ATTEMPTS TO LINK THE INTERRUPTIONS IN T5 BACTERIOPHAGE DNA WITH AN ENZYME FROM E. COLI

H. Moroson and A. Anello, Physical Biology Division,
Sloan-Kettering Institute for Cancer Research, Rye, New York

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The T5 bacteriophage DNA molecule has been demonstrated by Abelson and Thomas (1966) to contain gaps at specific locations in both of its polynucleotide chains. It has also been demonstrated by several workers that an enzyme from E. coli has the property of joining preformed polynucleotides in phosphodiester linkage. This joining enzyme has been used by Gellert (1967) to form covalent circles of λ DNA from hydrogen-bonded circles, by Olivera and Lehman (1967) to join synthetic preformed polynucleotides, and by Gefter, Becker and Hurwitz (1967) to repair λ DNA containing single strand breaks. These demonstrations have been extremely stimulating to many workers, and we decided to attempt to link the naturally present single-strand gaps in T5 bacteriophage DNA by means of this enzyme. A negative result would not necessarily mean the impossibility of achieving such repair, while a positive result could be helpful in understanding the nature of these gaps and would also supply an interestingly modified T5 phage for further genetic studies. Our preliminary results indicated this rejoining does not take place.

In this paper we describe attempts to convert T5 DNA containing single strand breaks to a form which would sediment in a manner resembling ungapped DNA in an alkaline sucrose gradient. Experiments were also carried out with T5 phage having additional breaks introduced in its DNA by P^{32} decay, or by treatment with methylhydrazine, as well as with T7 phage having breaks introduced into its DNA by X-rays.

Materials and Methods

Preparation of Enzyme Crude Extract

Crude enzyme extract (kindly donated by L. F. Cavalieri) was prepared from frozen E. coli cells and purified by streptomycin sulfate by the method of Olivera and Lehman (1967). The extract was a highly viscous material and was stored at -20°C prior to use. To 200 mg of this sample was added 2.0 ml phosphate-EDTA buffer and shaken till practically all dissolved. The enzyme incubation mixture followed that of Olivera and Lehman (1967) incorporating magnesium and DPN. Large amounts of enzyme were used and the incubation period was usually 2 hours at temperatures of 20-37°C. In some experiments, an incubation time of 2 days at 18°C was employed. For treatment of phage, to 0.6 ml of phage in saline-MgCl (.005M) buffer, was added .10 ml enzyme solution and .10 ml of DPN (1 mg/ml) followed by incubation. The action of the enzyme involves the formation of a phosphodiester linkage between a 5'-phosphomonoester group and a 3'-hydroxyl group when these are present at the site of a single-stranded break.

Zonal Centrifugation

Sedimentation of T5 DNA was carried out in a 5 - 20% sucrose gradient, according to the method of McGrath and Williams (1966), the sucrose being dissolved in .9 M NaCl, .1 M NaOH, and 10^{-3}M ethylenediamine tetraacetate. In a small triple gradient Buchler mixer, 7.2 cc of 5% alkaline sucrose and 6.6 cc of 20% alkaline sucrose are metered with a pump into three 5.0 ml cellulose centrifuge tubes. 0.1 ml samples of phage containing ∿20 ug DNA (20,000-40,000 cpm) are slowly layered on each tube, spread with a pin, and spun in the Spinco Model L centrifuge, SW39 rotor for 3 hrs at 35,000 rpm at 20°C. The bottom of the tube is pierced and 4 drop samples are immediately collected in copper planchets for ^{32}P counting or filter paper disks for ^{3}H counting. Because of the presence of Mg^{++} which caused hydroxide precipitation and consequent spinning down of all DNA to the bottom of the centrifuge tube, it was necessary to also add to the initial phage sample (.6 ml) 4 μg

EDTA prior to layering on the gradient. This had the result of overcoming the Mg^{++} precipitation effect and gave reproducible DNA profiles.

Calculation of DNA Molecular Weight

The $S_{20,w}$ values were calculated from the distance traveled of the main peak(s) using the equation $S_{20} = \frac{(6.45 \times 10^{10})D}{w^2 t}$ from Burgi and Hershey (1963) where D stands for the distance sedimented in a concentration gradient of sucrose. The calibration of molecular weight with $S^\circ_{20,w}$ of Studier (1965), $S^\circ_{20,w} = .0528\ M^{.400}$ for alkaline DNA was used, where the average distance traveled was calculated for each fraction as Σ fidi/Σfi, where fi is the fraction of total activity and di is the distance moved for each fraction.

Demonstration of Enzyme Activity

In order to insure the presence of ligase activity in the enzyme extract, single breaks (as well as double breaks) were introduced into T7 phage by irradiating a suspension of phage in saline-phosphate buffer with an X-ray dose of 40 Krads, shown by Freifelder (1965) to introduce about 20 single breaks in each phage particle. Irradiated and control phage were then incubated with enzyme extract prior to layering upon the alkaline sucrose gradient. The repair of a large fraction of X-ray introduced single breaks was demonstrated in this manner. Additional single strand breaks were also introduced into T5 phage by allowing ^{32}P decay, Abelson & Thomas (1966), and by incubating T5 phage with a 10^{-3}M concentration of methylhydrazine (MH) in saline phosphate buffer for two hrs at 25°C. Placque forming ability of T5 dropped to about 10% of its initial value after MH treatment, so MH is at least as toxic as 40 Krads X-radiation, although less reproducible, and appeared to introduce as many breaks.

Growth of Coliphage T7 and T5

Labeled T5 or T7 coliphage is grown in the following way (Adams, 1959):

Into each of 4-100 ml milk dilution bottles add 100 µc P^{32} (or 100 µc tritiated thymidine) in 0.25 µl to TPG buffer, and 2.0 µl of an overnight culture of E. coli B/rCSH. Grow at 37°C with aeration for 2-1/2 hrs, and

then add 0.6 ml of phage at a titer of 1 X 10^{11} phage/ml. After 3 hours for T5 or 1 hour for T7 additional incubation lysis occurs with clearing. Add 2 drops of chloroform, shake, and let stand for 10 min. Filter through a celite pack, and collect phage by centrifugation at 18,000 rpm for 30 min. Dissolve pellet in 5.0 ml of BPES buffer, add crystal of DNAase and incubate 2 hr at 37°C. Spin 10 min at 8000 rpm to remove debris, and 18,000 rpm for 30 min to pelletize the phage. Dissolve pellet in 2.5 ml saline - Mg^{++} buffer and assay for phage titer and radioactivity. Typical titer is 8 X 10^{11} phage/ml and 6000-7000 cpm of P^{32} per 10 λ in a Nuclear Chicago Gas Flow Counter. Ratio of P^{32} atoms per phage is less than .05.

Results and Discussion

Alkaline sucrose gradient profiles of T7 DNA are shown in Fig. 1a-d. The position of the peak for control T7 phage (1a) corresponding to $S^{\circ}_{20,w}$ of 36.4 and a single strand molecular weight of 12.8 X 10^6, is in close agreement with Studier's (1965) value of 13.5 X 10^6. Incubation of phage T7 with the enzyme extract for 2 hrs at 37°C results in a changed alkaline gradient profile (1b). The main peak is lowered, and a new peak, corresponding to a molecular weight of 5.9 X 10^5 appears, presumably due to nuclease activity in the crude extract. X-irradiation of T7 phage in buffer results in a shift of the DNA peak to a new position closer to the top of the gradient, corresponding to a $\overline{MW}$ of 3.5 X 10^5, (1c) compared with the expected value from Freifelder (1965) for this dose of radiation of ~ 6.4 X 10^5. Incubation of X-irradiated phage with enzyme extract however results in the reappearance of a high molecular weight peak ($\overline{MW}$ 9.1 X 10^6), and a reduction in the height of the low molecular weight peak ($\overline{MW}$ = 3.5 X 10^5) indicating rejoining of radiation produced single breaks as well as degradation of intact phage DNA (1d).

The effect of enzyme action on artificially produced breaks in T5 DNA

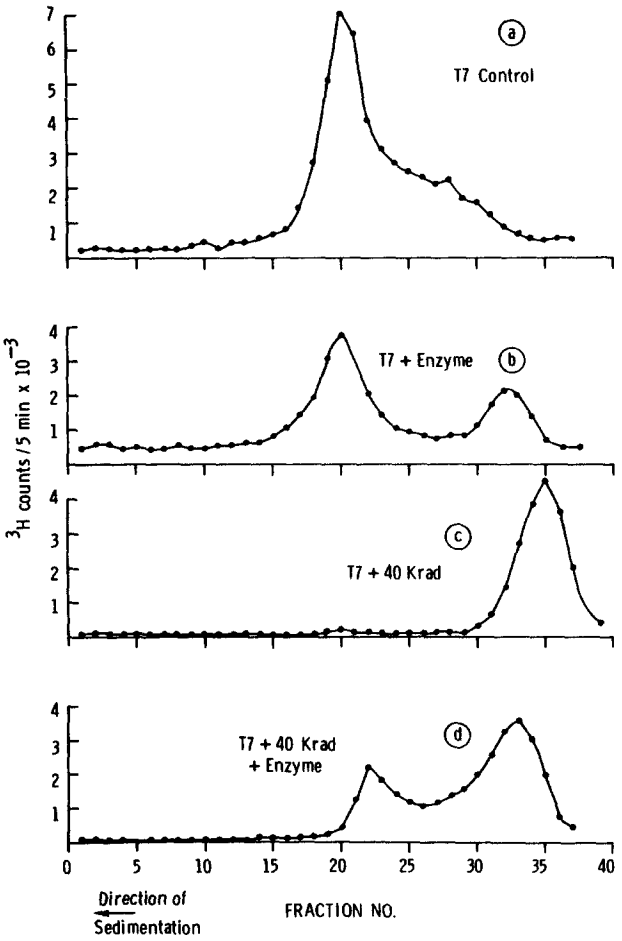

Figure 1 Demonstration of strand joining enzyme activity as well as strand breaking enzyme activity in T7 coliphage DNA. Alkaline sucrose density gradient profiles of T7 coliphage labeled with tritiated thymidine.

a. Control T7 DNA layered on alkaline sucrose gradient and spun in SW39 rotor at 35,000 rpm for 3.0 hrs.

b. T7 coliphage incubated with enzyme extract, DPN and Mg^{++} and incorporated for 2 hrs at 37°C prior to layering on gradient.

c. T7 coliphage receiving 40 Krads X-rays in buffer coliphage receiving 40 Krads X-rays followed by incubation with enzyme extract, DPN and Mg^{++} prior to layering on gradient.

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was investigated by treatment of T5 phage with methylhydrazine, a drug previously observed by us to introduce breaks into bacterial DNA, followed by enzyme incubation is shown in Fig. 2. The broad 4-peaked profile of control T5, is

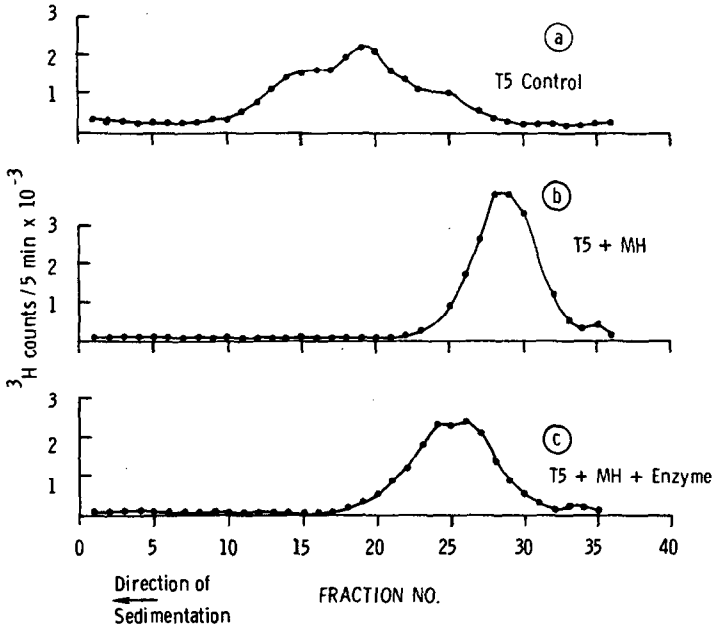

Figure 2 Demonstration of strand breakage in tritium labeled T5 coliphage by treatment methyl hydrazine and partial repair of these breaks by incubation for 2 hrs at 37°C with enzyme extract, DPN and Mg^{++}.

a. Control T5 coliphage layered on alkaline sucrose gradient and spun in SW39 rotor at 35,000 rpm for 3.0 hrs.

b. T5 coliphage incubated with 10^{-3}M methyl hydrazine for 30 min at 0°C prior to layering on alkaline sucrose gradient.

c. T5 coliphage incubated with 10^{-3}M methyl hydrazine for 30 min at 0°C followed by incubation with enzyme extract for 2 hrs at 37°C prior to layering on alkaline sucrose gradient.

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converted to a single narrow band at peak position corresponding to $S^\circ_{20,w}$ (2a) of 17.6, and an $\overline{MW}$ of 2.2×10^6 following incubation for 30 min with 5×10^{-3}M MH (2b). Incubation of this MH treated phage with enzyme extract results in a shift of the alkaline sucrose gradient profile to a new position corresponding to an $S^\circ_{20,w}$ of 25.5, and a $\overline{MW}$ of 5.4×10^6 (2c), i.e. more than twice the original molecular weight, strongly suggesting enzyme repair of single-strand breaks produced by treatment of T5 with MH.

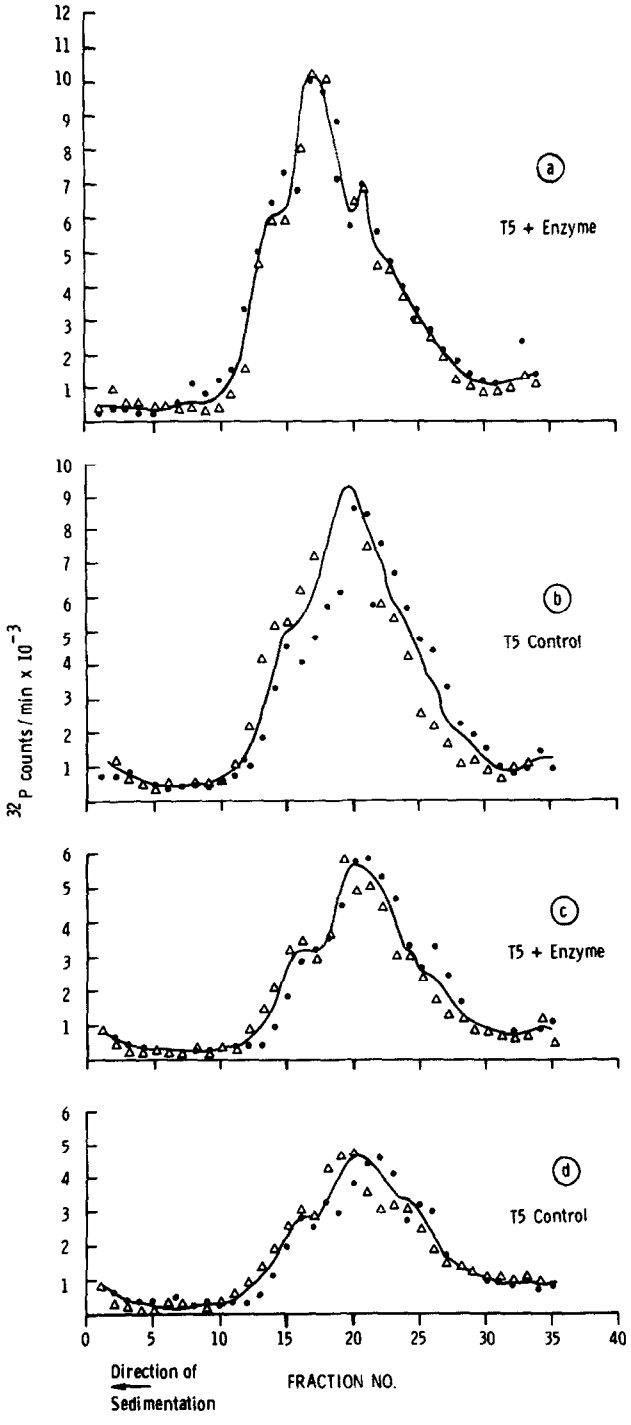

Figure 3 Demonstration of lack of rejoining activity of preexisting single strand breaks and ^{32}P decay produced strand breaks in T5 coliphage DNA.

a. T5 coliphage lightly labeled with ^{32}P incubated with enzyme extract, DPN and Mg^{++} for 2 hrs at 37°C prior to layering on alkaline sucrose gradient and spinning in SW39 rotor at 35,000 rpm for 3.0 hrs. The different symbols represent two different runs.

b. T5 coliphage control.

c. T5 coliphage treated as in a. followed by storage for 10 days at 10-15°C in the presence of enzyme extract, prior to layering and spinning on alkaline sucrose gradient.

d. T5 coliphage control, stored for 10 days at 10-15°C prior to layering and spinning on alkaline sucrose gradient.

The next step was to incubate control T5 phage (lightly labeled with ^{32}P) with enzyme extract in an attempt to seal the natural preexisting gaps in T5. If the gaps were to be sealed, the alkaline sucrose gradient profile obtained would be expected to lose its 4 peaked structure, and become sharper and narrower, resembling that of T7 (Fig. 1a). The results actually obtained are shown in Fig. 3. The profile did not sharpen and the fine structure remained after many trials with different incubation temperatures and incubation times. The peak positions for untreated control (3b) are at fractions no. 15, 20, 24, and 26, corresponding to $\overline{MW}$ of 17, 11, 5, and 3 X 10^6, while the enzyme treated T5 phage had peak positions at fractions no. 14, 18, 21, and 23 (3a). These differences are greater than the variation between control experiments, and were repeatedly obtained but are not believed indicative of any joining of preexisting gaps in T5 DNA. Although the sealing of gaps has not been clearly demonstrated with different incubation times, the incubation medium may not have been optimum for this demonstration, or enzyme may not have penetrated the phage. Jacquemin-Sablon and Richardson (1968) have found the interruptions in extracted T5 DNA are removed by the ligase reaction. Zonal gradient centrifugation was then repeated with the same T5 phage 10 days later, after sufficient ^{32}P decays had occurred to introduce additional breaks in the T5 DNA (Fig. 3c and d). Since Fig. 3c and 3d are virtually superimposable and show more breaks than Fig. 3b, it was concluded that ^{32}P breaks unlike X-ray breaks are not reparable by enzyme extract.

It has been shown that partial repair of large numbers of single strand breaks introduced into T7 DNA by X-radiation or T5 DNA by treatment with MH can be observed by treatment with a crude enzyme extract from E. coli. Under similar conditions it was not possible to observe repair of natural breaks in the DNA of T5 phage, or repair of breaks caused by ^{32}P decay in T5 phage.

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