The Integrity of Deoxyribonucleic Acid Extracted from *Escherichia coli* 15T⁻ after Thymine-less Death

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1. The integrity of DNA extracted from *Escherichia coli* strain 15T⁻ after thymine-less death was examined by studying the effects of treatment with aqueous alkali on the solubility in dilute acids and by viscosity and ultracentrifugal measurements, some of which were designed to detect single-strand breaks or inter-strand cross-links. None of the results showed that there was any modification or damage associated with thymine-less death.

Barner & Cohen (1954) discovered that thymine-less bacterial auxotrophs rapidly lose viability when thymine is withdrawn from their growth media. There is much circumstantial evidence to suggest that this loss of viability, known as thymine-less death, results from damage to the bacterial DNA. To summarize: (1) several authors (Dunn & Smith, 1958; McFall & Magasanik, 1962; Mennigmann & Szybalski, 1962a) have reported difficulties in extracting high-molecular-weight DNA from bacteria after thymine-less death; it has also been found (Barner & Cohen, 1954; McFall & Magasanik, 1962) that the DNA of *Escherichia coli* 15T⁻, unlike normal DNA, cannot be reprecipitated from alkali under the conditions of Schmidt & Thannhauser (1945); (2) both ³²P-‘suicide’ (Fuerst & Stent, 1957) and irradiation with ultraviolet light (Gallant & Suskind, 1960, 1961) damage DNA and co-operate with thymine deficiency in producing a loss of viability; (3) the specific transforming activity of DNA extracted from thymine-starved *Bacillus subtilis* is lower than that of DNA extracted from wild-type *B. subtilis* (Mennigmann & Szybalski, 1962b); (4) there is an increase in mutation rate during thymine deficiency (Coughlin & Adelberg, 1956; Wainright & Nevill, 1956; Kanazir, 1958; Kanazir, Barner, Flaks & Cohen, 1959); (5) cells are susceptible to thymine-less death only if they are synthesizing DNA at the time of thymine deprivation (Maaløe & Hanawalt, 1961); (6) lysogenic bacteriophages (Korn & Weissbach, 1962; Melechen & Skaar, 1962) and colicins (Sicard, 1961; Sicard & Devoret, 1962) are induced by thymine starvation. Other inducing agents include ultraviolet irradiation (Lwoff, Siminovitch & Kjeldgaard, 1950),

mitomycin C (Otsuji, Sekiguchi, Iijima & Takagi, 1959), nitrogen mustards (Jacob, 1952) and streptonigrin (Levine & Borthwick, 1963). Each of these agents is known to damage the DNA (Wacker, Dellweg & Jacherts, 1962; Iyer & Szybalski, 1963; Brookes & Lawley, 1963; Cohen, Shaw & Craig, 1963).

With several methods we have been unable to find any direct evidence for changes to the DNA of *E. coli* 15T⁻ during thymine-less death.

MATERIALS AND METHODS

Bacterial cultures. *E. coli* 15T⁻, obtained from Dr S. Cohen, was maintained on agar slants containing 10 mg. of thymine/l. at 4°. Bacteria were grown with aeration at 37° in a liquid minimal medium which contained inorganic salts, as used by Cohen & Arbogast (1950), 0.1% of mannitol as a carbon source and 16 mg. of thymine/l. The mean generation time of the bacteria in this medium was about 55 min. Overnight cultures were diluted tenfold into fresh, warm medium and aerated at 37° until the extinction was 0.3 as measured in 1 cm. cells of a Beckman DU spectrophotometer at 650 mμ. This corresponded to a cell density of about 5 × 10⁸ viable cells/ml. Cultures were then chilled in an ice-bath and centrifuged at 5400g for 10 min. at 4°. The bacterial pellet was washed twice by resuspending in ice-cold saline (0.1 M-NaCl–0.01 M-MgCl₂) and centrifuging before finally resuspending in an equal volume of prewarmed fresh medium containing no added thymine. On aeration at 37° thymine-less death is first evident at 30–40 min. Cells were harvested after 1.5 or 3 hr. of thymine deficiency (about 2% or 0.3% survival respectively). The DNA extracted from these cultures is called ‘after-death’ DNA; DNA extracted from bacteria grown with 16 mg. of thymine/l. is called ‘before-death’ DNA.

Preparation of DNA. Parallel preparations were made by each of three methods from thymine-starved bacteria (0.3% survival after 3 hr.) and from *E. coli* 15T⁻ which had not been starved of thymine.

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Table 1. *Yields and analysis of Escherichia coli 15T⁻ deoxyribonucleic acid isolated by the method of Marmur (1961)*

RNA was measured as ribose assayed by the orcinol reaction and is given as a percentage of the total sugar present (ribose+deoxyribose). A correction was made for the orcinol colour given by the deoxyribose present.

DNA	Yield of bacteria (g. wet wt.)	Yield of DNA (mg.)	RNA content (% of total sugar)	Protein (% w/w, of DNA)	Purine-bound deoxyribose/ phosphorus (molar ratio)
Wild type	8.1	27	1	0.1	0.47
Before-death	8.0	13	0.5	0.2	0.45
After-death	8.0	25	1	0.2	0.45

Method 1. This was that of Marmur (1961). Table 1 shows the yields and compositions of these preparations, the yields being similar for the thymine-starved and for the unstarved bacteria. Each preparation was dissolved in 0.01M-NaCl at pH 7.

Method 2. This involved deproteinization of whole bacterial lysates by phenol. Cells were washed in saline-EDTA (Marmur, 1961) and resuspended in 0.1M-tris, pH 8. The cell walls were treated with 200 µg. of lysozyme/ml. at 30° for 10 min. and the cells were then lysed by adding 1% of Duponol at 37°. As soon as lysis was complete (3-4 min.) the lysate was chilled in an ice bath and rocked gently for 1.5 hr. at 4° with an equal volume of cold, water-saturated, redistilled phenol. The phenol and aqueous layers were separated by centrifuging at 2500g for 5 min. and the phenol layer was removed and discarded. The aqueous layer was rocked gently with five lots of ether to remove the last traces of phenol, and the ether was removed by incubating at 37° and blowing air across the surface of the solution. RNA was hydrolysed by adding ribonuclease (50 µg./ml.) and incubating at 37° for 30 min. Papain was then added to destroy the ribonuclease. The DNA preparation was then centrifuged in CsCl (mean density 1.71 g./ml.) at 160000g for 24 hr. Drops were collected from a hole pierced in the bottom of the tube. The DNA was found to have been in a band at the centre of the tube and the drops containing DNA were pooled and dialysed against a solution of 5 mm-phosphate, pH 7.2, containing 0.1M-NaCl.

Method 3. Batches of cells were extracted after 0, 1.5 and 3 hr. of thymine starvation. The DNA was purified by phenol deproteinization as in method 2 but the products of ribonuclease and papain digestion were removed by dialysis for 72 hr. at 4° against three changes of 1.5 l. of 0.01M-phosphate buffer, pH 7.2, containing 0.01M-NaCl. The RNA which remained after this treatment was removed by activated charcoal (Zamenhof & Chargaff, 1951). Sedimentation analysis of these DNA preparations at a concentration of 15 µg./ml. in dilute saline-citrate (Marmur, 1961) gave a $S_{20,w}$ value 30.5S. This corresponds to a molecular weight of about 40×10^6 (Rubenstein, Thomas & Hershey, 1961).

Other materials. Caesium chloride was supplied by British Drug Houses Ltd., Poole, Dorset, and was designated for ultracentrifuge work. Papain was used as supplied by British Drug Houses Ltd. and was diluted (1:2, v/v) with 0.01M-cysteine. Ribonuclease was obtained from Sigma

Chemical Co., St Louis, Mo., U.S.A. Before use the enzyme was heated at 80° for 5 min. in tris or phosphate buffer, pH 7.0, to inactivate any residual deoxyribonuclease activity.

Viscometry. All measurements were performed at 25° with a viscometer described by Zimm & Crothers (1962).

Sedimentation analyses. These were performed at a concentration of 15 µg./ml. at 20° and at 39460 rev./min. in a standard 12 mm. cell in the Spinco model E Analytical Ultracentrifuge. Photographs of the sedimenting boundaries were taken every 4 min. with 15 sec. exposures. The film was examined in an Analytrol integrating densitometer (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) and the sedimentation constants were calculated graphically by standard procedures (Schachman, 1959).

Determination of band-widths in caesium chloride. The methods used were those of Thomas & Berns (1961a,b) and of Thomas & Pinkerton (1962). The concentration of CsCl was adjusted to give a density of 1.710 g./ml. as determined by a differential refractometer (Vinograd & Hearst, 1962). The mixture for ultracentrifugation contained *E. coli* DNA (5 µg./ml.) together with 50 mM-phosphate, pH 7.2, 12.4 mM-NaCl, CsCl and DNA from *Micrococcus lysodeikticus* (2 µg./ml.) added as a density marker. After centrifuging for 30 hr. at 44770 rev./min. and 25°, photographs were taken with 30 sec. exposures.

Determination of cross-linking in DNA. The method of Iyer & Szybalski (1963) was used. DNA prepared by method 3 was heated for 6 min. at 100° in a solution of 15 mM-NaCl and 1.5 mM-sodium citrate, pH 7, containing 1% of formaldehyde. The solution was cooled by immersing the test tube in ice-water. If the DNA contained heat-stable cross-links, it would re-nature on rapid cooling. The DNA solutions were examined for this possibility in the analytical ultracentrifuge. The mixture for centrifugation contained 4 µg. of formaldehyde-treated DNA/ml., 0.6 mM-NaCl and 0.06 mM-sodium citrate together with CsCl and *M. lysodeikticus* DNA as a density marker.

Chemical estimations. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as a standard. DNA was estimated by the method of Burton (1956), RNA by the method of Ogur & Rosen (1950) and total phosphorus according to Chen, Toribara & Warner (1956) except that the samples were ashed as described by Burton & Petersen (1960).

RESULTS

Precipitability of Escherichia coli 15T⁻ DNA after treatment with alkali

It has been reported that DNA from thymine-starved 15T⁻ is unusual in not being reprecipitated by adding excess of acid after incubation in alkali (Barner & Cohen, 1954; McFall & Magasanik, 1962). We have confirmed that this behaviour occurs when the washed bacteria are treated with alkali. To study this further, we extracted DNA by the method of Marmur (1961) and examined the effects of alkali on the DNA at each stage of the isolation. Sodium hydroxide was added to a final concentration of 1N and the solution was heated for 1 hr. at 70°. (Heating bacteria at 70° for 1 hr. gave similar results to heating at 37° overnight.) The alkali was neutralized with a slight excess of 5N-hydrochloric acid and the mixture was centrifuged at 2500g for 10 min. Controls were performed by treating identical DNA preparations with N-alkali followed by immediate neutralization. The amount of DNA which can be reprecipitated at these various stages is shown in Table 2.

This clearly suggested that the DNA was not abnormally labile to alkali but that, after the treatment with alkali, another component present in the bacteria prevented the reprecipitation of the DNA by acid. To test this, some herring-sperm DNA, which is stable to alkali, was added to the acid-washed bacterial pellets at the same time that the alkali was added. After incubation at 70° for 1 hr., addition of hydrochloric acid failed to reprecipitate either the *E. coli* DNA or the herring DNA, although both DNA samples could be precipitated quite normally in the unheated controls.

Thus it is clear that the apparent alkali-lability

of the DNA is not due to an abnormality of the DNA, nor does it have anything to do with thymine-less death as has been suggested (McFall & Magasanik, 1962b), since the apparent alkali-lability can be observed before death occurs. No apparent lability is observed with the DNA of the thymine-less mutant *E. coli* B₃ under the same conditions, nor with the DNA of strain 15THU, which is an independently isolated thymine-less derivative of *E. coli* 15 (Stern, Sekiguchi, Barner & Cohen, 1964).

Possibility of single-strand breaks in the DNA

The existence of single-strand breaks might result in low-molecular-weight DNA extracted after thymine-less death, and increased susceptibility of the DNA to shear.

Viscosimetric analysis. With the DNA samples obtained by method 1, the intrinsic viscosity was found to be 12.51/g. for both 'before-death' DNA and 'after-death' DNA (Fig. 1). This indicated that there was no difference in the molecular weights of the DNA samples extracted before and after thymine-less death in *E. coli* 15T⁻.

To investigate the susceptibility of the DNA to further shear, solutions of 46 µg./ml. were forced through a 2 in. no. 19 needle, by the use of a 2 kg. weight acting downwards on the barrel of a hypodermic syringe. This treatment did not affect the viscosity of the DNA samples, but more vigorous forcing of the solutions through the same needle twice, by hand, resulted in a fall in specific viscosity of 20% for 'after-death' DNA and of 25% for the 'before-death' DNA.

It has been reported (Mennigmann & Szybalski, 1962a), however, that the viscosity of DNA extracted by a phenol deproteinization from *E. coli*

Table 2. *Precipitability of Escherichia coli 15T⁻ deoxyribonucleic acid after thymine-less death at various stages of extraction of the acid*

15T⁻ DNA was isolated after thymine-less death by the method of Marmur (1961). Samples from each stage of the extraction were incubated in N-alkali at 37° overnight before neutralization with HCl. The percentage of the DNA which was precipitable and non-precipitable at these various stages is shown. Controls were performed by neutralizing the alkali-treated samples immediately without incubation. The amount of DNA present in each fraction at the various stages of the purification is expressed in terms of the percentage of the amount present in the washed bacterial pellet.

Stage of purification of DNA	Unheated control		Heated in alkali		Amount of DNA (%)
	Not precipitated by acid	Precipitated by acid	Not precipitated by acid	Precipitated by acid	
Washed bacterial pellet	9	91	96	4	100
Crude lysate	11	89	56	44	100
Aqueous layer after first deproteinization	17	83	33	67	80
Protein interface from first deproteinization	0	100	0	100	20
DNA precipitated by ethanol from aqueous layer	0	100	4	96	79

Fig. 1. Viscosity of DNA samples from *Escherichia coli* 15T⁻ before and after death, prepared by method 1. The specific viscosity of the DNA was measured at different DNA concentrations and the intrinsic viscosity obtained from the intercept on the ordinate. The solvent was 0.01 M-NaCl, pH 7.0. ○, 15T⁻ DNA before death; ●, 15T⁻ DNA after death.

15T⁻ after thymine-less death was only 1% of that from unstarved 15T⁻. Differences between phenol- and chloroform-deproteinized DNA samples have previously been reported (Ginoza & Guild, 1961) and have been attributed to the fact that chloroform always leaves a residual quantity of protein in the preparation which can be subsequently removed by phenol. Since this residual protein may have been functioning as a support for the more fragile DNA from bacteria after thymine-less death, the chloroform-deproteinized DNA samples prepared by method 1 were further deproteinized by phenol. Measurement of the intrinsic viscosities of DNA samples treated in this way gave identical values for the 'before-death' and 'after-death' DNA. There was no alteration in their susceptibilities to shear.

It therefore seems that the extensive degradation of DNA as found by Mennigmann & Szybalski (1962a) in 15T⁻ is not a necessary consequence of thymine-less death. It has also been reported by Maaløe (1963) that D. Freifelder, in unpublished experiments, has been unable to find any evidence for single-strand breaks in the DNA of *E. coli* 15TAU (a derivative of 15T⁻) after thymine-less death. It is not ruled out, of course, that the molecular mechanism of thymine-less death in *B. subtilis* inhibited with 5-fluorodeoxyuridine is due to the multiple fragmentation reported by Mennigmann & Szybalski (1962a).

Sedimentation analysis. The existence of single-

Table 3. Sedimentation coefficients of *Escherichia coli* 15T⁻ deoxyribonucleic acids before and after death, before and after heating in 1% formaldehyde

The DNA samples were dissolved (15 μg./ml.) in 14 mM-phosphate, 8 mM-NaCl, pH 7.2. The sedimentation coefficients are expressed as $S_{20,w}$ and are not corrected to zero concentration.

	$S_{20,w}$ (s)			
	Heated at 93° for 3 min.		Unheated	
	'Before-death' DNA	'After-death' DNA	'Before-death' DNA	'After-death' DNA
Determination 1	16-18	18	22	21
Determination 2	17	16-18	23	21

strand breaks would result in different sedimentation patterns in the analytical ultracentrifuge after the DNA has been heated in 1% formaldehyde to separate the strands. Sedimentation velocity determinations on 'before-death' and 'after-death' DNA from *E. coli* 15T⁻ (prepared by method 1, chloroform- and phenol-deproteinized) with and without heating in the presence of 1% formaldehyde gave the results shown in Table 3. Only sedimenting boundary was present in each case and there is no difference in the sedimentation coefficients of the 'before-death' and 'after-death' DNA samples. This suggests the absence of single-strand breaks in DNA associated with the process of thymine-less death.

Caesium chloride gradient equilibrium centrifugation studies. To confirm the absence of many single-strand breaks in the DNA after death, DNA samples were heated in 1% formaldehyde and rapidly cooled. The widths of the bands formed by the DNA samples in a caesium chloride gradient were then measured. Since the band-width is an inverse measure of the molecular weight (Thomas & Berns, 1961a,b; Thomas & Pinkerton, 1962) it might be expected to be broader for the 'after-death' DNA than for the 'before-death' DNA. DNA samples prepared by method 2 were used at a concentration of 4 μg./ml. and buffered in 0.01 M-phosphate, pH 7.2, during heating at 93° for 3 min. in 1% formaldehyde. The samples were cooled rapidly in ice-water and caesium chloride was added to give a density of 1.710 g./ml. An unheated sample of *Micrococcus lysodeikticus* DNA was included as a density marker in all runs.

The density of the native *E. coli* DNA was identical for 'before-death' and 'after-death' DNA and agreed with the published value (Schildkraut, Marmur & Doty, 1961) of 1.710 g./ml. This confirms the observations of Luzzatti & Revel (1962).

Fig. 2. Widths of DNA bands obtained in equilibrium density-gradient centrifugation. For each band, values of band-width (W) at various heights (h) were obtained from the densitometer trace (see the text). ∇ , 'Before-death' DNA before heating; $\circ$, 'before-death' DNA after heating; $\blacktriangledown$, 'after-death' DNA before heating; $\bullet$, 'after-death' DNA after heating.

The distribution of DNA molecules in the band can be expressed in terms of the following equation (Thomas & Berns, 1961a,b):

$$h/h_{\max} = \exp \frac{-W^2 M}{8\beta}$$

where $h_{\max}$ is the maximum height of the trace above the base line on the densitometer trace of the DNA bands, h is the height above the base line of the particular point on the densitometer trace, W is the band-width at this point, β is an expression which includes the density, density gradient and the centrifugal force (Thomas & Berns, 1961a; Cummings, 1963) and M is molecular weight for a uniform population of molecules.

Fig. 2 shows the plots of $\log(h/h_{\max})$ against W^2 for DNA from normal cells and after thymine-less death. In each case, the value of β is virtually identical, so the slopes of the lines should be proportional to the molecular weight. For both the native and denatured DNA there is no difference between results obtained with normal ('before-death') DNA and the DNA from cells which have suffered thymine-less death. It can therefore again be concluded that there is no evidence that single-strand breaks are induced in the DNA by thymine-less death.

The light-source for the photographs was obtained from the standard Spinco optics arrangement which employs a chlorine-bromine filter. Cummings (1963) has shown that the wavelength of the light produced under these circumstances results in artificially narrow band-widths, especially with denatured DNA. Accordingly, the relative slopes of the two lines in Fig. 2 are approximately those expected if the molecular weight is halved on denaturation.

Fig. 3. Densitometer tracings of *Escherichia coli* 15T- DNA in a caesium chloride gradient after heating in 1% formaldehyde followed by rapid cooling. DNA concentration, 5 μ g./ml. Solvent, 0.6 mM-NaCl-0.06 mM-trisodium citrate, pH 7.0. DNA of *Micrococcus lysodeikticus* was present in each case as a density marker at 1.731 g./ml. Native *E. coli* DNA has a density of 1.710 g./ml. Denatured *E. coli* DNA has a density of 1.725 g./ml. (a) 'Before-death' DNA; heated in 1% formaldehyde. (b) DNA isolated after 1.5 hr. thymine starvation; heated in 1% formaldehyde. (c) DNA isolated after 3 hr. thymine starvation; heated in 1% formaldehyde. Base-line and extinction scales are in arbitrary units.

Possibility of cross-linking in *Escherichia coli* 15T- DNA after thymine-less death

Mitomycin C, ultraviolet irradiation and nitrogen mustards can each cause the formation of cross-links between the two DNA strands. These agents and thymine-less death also induce lysogenic phage

and are lethal to the cell. This suggested that the lethal event in thymine-less death might be a cross-linking of the DNA. To investigate this possibility DNA was prepared by phenol deproteinization (method 3). The DNA samples were heated in dilute saline-citrate (Marmur, 1961) for 6 min. at 100° and cooled rapidly in ice-water. The denatured DNA (5 µg./ml. final concentration) was then buffered with phosphate, pH 7.2, and subjected to caesium chloride density centrifugation. If cross-links exist which are heat-stable then re-naturation of the denatured DNA strands would be expected on rapid cooling. The densitometer tracings of the bands obtained are shown in Fig. 3. DNA of *Micrococcus lysodeikticus* was present as a density marker.

The DNA obtained after 1.5 hr. of thymine deficiency did not re-nature to give a detectable band at the density of native *E. coli* DNA. Although 98% of the bacteria were killed, there is thus no evidence for any heat-stable cross-linking. Even after thymine deficiency for 3 hr. there is no definite evidence for cross-linking, but the unevenness of the base line makes it impossible to rule out 1% of the DNA having been re-natured in this sample. The molecular weight of the DNA was at least 3×10^7 , so this degree of re-naturation would correspond to an average of about one cross-link per molecule of weight 3×10^9 . This is approximately the amount of DNA in the whole chromosome of *E. coli* (Cairns, 1963).

In considering this experiment, it is important to note that the previous measurements (Fig. 2) as well as those of Thomas & Berns (1961b) and Cummings (1963) provide evidence against any appreciable degradation of the primary structure of the nucleic acid during the short period of heating.

DISCUSSION

Because the yields of DNA from thymine-starved bacteria have always been similar to those from normal bacteria, it is reasonable to expect that the purification methods have not selected a small proportion of the intact DNA from a larger amount of damaged DNA. Provided that sufficient deproteinization steps are performed to remove all of the extra protein produced by unbalanced growth, the DNA can be precipitated by ethanol and spooled quite normally on to a glass rod. Our results clearly rule out the possibility that extensive fragmentation of the DNA is necessarily associated with thymine-less death. In fact, we have not found any differences between the DNA extracted from viable bacteria and that from the killed cultures. Moreover, it appears that our techniques would have detected more than 100 single-strand breaks per chromosome and possibly a single cross-link

between the two complementary strands. However, it is still possible that degrees or types of damage not detected by our methods may still be involved in the process of thymine-less death.

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