DNA were almost entirely (96 to 97%) located above the M-band in the sucrose gradients. The result of a pulse-chase experiment was consistent with the M-band DNA being a precursor of top-fraction DNA (Fig. 10). Disruption of the M-band with Brij and deoxycholate followed by density gradient sedimentation analysis revealed the presence of both fsf and ssf DNA components. The
top-fraction radioactivity was mainly in the form of heads and phage, while the small amount of free DNA present was of lower mol. wt. than ssf.

The ssf DNA of the M-band was not formed by shear degradation of the fsf because a double-label pulse-chase experiment showed that the specific activities of the two species were quite different, and resembled those found when the two components were obtained directly from a Brij lysate of the same culture.

**The intracellular DNA of phage T2-infected bacteria**

T2 is a phage whose particles consist of a mixture in which each virus particle contains one DNA molecule, the sequence of which is a circular permutation of the other sequences in the collection. We wanted to know whether after Brij lysis individual genomes could be detected as free intracellular DNA molecules corresponding to the ssf of T5 DNA.

A culture of *E. coli* b was infected with T2 and 12 min later a 20 s pulse of [3H]-thymidine was given. Samples were removed at 13 and 17 min and lysed by the Brij method. Analysis of these lysates showed that they contained little or no [3H]-labelled material which sedimented with [32P]-labelled DNA from intact T2 phage. This result agrees with that of Frankel (1966) who showed that after lysis with lysozyme and SDS no mature virus DNA could be detected in bacteria infected with T4 phage.

**Effects of inhibitors on phage T5 replication**

**Chloramphenicol**

This antibiotic blocks T5 phage DNA synthesis, indicating that the process depends on concomitant protein synthesis (Crawford, 1959). We used the double-label technique and Brij lysis to examine the fate of a [3H]-thymidine pulse at 30 min after the addition of chloramphenicol (45 μg/ml) at 26 min after infection. After 32 min there were no changes
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Fig. 10. Specific activity changes in the M-band and top fraction during a 'pulse-chase' experiment. The culture was continuously labelled with [35P]-phosphate and a 20 s 'pulse' label of [3H]-thymidine given at 25 min after infection. After separation by the sarkosyl method the specific activities were measured from the [3H]/[35P] ratios of the alkali- and acid-insoluble material in the two fractions. ○--○, M band; ●--●, top fraction.

Fig. 11. (a) Specific activity changes in fsf and ssf DNA following addition of chloramphenicol at 26 min after infection and [3H]-thymidine at 30 min after infection. ○--○, fsf; ●--●, ssf. (b) Changes in total amounts of components, after chloramphenicol addition as in (a). □--□, [3H]; ●--●, total DNA-deoxyribose; △--△, fsf DNA-deoxyribose; ○--○, ssf DNA-deoxyribose.

in the specific activities of the fsf and ssf components, although total DNA synthesis did not stop until 35 min after infection (Fig. 11). We concluded that the conversion of fsf to ssf requires continual protein synthesis. The results cannot indicate whether DNA synthesis requires continual protein synthesis independently of that needed for the formation of the ssf.

Rifampicin

This antibiotic prevents RNA synthesis by combining with the E. coli DNA-dependent RNA polymerase (Wehrli et al. 1968). We found that with T5-infected bacteria, rifampicin concentrations between 25 and 50 μg/ml reduced the phage burst size to one. The effect on DNA synthesis of adding rifampicin at different times after infection is shown in Fig. 12.
A separate experiment showed that when rifampicin was added at 23 min after infection RNA synthesis ([3H]-uracil uptake) stopped 2 min later, and protein synthesis ([35S]-sulphate uptake) 7 min later. Hence, the reduction in DNA synthesis at 35 min (Fig. 11) was probably caused by the reduced protein synthesis and not by a direct effect of rifampicin on DNA synthesis.

After addition of rifampicin (33 μg/ml) at 23 min, and of [3H]-thymidine for 20 s at 30 min, a Brij lysate was made at 38 min after infection and analysed on a sucrose density gradient. The rifampicin-inhibited culture contained a [3H]-labelled component (X) which sedimented slightly faster than mature phage. Analysis of lysates of infected bacteria uniformly labelled with [32P]-phosphate and grown in the absence and presence of rifampicin (added at 23 min) showed that the decrease in the amount of phage present was almost entirely compensated for by the increase in X. Component X was occasionally found in wild-type infections, but not during infections by mutants T5.B2 and T5.N1 when phage heads accumulate. It was tentatively concluded that X is an intermediate between heads and mature phage.

**Characterization of amber mutants of phage T5**

Complementation tests showed that the four mutants examined here were defective in different cistrons. Using the su− host *E. coli* c3000, each mutant was examined for its ability to induce functions typical of the three main classes of T5 proteins described by Lanni (1968), and McCorquodale & Buchanan (1968), and which appear sequentially during infection. The results are summarized in Table 1 which shows that the mutants fall into three categories: T5.B3 development is blocked at an early stage since no virus DNA is made; T5.B2 and T5.N1 are blocked at late stages in development since no mature phage are made, although phage heads accumulate.
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Fig. 13. ‘Pulse-chase’ experiment after infection of Escherichia coli c3000 with T5 B1. A 20 s pulse of [3H]-thymidine was given at 30 min after infection. Brij lysates were made at (a) 30 min 20 s and (b) 40 min 20 s and analysed on neutral sucrose gradients. Sedimentation was at 40000 rev/min for 105 min. † indicates position of [32P]-labelled T5 DNA marker.

The most interesting mutant for the studies here proved to be T5 B1. Usually with this mutant virus DNA synthesis stopped at about 30 min although in some instances synthesis continued until about 35 min after infection. A pulse-chase experiment showed that although fsf DNA was formed it was not converted to ssf DNA, nor to phage heads. The fsf DNA made initially showed a broad sedimentation profile with considerable material in the low

Table 1. T5 phage amber mutants

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<th>Growth on E. coli c3000 (su⁻)</th>
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<tbody>
<tr>
<td></td>
<td>Host DNA breakdown</td>
</tr>
<tr>
<td>T5. am B3</td>
<td>+</td>
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<tr>
<td>T5. am B1</td>
<td>+</td>
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<tr>
<td>T5. am B2</td>
<td>+</td>
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<td>T5. am N1</td>
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+, Levels same as on Escherichia coli CR63 (su⁺); (+), ≤ 10% as on E. coli CR63 (su⁺); -, no detectable amounts.
mol. wt. range. After the chase, less slow-sedimenting material was present resulting in a more symmetrical peak of the fsf (Fig. 13).

The colorimetric assay for T5-induced DNase appearing 10 min after infection and described by Stone & Burton (1962) showed that although T5. B1 induced normal levels of this enzyme in the su + host *E. coli* cr63, no increased levels of DNase appeared when su - hosts such as strain c3000 or b/2 were infected. (These experiments were done by Mr R. F. Holcombe.)

These results confirm that the conversion of fsf to ssf DNA is a T5 specific function, and imply that the phage-induced exonuclease is required for the process.

**DISCUSSION**

These results show that bacteria infected with wild-type T5 contain mature phage, heads and free DNA which can be resolved into the fsf and ssf on density gradients. For the formation and incorporation of DNA into intact virus particles, the results of pulse-chase experiments (this paper, and Lunt & Kay, 1968) are consistent with the sequence

\[
\text{fsf} \rightarrow \text{ssf} \rightarrow \text{heads} \rightarrow \text{phage}. 
\]

This sequence is supported by the analysis of intracellular components which accumulated in su + host bacteria infected with the T5 amber mutants examined here. The results with the mutant T5. B1 were particularly useful for indicating that fsf is a precursor of ssf, since the latter is not formed, although fsf accumulates during these infections.

The properties of the fast sedimenting intracellular DNA resemble those of concatemer type DNA found in bacteria after infection by other viruses: these properties are a high sedimentation coefficient on neutral sucrose gradients and extreme sensitivity to shear. We also observed strong binding to nitrocellulose, a property shown by replicating DNA from bacteria (Oishi, 1968), and believed to be associated with the presence of single-stranded regions. In addition, one preparation of fsf DNA was examined with the electron microscope when branched molecules up to twice the length of mature T5 phage DNA were found (unpublished observations with Dr R. Millward, Imperial Cancer Research Fund). The fsf thus appears identical with the fast-sedimenting replicating T5 DNA first described by Smith & Burton (1966) and Smith & Skalka (1966).

The results of the pulse-chase experiment described in Fig. 1 indicate that rapidly labelled replicating wild-type T5 DNA is very heterogeneous in size. After about 2 min of the chase period, the radioactivity is more symmetrically distributed about the peaks characteristic of the fsf and ssf. With the mutant T5. B1, when ssf is not formed, the pulse-chase more obviously shows the conversion of slower sedimenting, rapidly labelled DNA to pieces sedimenting in the fsf region (Fig. 13). The origin of the rapidly labelled short pieces is not clear. Possibly they arise through breakage near branch points in newly synthesized DNA, the label of which is transferred to more stable regions during the chase period.

The results of sedimenting fsf DNA through alkaline gradients suggest that many single-strand nicks are present, some of which are repaired while the DNA is still in the fsf. Interestingly, Hayward & Smith (1972a) have shown that in DNA from intact T5, besides the pattern of ‘major’ single-strand nicks which is characteristic of all the DNA molecules in a collection, there are additional nicks which are probably varied over the population as a whole, but which provide a reproducible pattern of minor fragments in denatured DNA preparations. Possibly these minor fragments result from unrepaired nicks originally present in the replicating phage DNA and which persist into the virus DNA.
Replication of phage T5

The structure of the ssf is not clear. It resembles DNA from mature phage in its resistance to shear and in its rate of sedimentation through neutral sucrose gradients. Consistent with this, preliminary results with electron microscopy indicate that ssf DNA is the same length as DNA from mature phage. However, unlike mature phage DNA, the ssf component is retained by nitrocellulose, although slightly less than is fsf DNA. The extent of the binding, 86% at the end of a 75 min chase, made it seem unlikely that this resulted simply from contamination with fsf fractions, or with rapidly labelled DNA present in the ssf region. Although our annealing conditions may have been unsuitable for their detection, it appears that complementary single-stranded ends are absent. The result in Fig. 9 shows that ssf DNA sediments heterogeneously on alkaline sucrose gradients. The distribution is similar, although not identical with that of DNA from intact T5 phage, the difference being the presence of a shoulder rather than a peak in the slower sedimenting region of the ssf profile. The effect is a slight one, but was reproducible in several experiments. In conclusion, the properties of the ssf suggest that it may possess single-stranded regions and a pattern of nicks slightly different from that in virus DNA.

The differences between ssf and mature virus DNA make it seem unlikely that ssf is a breakdown product of partially formed head structures. In addition, analyses of sarkosyl lysates show that ssf remains associated with the M-band, suggesting that ssf was present intracellularly as free or membrane-bound molecules, and not condensed into compact structures which would be expected to escape into the top fraction.

The existence of ssf as a free DNA component which can be released by gentle lysis of infected bacteria supports the postulate that collections of T5 phage DNA molecules are excised directly from concatemers before being incorporated into phage heads. However, the mechanism whereby ssf is produced from fsf remains obscure. The scheme proposed by Kelly & Thomas (1969) could apply to T5 and involves an initial DNA ‘nickase’ attack followed by DNA polymerase action, thereby generating molecules with terminal redundancy corresponding to the DNA length between the initial nicks. Certainly the formation of unique pieces of DNA from concatemers would appear to demand the action of a sequence-specific endonuclease. However, it is difficult to envisage plausible mechanisms which require the participation of the phage-induced 5’ exonuclease for the conversion of fsf to ssf DNA, and which is indicated by the experiments with the mutant T5.B1 in non-permissive hosts. In interpreting these experiments we assume that the T5 DNase we detected by the colorimetric assay of Stone & Burton (1962) is identical with the enzyme purified by Paul & Lenman (1966) and more recently by Frenkel & Richardson (1971a). The latter authors have shown that the enzyme acts as an exonuclease which removes nucleoside 5’ monophosphates and acid-soluble 5’ phosphoryl terminated oligonucleotides from the 5’ ends of single-or double-stranded DNA.

The results with T5.B1 show that the absence of the 5’ exonuclease is associated with inability to convert fsf to ssf and with a premature halt to net DNA synthesis. Our results with chloramphenicol added at 26 min after infection indicated that the conversion of fsf to ssf stopped about 3 to 4 min before DNA synthesis stopped. It therefore seems unlikely that continual DNA synthesis is essential for the conversion. Normally the 5’ exonuclease is formed at 12 to 15 min after infection (Paul & Lehman, 1966; Fielding & Lunt, 1970) when T5 DNA synthesis begins. During infections with T5.B1 no exonuclease is present, yet T5 DNA synthesis is initiated and continues until about 30 min after infection, although no ssf can be detected. Together with the chloramphenicol experiment this result supports the possibility that accumulation of fsf precedes and could even be responsible for the eventual halt to DNA synthesis. The results throw no light on the part played by the 5’
exonuclease. These problems have also been discussed by Frenkel & Richardson (1971b) who have isolated a T5 mutant with a temperature-sensitive 5' exonuclease and which at non-permissive temperatures shows similar behaviour to T5.B1.

In conclusion, our results indicate that T5 phage maturation involves the formation of phage-size pieces of DNA, probably of unusual structure as intermediates between replicating DNA and phage heads. The T5 phage-induced 5' exonuclease appears to be required for this process but its role is unknown.

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REFERENCES


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