Morphological Changes in *Escherichia coli* Strain c Produced by Treatments Affecting Deoxyribonucleic Acid Synthesis

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(Accepted for publication 1 May 1967)

**SUMMARY**

Unusual swollen and branched cell forms were produced in a thymineless mutant of *Escherichia coli* strain c by treatment with mitomycin C, and to lesser extents, by incubation without thymine and by exposure to ultraviolet light. In the case of mitomycin C treatment the morphological changes were accompanied at first by degradation of deoxyribonucleic acid (DNA), later by degradation of ribonucleic acid (RNA) and inhibition of protein synthesis, and throughout by inhibition of DNA synthesis and extensive loss in viability. Thymineless incubation prevented DNA synthesis and also resulted in extensive killing. Cultures incubated after exposure to ultraviolet light exhibited a small amount of DNA degradation and a lag in DNA synthesis. Upon prolonged incubation with mitomycin C or without thymine many of the abnormal forms became very enlarged and eventually lysed. No evidence of bacteriophage or bacteriocin could be detected in the treated cells.

**INTRODUCTION**

Strain c of *Escherichia coli* is quite distinct from others of the species in that its shape is more spherical than rod-like, and its nuclear matter is peripherally distributed in the cell (Lieb, Weigle & Kellenberger, 1955). In experiments with a thymineless auxotroph derived from this strain (Hewitt, Suit & Billen, 1967), it was noticed that prolonged incubation of the organism without thymine resulted in the production of swollen and branched forms different from the filamentous 'snakes' produced by other *E. coli* strains (Barner & Cohen, 1954). Upon further investigation it was found that incubation of *E. coli c* with the antibiotic mitomycin C was particularly effective in producing such abnormal forms, again quite different from the long non-septate filaments formed by *E. coli* strains B and K12 during incubation with mitomycin C (Reich, Shatkin & Tatum, 1961). The present report describes the morphological abnormalities and other changes that take place in cultures of *E. coli c* incubated with mitomycin C, without thymine, and following exposure to ultraviolet light.

**METHODS**

*Organisms.* *Escherichia coli* strain c was kindly supplied by Dr R. L. Sinsheimer. Derivation of the mutant designated *thy*-321 has been described by Hewitt, Suit & Billen (1967). The cultures were maintained at room temperature in sealed 0.6% (w/v) nutrient agar stab tubes.
Conditions for growth. Escherichia coli c thy-321 was grown in a minimal medium (MM) containing 0.7% K$_2$HPO$_4$, 0.3% KH$_2$PO$_4$, 0.05% Na citrate, 2H$_2$O, 0.01% MgSO$_4$·7H$_2$O, 0.1% (NH$_4$)$_2$SO$_4$, and 1% (w/v) glucose and supplemented with 2 µg./ml thymine. Two ml media were loop inoculated from slants or stabs and shaken overnight at 37°. Next day, the cultures were diluted to an optical density (at 660 mp in a Bausch and Lomb Spectronic '20' spectrophotometer) of 0.05-0.1 (2-4 × 10$^8$ cells/ml. by viable count) with fresh media and incubated at 37° with forced aeration by sparging to produce either large inoculum cultures or experimental cultures. Inoculum cultures were grown to an optical density of 0.35, harvested by filtration (Schleicher and Schuell membrane filters, 0.45 µm pore size, 47 mm diam.), washed, concentrated threefold in unsupplemented MM, and stored in the refrigerator for up to 5 days’ use. Experimental cultures were grown from diluted overnight or inoculum cultures to an optical density of 0.25-0.3 (about 1 × 10$^8$ cells/ml.) and subjected to the experimental treatments described below. The normal doubling time for E. coli c thy-321, as measured by optical density increase, was 58 min. in MM.

Viable counts. Viable counts were made by spreading 0.1 ml of appropriate dilution of the cultures on the surface of nutrient agar plates [1% (w/v) agar, 1.3% (w/v) tryptone, 0.8% NaCl, 0.2% Na citrate, and 0.3% glucose]. The colonies were counted after overnight incubation at 37°.

Thymineless incubation. Experimental cultures were grown in fully supplemented MM, harvested, washed, resuspended at the same concentration in MM containing no thymine, and returned to aeration at 37°. In some cases, in order to ensure that small amounts of thymine, possibly available from internal pools, would not be utilized by the organism, 50 µg./ml. uridine was added to the culture (Freifelder, 1965).

Incubation with mitomycin C. Mitomycin C (obtained from Calbiochem) was added to experimental cultures growing in fully supplemented MM at final concentrations of 0.1-5 µg./ml.

Irradiation with ultraviolet light (u.v.). Experimental cultures were grown in MM, harvested, washed, and resuspended in unsupplemented MM at a concentration of 1 × 10$^8$ cells/ml. Layers of the suspension, 2 mm. deep, were exposed while being stirred in sterile glass Petri dishes, to two Westinghouse germicidal u.v. lamps at a distance of 95 cm., giving an average incident dose of 6.4 ergs/mm.$^2$/sec. Exposure for 60 sec. reduced the viable count to 15-20%. The suspensions were then diluted into supplemented MM and returned to aeration at 37°.

Phase microscopy. Cultures were examined microscopically with a Leitz Ortholux Research Microscope equipped with dark phase optics and illuminated by a 60 W light source manufactured by the W. H. Talley Co., Houston, Texas. Coverslip impressions were made from drops of the cultures which had been allowed to soak into a minimal agar plate. The coverslips were placed on microscope slides coated either with 1% (w/v) agar or with a mixture of 1% (w/v) agar and 25% (w/v) gelatin in peptone broth, in a manner similar to that described by Alder & Hardigree (1965). The gelatin preparation enhanced visualization of the nuclear material (Mason & Powelson, 1956). It was filtered through an 0.45 µm Millipore filter immediately before use.

Chemical analyses. Ten-ml. samples were removed from incubating cultures at intervals and the cells pelleted by centrifugation at 12,000 g for 15 min. The pellets were extracted by the method of Ogur & Rosen (1950), and the extracts were analysed...
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for deoxyribonucleic acid (DNA) by the method of Burton (1956) and for ribonucleic acid (RNA) by the method of Visser & Chargaff (1948). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Changes in morphological appearance during treatment. The changes in morphological appearance of Escherichia coli c thy-321 that occurred during incubation with mitomycin C are shown in Plate 1. Within 30 min. of incubation with mitomycin C the cells began to enlarge, both in length and girth. At early times 'y' or knobbed forms were frequently observed, and later some obviously branched forms appeared. As incubation continued, the cell type most often observed was grossly swollen in the centre with two or three elongated 'arms'. The cultures were never uniform in appearance, however, and cells of all shapes and sizes could be seen throughout the incubation period.

The cells in Pl. 1, a–c, were mounted on gelatin-coated slides in order to provide a background with a refractive index near that of the cytoplasm of the cells, allowing better resolution of the nuclear material (Mason & Powelson, 1956). The nuclear material appears white or lighter in the photographs. The nuclear material in the normal cells did not show up distinctly with the gelatin technique; we assume that this was because of its peripheral distribution (Lieb, Weigle & Kellenberger, 1955). Upon mitomycin C treatment the nuclear material first became more visible, as if coalesced, and almost filled the cell; later, it became diffuse or patchy. However, in the cell shown in Plate 1, f, a branched form at 4 hr, the nuclear material was still obvious and condensed in one area.

Similar morphological changes were observed in cells incubating without thymine or following u.v. irradiation (Pl. 2, a–f). In the case of thymineless incubation the changes developed more slowly than with mitomycin C treatment. For some time most of the cells were more elongated than swollen, but eventually (4–5 hr) huge, swollen and branched forms were frequently observed. The changes seen during the post-irradiation incubation were never as extensive as with the other two treatments.

After 3–4 hr of mitomycin C treatment, and to a lesser extent after 5 or more hr of thymineless incubation, 'ghosts' of the swollen branched forms were seen (Pl. 2, g–i) as well as clumped fragments of membrane-like material, suggesting that as the abnormal cell forms enlarged they became fragile and eventually lysed.

Effect of treatments on growth and viability. The treated cultures were examined for other changes which might have been related to the changes in morphological appearance.

Figure 1 shows that incubation with mitomycin C brought about rapid and extensive killing of the organism after about 20 min. After a somewhat longer period extensive killing was also produced by thymineless incubation. The survivors of u.v.-exposure remained viable and began to divide after a lag period of an hour or more.

Even the cultures that were declining in viability increased substantially in mass for a time during the incubation (Fig. 2). This is probably a reflexion of the enlargement of the abnormal forms described above. The addition of mitomycin C or removal of thymine did not appear to affect the rate of increase in optical density significantly for about 30 min. Then the rate slowed and the increase gradually came to a halt.
within the subsequent 2–3 hr of incubation. After this, little further change was observed for up to 6 hr of thymineless incubation (not shown in figure). However, in the case of mitomycin C, the optical density of the culture began to decrease after about 3 hr. This was the time when microscopic examination began to reveal ghosts and debris in the culture. The time and extent of lysis produced by mitomycin C were variable from experiment to experiment. Each of four concentrations tested (0.1, 0.5, 1.0 and 5.0 μg./ml.) produced some lysis, but the onset was earliest with 5 μg./ml.

Fig. 1. Effects of incubation with mitomycin C (MC, 5 μg./ml.) incubation after ultraviolet light (u.v.)-irradiation, and thymineless (−thy) incubation with or without subsequent readdition of thymine (+thy, 2 μg./ml.) on the viability of Escherichia coli c thy-321. Cultures were grown in minimal salts media and treated as described in Methods. O—O, −thy; ■—■, +MC; ▲—▲, u.v. exposed; ▼—▼, −thy, 120 min.

Fig. 2. Effects of various treatments on the growth of Escherichia coli c thy-321. The data shown for +MC, u.v.-exposed, and −thy, 120 min., were obtained from the cultures shown in Fig. 1. ▲—▲, Logarithmically growing cultures.

Mitomycin C had the most drastic effect on the synthesis of DNA, RNA, and protein of any of the treatments (Fig. 3). There was no net increase in DNA (diphenylamine-reacting material) in the presence of 5 μg. mitomycin C/ml. and almost 30% of the DNA initially present disappeared from the cold acid-precipitable fraction within the first 30 min. of incubation. During the first 30–45 min. of incubation with mitomycin C, there were 40% net increases in RNA and protein. Subsequently, RNA was degraded, but the protein content of the culture remained constant. The period of time covered by the sampling in these experiments preceded the onset of visible lysis in the culture.

Thymineless incubation, with or without 50 μg. uridine/ml., also prevented net DNA increase, but significant DNA degradation was not observed until after an hour or more. RNA and protein synthesis in cultures incubated without thymine continued at linear rates for almost 90 min. In the case of u.v.-exposed cells, a 12%
Fig. 3. Effects of various treatments on synthesis of (a) DNA, (b) RNA, and (c) protein; by *Escherichia coli* c thy-321. Cultures were grown in minimal salts media and treated as described in Methods. Final concentrations of mitomycin C (MC), thymine (thy), and uridine (uri) were 5, 2 and 50 μg/ml., respectively. The u.v. exposure reduced the numbers of colony formers to 23%. ••••, +thy; ▲▲▲▲, −thy; ■■■■, −thy+uri; ○○○○, +thy+MC; △△△△, +thy (u.v.-exposed).
loss in DNA was observed during the first 15 min. of post-irradiation incubation. DNA synthesis then commenced and continued throughout the remainder of the incubation. The synthesis of RNA was depressed by u.v. exposure; about 80 min. of the incubation elapsed before the RNA content of the culture doubled. Protein synthesis was less affected, even appearing to be somewhat elevated in rate, during the first 30 min. of incubation.

Search for induction of bacteriophage or bacteriocin. Since incubation with mitomycin C, thymineless incubation and u.v.-irradiation are treatments known to induce prophage carried by lysogenic bacteria (cf. Korn & Weissbach, 1962; Lwoff, Siminovitch & Kjeldgaard, 1950), and since mitomycin C produced obvious lysis of Escherichia coli c thy-321 (Fig. 2) the treated cultures were examined for evidence of bacteriophage or bacteriocin. Tests of treated 'shift-down' cultures for stimulated messenger RNA synthesis (Frampton & Brinkley, 1965), tests of supernatant fluids and artificial lysates of the treated cultures for lytic activity against various indicator strains, and examination of these materials in the electron microscope for the presence of complete or incomplete phage particles were all negative. Therefore, it appears that no readily inducible agent of this sort is carried by the organism. The lysis that occurs during incubation with mitomycin C can be explained as the result of the breakdown of the abnormal swollen cell forms that are produced by the treatment.

DISCUSSION

In other strains of bacteria mitomycin C, thymineless incubation, and exposure to ultraviolet light usually cause the development of long, non-septate filaments otherwise superficially normal in appearance (Hughes, 1956; Reich et al. 1961). Thus, a major effect of the treatments is the prevention of septation and cell division. The effects of the treatments on Escherichia coli c thy-321 are probably qualitatively the same as that on other organisms, and the differences between the morphological aberrations produced in it and in other organisms may be only extensions of the normal morphological differences between it and other cells; e.g. its spherical rather than rod-like shape.

The inhibition of cell division by the treatments can be explained as a secondary result of their effects on DNA. Each of the treatments affects the integrity of DNA and/or inhibits DNA synthesis (cf. Kelner, 1953; Barner & Cohen, 1954; Shiba, Terawaki, Taguchi & Kawamata, 1959; Iyer & Szybalski, 1963). According to the replicon model (Jacob, Brenner & Cuzin, 1963), septum formation is dependent upon the synthesis of the cell membrane at a site where the DNA molecule is attached, but in each cell generation synthesis of that membrane must await completion of a round of DNA replication. Thus, the inhibition of DNA replication could bring the entire sequence of events to a halt, and might result in lesions in the cell wall and membrane so that the cells would swell and become fragile as they increase in mass. This is the case with cells that are specifically prevented from cell-wall synthesis, e.g. during spheroplast formation (Lederberg & St Clair, 1958). As overall control of the normal sequential steps in growth and cell division is lost, growth may be attempted at several points resulting in the production of branched forms.

The reason that u.v.-irradiation was much less effective than the other treatments in producing extensive morphological changes is probably because the DNA synthetic
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capacity of the treated cells soon recovered upon incubation in growth medium (Fig. 3). Greater doses would probably produce more drastic changes. The fact that thymineless incubation, while completely inhibiting DNA synthesis, still resulted in fewer swollen and branched forms than did treatment with mitomycin C suggests that the more extensive degradation of DNA brought about by the drug may be an accelerating factor in producing the hypothetical membrane and wall lesions.

Other than the striking differences in morphological changes that have been discussed, the effects of the treatments on *Escherichia coli c thy-321* that we observed are similar in many respects to those reported by other investigators with other organisms. In the case of incubation with mitomycin C, DNA synthesis was completely inhibited and some DNA was degraded, although the loss was less extensive than that observed by Reich, Shatkin & Tatum (1961) in *E. coli 15T*- . In *E. coli 15T*- , treatment with 0.5-20 µg. mitomycin C/ml. brought about degradation of DNA to acid-soluble fragments at a linear rate for at least 24 hr. The conditions found necessary for the breakdown, conditions which permit growth, were met in our experiments. However, DNA measurements in our experiments were made only during the first 2 hr of incubation because some lysis usually began shortly thereafter. The visible changes that suggest fragmentation and dispersion of the nuclear material were most obvious after 2 hr and may have been accompanied by further DNA degradation. The changes in the appearance of the nuclear material that we have observed are quite similar to those described by Reich and associates for *E. coli b*.

Sekiguchi & Takagi (1960) found that the effects of mitomycin C treatment on RNA and protein synthesis in *Escherichia coli b* changed with different concentrations of the drug and with different stocks of the organism. The effects that we have observed with *E. coli c thy-321* are similar to those obtained with the more sensitive *b*, although our use of a different concentration of mitomycin C precludes a strict comparison. With an unspecified strain of *E. coli* Smith-Kielland (1966) observed a stimulation of RNA synthesis, chiefly that of sRNA, during the first 30 min. of treatment with 10 µg./ml. mitomycin C. She reported that longer periods of incubation resulted in RNA degradation, as did Suzuki & Kilgore (1964) in the case of *E. coli b*. The latter investigators found that 50 S ribosomes were especially sensitive and were almost completely degraded after 60 min. incubation with 5 µg./ml. mitomycin C. We have found that RNA is lost from *E. coli c thy-321* after 30 min. incubation with mitomycin C (Fig. 3) but have not determined what kind of RNA is most affected. The fact that our mitomycin C-treated cultures demonstrated no net increases in RNA or protein after the first 30 min. and actually showed some degradation of RNA is surprising, for the extensive enlargement of the treated cells appears to involve a substantial increase in mass (Fig. 2). It is likely that turnover is taking place, with some of the dying cells undergoing lysis and degradation while others are carrying out some synthetic activity.

The extensive thymineless death exhibited by the organism is of interest in view of the suggestion that the major cause of thymineless death is phage or bacteriocin induction (Mennigmann, 1964; Ishibashi & Hirota, 1965). This is not obvious in the present case at least. The lag in death is somewhat longer than that usually observed with *Escherichia coli 15T*- (Barner & Cohen, 1956). This does not seem to be because thymine from internal pools is available to the organism, for it was found that incubation without thymine and with or without 50 µg. uridine/ml. gave identical
kinetics of thymineless death. Freifelder (1965) showed that ribotides antagonize the utilization of small amounts of thymine by thymineless organisms.

It would appear that the events responsible for thymineless killing in *Escherichia coli* c thy-321 remain reversible by thymine for some period of time. Readdition of thymine to a dying culture after 2 hr of starvation stopped death immediately (Fig. 1) and seemed to rescue some of the cells as plating centres because the viable count increased more than fivefold in 90 min. (The normal doubling time of viable count in this media is 54 min.) The rate of increase was erratic. Somewhat different results were obtained by Barner & Cohen (1956) with *E. coli* 15r-. Following readdition of thymine to a starving culture, there was a lag of 30–45 min., and then the survivors began to multiply at the normal rate. Strain 15 may sustain more extensive damage from the attempted repair of single strand breaks in the DNA (Pauling & Hanawalt, 1965) during thymine deprivation than strain c. It would be of interest to examine strain c for the abnormal features of DNA replication that have been observed in *E. coli* 15 derivatives after thymine starvation and have been implicated in the mechanism of thymineless death (Pritchard & Lark, 1964; Pauling & Hanawalt, 1965).

The authors wish to thank Dr R. Hewitt for helpful discussions and assistance with the u.v.-irradiation, Dr A. Cole for use of the electron microscope, and in particular Dr B. R. Brinkley for preparation and examination of materials in the electron microscope. The work has been supported in part by grant RO1 GM12433 from the U.S. Public Health Service. One of the authors (J.C.S.) is a Faculty Research Associate of the American Cancer Soc., Grant no. PRA-19.

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**EXPLANATION OF PLATES**

**PLATE 1**

*Escherichia coli c thy-321* incubated with 5 µg mitomycin C/ml.: a and h, 0 min.; b and i, 30 min.,
c and j, 1 hr; d and k, 2 hr; e and f, 3 hr; f and m, 4 hr; g and n, 5 hr. Cells were incubated in minimal
salts media and mounted on microscopic slides coated with gelatin (a-g), or with agar (h-n) as
described in Methods. × 2400.

**PLATE 2**

*Escherichia coli c thy-321* incubated: a-c, after u.v. exposure; d-f, without thymine; g-i, with
5 µg mitomycin C/ml. a, 1 hr, agar mount; b, 14 hr, agar mount; c, 2 hr, gelatin mount; d, 1 hr,
agar mount; e, 4 hr, gelatin mount; f, 4 hr, agar mount; g, 4 hr, agar mount; h, 4 hr, gelatin mount;
i, 5 hr, wet mount. × 2400.