# CYTOLOGICAL ANALYSIS OF ULTRAVIOLET IRRADIATED *ESCHERICHIA COLI*

## I. Cytology of Lysogenic *E. Coli* and a Non-Lysogenic Derivative[1]

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A number of observations are on record which indicate that bacteria respond to several physical and chemical agents by a rather characteristic sequence of cytological changes. These changes deviate markedly from the usual operations of reduplication and distribution of the bacterial chromatin. Citation of relevant observations will be made in the present and a subsequent communication. Also it may suffice to indicate that a study, by light and electron microscopy, has been made by Kellenberger (1953) of the sequence of cytological changes induced in *Escherichia coli* by ultraviolet radiation and by the action of the radiomimetic agent methyl-bis-($\beta$-chloroethyl)-amine.

The present report describes electron and light microscopic observations on growing cells of a lysogenic strain of *E. coli* and a non-lysogenic derivative. In addition, changes undergone by the non-lysogenic cells following treatment with ultraviolet radiation are noted. The accompanying report deals with responses to ultraviolet induction of lysogenic cells (Payne *et al.*, 1955). Terminology concerning lysogeny follows that adopted by Lwoff (1953).

### METHODS AND MATERIALS

Table 1 lists the strains of *E. coli* used in this and the following report (Payne *et al.*, 1955) with references giving further descriptions. The tryptone-yeast-extract-glucose medium of Morton and Engley (1945) was used in most experiments, but nutrient medium (Difco) gave comparable results where tested. The synthetic medium employed was that of Gots and Chu (1952) as modified by Gots and Hunt (1953). Weigle and Delbrück's (1951) buffer was used.

The method of ultraviolet treatment and cell growth was essentially as outlined by Gots and Hunt (1953). Log phase cells were centrifuged, washed once in buffer, resuspended in buffer, irradiated, and diluted into the appropriate growth medium. Two ultraviolet doses were used.[3] The first dose (40 sec) was comparable to that giving maximal induction of *E. coli* (Payne *et al.*, 1955). The second dose was for 10 sec.

Nuclear cytology was studied by DeLamater's (1951, 1953*a*) procedure and by the methods described by Hartman and Payne (1954). Aliquots from some cultures were diluted into a small amount of a distilled water solution of neotetrazolium chloride (final concentration 0.03 to 0.1 per cent—General Biochemicals, Inc.). Reduction of the tetrazolium salt was allowed to continue for several minutes before the cells were fixed in the vapors of 2 per cent osmium tetroxide solution and then observed in wet mounts. Light optics and the photographic technique have been described previously (Hartman and Payne, 1954; Davis *et al.*, 1953).

Preparations for electron microscopy were made by the technique of Beutner *et al.*, (1953). Shadow-casting was in all cases done with chromium at a thickness of several Angstroms and with a shadow length-to-height ratio of about 5:1. This ratio varied at different places on the specimens due to sagging of the membrane on the grid. Final microscopic examinations and photography were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, by Delbert E. Philpott.

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[3] Irradiation under the experimental conditions, measured by a General Electric germicidal ultraviolet intensity meter #16522 which is sensitive almost solely to 2537 A radiation, was 105 u.v. milliwatts per sq ft (1130 ergs/sec/cm$^2$).

TABLE 1
*Microorganisms used*

| Organism | Strain | From Whom Obtained | Derivation | Properties | References |
|---|---|---|---|---|---|
| *E. coli* | K12 (wild type) | Dr. J. S. Gots | | Lysogenic for lambda⁺ | Lederberg (1951); Lederberg *et al.* (1951); Weigle and Delbrück (1951) |
| *E. coli* | K12S | Dr. E. M. Lederberg | Ultraviolet survivor of K12 wild type (denoted W1485) | Non-lysogenic, sensitive to lambda phages listed below | Lederberg and Lederberg (1953) |
| *E. coli* | K12-i1 | Dr. R. K. Appleyard | Derived from wild-type K12 | Defective lysogenic; "immune" | Appleyard (1954*a*, 1954*b*) |
| *E. coli* | K12-i2 | Dr. R. K. Appleyard and designated C60 | Derived from C6 | Defective lysogenic; "immune" | Appleyard (1954*a*, 1954*b*) |
| *E. coli* | *Lambda*⁺ | | Irradiated K12 | Temperate | Weigle and Delbrück (1951) |
| *E. coli* | *Lambda* v1 | Dr. R. K. Appleyard | Growth on K12S | Weakly virulent | Appleyard (1954*a*, 1954*b*) |
| *E. coli* | *Lambda* v2 | Dr. R. K. Appleyard | Growth on K12S | Strongly virulent | Lederberg and Lederberg (1953); Appleyard (1954*a*, 1954*b*) |

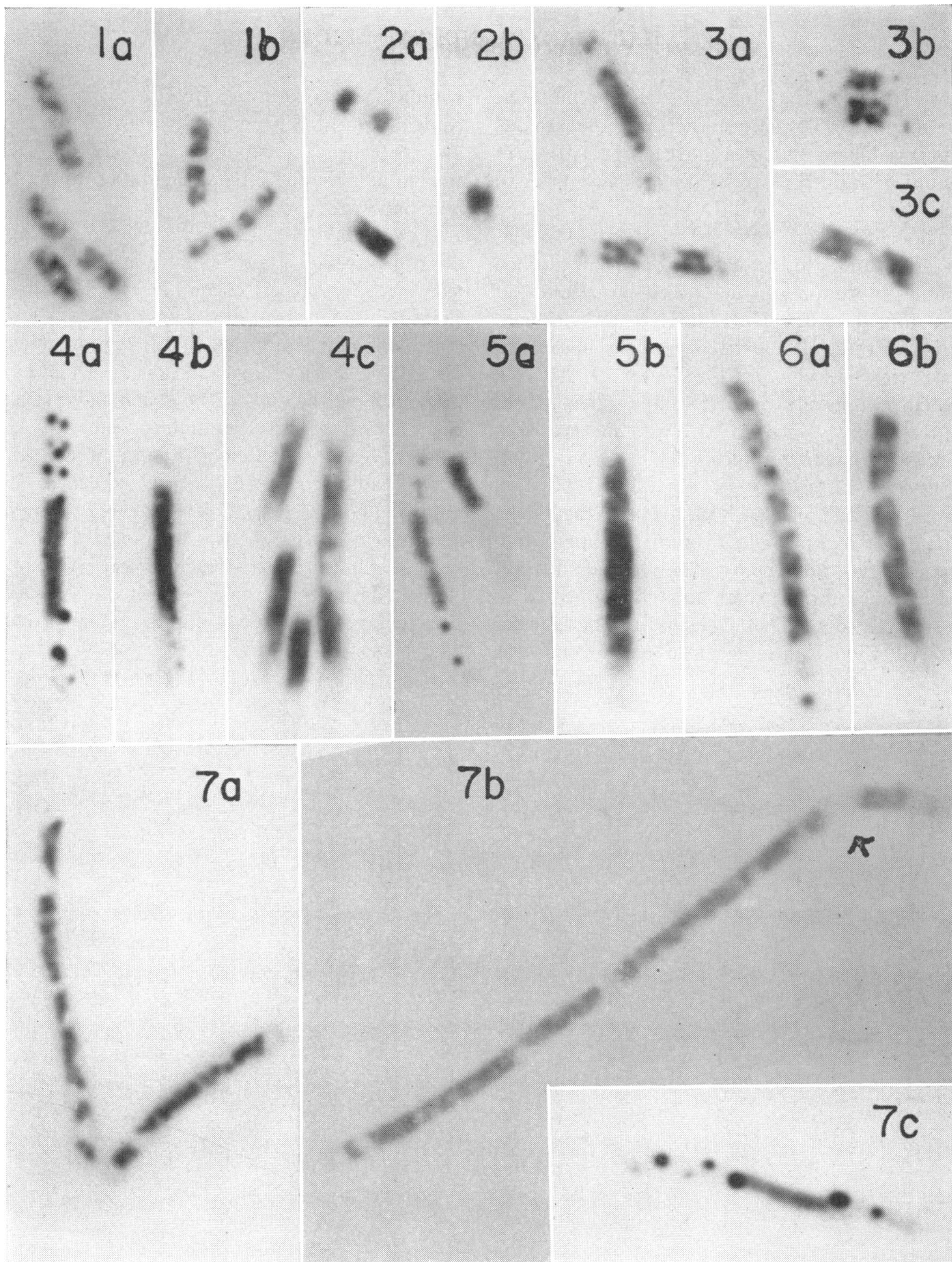
## RESULTS

*Nuclear cytology of normal, growing cultures of lysogenic strain K12 and non-lysogenic strain K12S.* The nuclear configurations of log phase broth cultures of *E. coli* strain K12 and the non-lysogenic mutant K12S were essentially similar (compare figures 1a and 1b with figures 1a and 1b of Payne *et al.*, (1955)). The nuclei were almost all representative of divisional stages. There were two or four nuclei in most cells, very infrequently one or eight. The nuclear appearances were similar with the DeLamater and the May-Greenwald-Giemsa procedures (compare figure 1a with 1b and figure 1a with 1b in the following paper (Payne *et al.*, 1955)). The stained material in the nucleus was arranged in rodlets and granules. These were sometimes joined in a lobed arrangement or were adjacent and numbered up to four per nucleus, but usually one to three. Their extremely small size and closely packed arrangement made it impossible to discern the exact number in many nuclei. Occasionally, cytoplasmically located perinuclear granules, numbering one per nucleus (rarely two), were visible. In DeLamater preparations, residual cytoplasmic granules might be located peripherally in the cell, but with the hydrolysis time used in this study most of these structures were removed. The nuclear configurations of log phase cells grown on salt-glucose medium were the same as those of broth-grown cells. Growing cultures contained a very small number ($10^{-3}$ or less) of cells whose nuclear configurations as determined by light cytology were suggestive of certain nuclear stages after irradiation as described below.

By electron microscopy normal cells of K12 and K12S showed dark and light patterning—the less dense areas corresponding with nuclear sites demonstrated by light microscopy.

*Effect of ultraviolet on non-lysogenic strain K12S.* The 40-sec exposure to ultraviolet permitted a survival of 10 to 20 per cent of the cells of strain K12S, whereas no killing was detected after the 10-sec exposure. The chromosomes of the nuclei of irradiated cells appeared to coalesce slightly so that their visualization as individual entities was more difficult than in non-irradiated cells. The chromosomes of pairs of adjacent daughter nuclei flowed together into one mass so that, instead of 2 or 4 chromosomal clusters, there were 1 or 2 clusters per cell (figures 2a and 2b). Single chromatinic masses lay centrally or, if there were two, slightly closer to the center than to the poles of the elongating cells. These changes were progressive and did not occur synchronously in the entire population or even in all nuclei of the same cell. Almost all nuclei had reached the fusion stage 15 min after irradiation. In many cells, especially those containing two chromosomal masses, an extranuclear granule, usually located peripherally, could be seen between the two bodies. The areas about the poles were always clear in ribonuclease-treated cells; however, in acid-hydrolyzed preparations, frequently one or several intensely-stained granules were present. These polar granules appeared as soon as 5 min following irradiation and were absent in the non-irradiated controls.

By 30 min (figures 3a to 3c) most of the nuclei appeared to have been derived from the fusion of two adjacent nuclei; nuclear counts



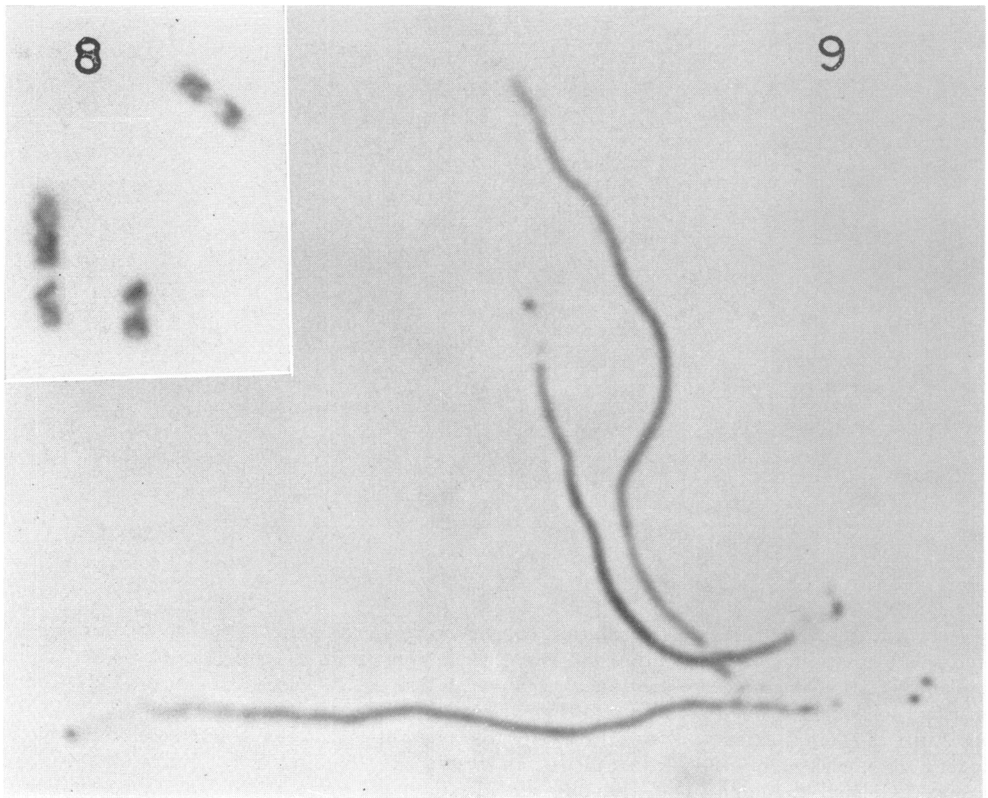

*Figures 1–7*. Effect of ultraviolet radiation on non-lysogenic *Escherichia coli* K12S. All figures are reproduced here at a final magnification of 3,480 ×. Cells were grown in broth, irradiated 10 sec (except where noted) in buffer suspension, and incubated post-UV in broth. Times denote post-UV incubation. Uncrushed specimens stained according to the nuclear staining procedures of DeLamater (1951, 1953*a*) using Azure A-thionyl chloride after acid hydrolysis, and the method of Hartman and Payne (1954), following ribonuclease digestion. *1:* Log phase non-irradiated cells of K12S. (a) Azure A stain. (b) May Greenwald-Giemsa. *2:* 15 min (incubation after irradiation). (a) Azure A. (b) May Greenwald-Giemsa. *3:* 30 min. (a, b) Azure A. (c) May Greenwald-Giemsa. *4:* 45 min. (a, b) Azure A. (c) May Greenwald-Giemsa. *5:* 60 min. (a) Irradiated 40 seconds. Azure A. (b) May Greenwald-Giemsa. *6:* 75 min. (a) Azure A. (b) May Greenwald-Giemsa. *7:* 90 min. (a) Azure A. (b) May Greenwald-Giemsa. (c) Irradiated 40 sec. Azure A.

per rod showed that in some cases as many as four may have flowed together and appeared as one mass. There did not appear to be any greater amount of stained material per cell than prior to irradiation, although cell division had ceased and cell elongation had increased to about 1½ times the length prior to irradiation. Refractile stained granules were present in the polar regions of cells stained by the DeLamater technique; contrariwise, the polar areas were clear in ribonuclease treated cells. In the center of the cell, located on opposite sides at the periphery, there were frequently present two perinuclear ribonuclease-resistant granules.

Between 45 (figures 4a to 4c) and 60 min (figures 5a and 5b) post-irradiation, the nuclei of many cells appeared to contain more chromatinic material than those of the original cultures. The chromosomes in some instances appeared to be subdivided into smaller units. The nuclear material, though granulated in many cells, was confined to one or two discrete loci in the cell, centrally located when only one. No normal-looking cells were found in the culture. There was no increase in viable cell number during this hour interval.

By 75 min the cells which received the lower dose of ultraviolet, producing non-lethal effects, were over twice their normal length. Disintegration of chromatinic material progressed only to a stage where the units were still microscopically discernible as discrete elements. In DeLamater preparations there were now generally several ribonuclease-sensitive granules visible at the poles of each cell. The chromosomes of some nuclei had been reconstituted, segregated into groups, and these clusters separated from the main nuclear mass (figures 6a and 6b).

At 90 min the lightly-irradiated cells were more elongated and the large chromatinic masses consisted of groups of chromosomes; many nuclei had separated from the main chromosomal mass



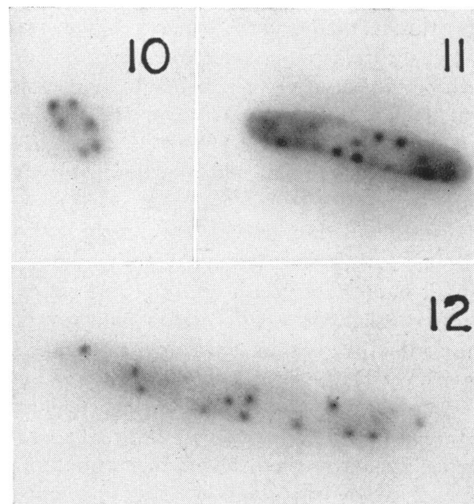

*Figures 8 and 9.* See legend for figures 1–7. × 3,675. *8:* 105 min. Azure A. *9:* 120 min. Azure A. Irradiated 40 sec.

and segregated along the rod (figures 7a and 7b). The peripheral granules were now absent in many cells. The first increase in viable cell count occurred at about this time. At 105 minutes many normal-appearing short rods with two to four nuclei each were present (figure 8). These small cells seem to have been derived by separation of the terminal portions of the long rods (see arrow in figure 7b), and the subsequent division of these cells. Some of the small cells had complex nuclear structures; others contained nuclei characteristic of normally dividing cells. Many long cells were still present; they contained chromosomes in clusters along the rods. After 2½ hours the resumption of cell division produced a large population of normal-appearing cells, leaving only a small per cent of the long multinucleate rods remaining at 3½ hours. The fate of these cells is unknown.

In the culture irradiated with the higher ultraviolet dose, the nuclear changes were similar to those described for the more lightly irradiated cells through the first hour after treatment. By 75 min, however, chromosomal disintegration had progressed to a stage where the stain showed only as a diffuse area; figure 7c shows such a cell 90 min following treatment. These cells continued to elongate and to accumulate cytoplasmic material to a greater extent than those lightly irradiated; cell division was not resumed; and the peripheral ribonucleic acid-containing granules became more evident (figure 7c). In addition, at 90 min some cells showed signs of lysis (loss of chromatin) and others contained chromatin in axial threads. The majority of the cells contained finely granular chromatin still localized in discrete patches along the cell. Many cells reached a length over 20 times that of the original population by 105 min, and contained finely granular chromatin and prominent polar bodies (figure 9). There were many "ghost" cells by 3 hours after irradiation. The cells were diffusely stained in those restricted areas containing chromatin along the length of the cell. There were multiple indentations in the chromatinic material as if it were being unsuccessfully partitioned. At the poles, and peripherally in cells stained by the DeLamater procedure, refractile granules were present. A few cells neither became extremely elongated nor contained diffuse nuclear masses. These cells resembled those seen in populations capable of recovery from ultraviolet exposure and appeared to be the origin of an increasing number of normal cells seen in the heavily irradiated population at 3 and 3½ hours.

The changes undergone by normal cells of K12S were examined electron microscopically following 10 and 40 sec irradiation and incubation on broth-containing agar. With the lower dose at 30 min there was usually a single, less electron-scattering, central area in the cell which appeared granular. At 60 min the cells were more elongated and the less opaque area had increased in size. At 2 hours the cells had elongated to a considerable extent (more than observed in broth cultures) and were intermixed with cells of normal length but not showing the sharp dark and light patterning of the cells noted before ultraviolet. In cells of the more heavily irradiated population 2 hours after ultraviolet no short cells were present, but they began to appear at 3 hours. Only a few were normal in appearance; most of them were vacuolated, granular, partially lysed, or electron transparent. In some areas it was clearly indicated that the small cells originated from the ends of the filaments. Since our electron pictures are essentially in confirmation of the beautiful pictures published by Kellenberger (1953), ours are not reproduced.

Neotetrazolium chloride formazan was localized as peripheral granules in normal cells




*Figures 10–12.* Wet mounts of living cells exposed to neotetrazolium, showing persisting mitochondrial granules. × 3,675. *10:* Log phase non-irradiated cell. *11:* 30 min after irradiation. *12:* 60 min after irradiation.

(figure 10), though in many cells the largest granules were usually polar. There was an increase in the number of these loci as the cell elongated at 30 min (figure 11) and 60 min (figure 12) post-irradiation. This increase was approximately proportional to the increase in the length of the cells. With the high dose of irradiation, the reduction of neotetrazolium by the long filaments and their progeny was quite unequal and some were unable to reduce the dye at all. These tetrazolium-reducing peripheral granules appear to correspond to the enzymatically active granules described as bacterial mitochondria (Mudd, 1953*a*, 1953*b*, 1953*c*, 1954; Davis *et al.*, 1953; Hartman *et al.*, 1953).

One additional feature is demonstrated in figure 11—namely, the lesser optical density of the fusion nucleus 30 min post-irradiation. The edges of this area can be seen in living cells to be clear, i.e. the boundary of the nuclear vesicle remains sharp and distinct.

## DISCUSSION

The observations on nuclear structure of normal cells are in agreement with the various structures of *E. coli* described by DeLamater (1953*a*, 1953*b*). However, the accessory structures (spindles, centrioles) which he described had largely been hydrolyzed away in our preparations. The correspondence of the light areas in electron micrographs with bacterial nuclei demonstrable by light cytology and electron microscopically has been noted repeatedly (Robinow and Cosslett, 1948; Hillier *et al.*, 1949; Mudd and Smith, 1950; Mudd *et al.*, 1950, 1953; Winkler *et al.*, 1951; Winkler and Knoch, 1951; Steinberg, 1952; Kellenberger, 1952; Hartman *et al.*, 1953)

It was noted that growing cultures of both the lysogenic and the non-lysogenic strains contained a small number of cells whose nuclei were cytologically identical with various configurations found after irradiation. These peculiar rare cell types have been described before, appearing in the illustrations of several workers including Zettnow (1899). Robinow (1944) (figure 3) specifically noted these forms in cultures of *E. coli* and avoided speculation as to their nature. The presence of these forms in cultures of K12S and in *E. coli* strain B seems to rule out their participation in known lethal syntheses (bacteriophage, bacteriocin). Bisset (1950) attributed this type of configuration to "complex vegetative reproduction" of "fusion cells." Our observations afford no basis for assuming any such complex reproductive process. There are, however, two processes which suggest possible mechanisms by which this cell type may arise. One mechanism is the response to injury, such as ultraviolet light damage, whereby aggregation of chromatin is initiated by the flowing together of daughter nuclei. The second mode of formation would be that produced by the direct inhibition of normal nuclear divisional, but not synthetic, processes as described for chemical and antibiotic treated bacteria by DeLamater (1953*b*) and DeLamater *et al.*, (1955).

Rita and Orsi (1952) attribute the appearance of ultraviolet treated K12 to an increase in the chromatinic substance and its arrangement along the axis of the cell. Wyss *et al.* (1950) interpreted it as involving nuclear fusion. Kellenberger (1953), in a thorough study, suggests the early "polychromosomic" state arises *both* by nuclear fusion and chromosomal replication. We find that, during the earlier stages following ultraviolet treatment, the aggregation of existing nuclear material is the initial mechanism. Later, actual reduplication increases the number of chromosomes beyond the original level per cell.

The chromosomes appear to be "frozen" in contracted form for a time at divisional stages analogous to metaphase and anaphase and flow together while in the contracted state. This interpretation is supported by the observation of a single extra-nuclear granule at 15 min and two such granules at 30 min post-ultraviolet. Extranuclear granules are occasionally noted in ribonuclease-treated cells and they are stained by a procedure which is specific for deoxyribonucleoprotein in *E. coli* (Hartman and Payne, 1954). These granules we believe correspond to the centrioles of DeLamater (1953*a*, 1953*b*), interpreted by him to be charged with appreciable nucleoprotein only during metaphase and anaphase. Thus their presence and persistence in ultraviolet-inhibited cells indicates that some mechanism involved in nuclear division is very sensitive to ultraviolet radiation. Should deoxyribonucleic acid synthesis (Barner and Cohen, 1955) occur only in the intermitotic phase, as is the case in all cells in which this process has been analyzed (Hughes, 1952; Swift, 1953), it is clear that an inhibition in deoxyribonucleic acid synthesis would be expressed in the mitotic cycle. Our preparations suggest a delay in any cytologically observable increment in chromatin for

about 40 min post-ultraviolet exposure; it is at about this same time that the extra-nuclear deoxyribonucleic acid granules disappear.

Kelner (1953) has shown that an immediate effect of ultraviolet radiation on *E. coli* is inhibition of deoxyribonucleic acid synthesis. This observation has been confirmed by Kanazir and Errera (1954), who found that the duration of the inhibition of deoxyribonucleic acid synthesis was approximately proportional to the dosage of the radiation. Ribonucleic acid synthesis was not inhibited. Cohen and Barner (1954) have presented additional data and advanced the hypothesis that the lethal consequences of ultraviolet radiation may be consequent to the unbalanced growth resulting from inhibition of deoxyribonucleic acid synthesis without corresponding inhibition of ribonucleic acid synthesis and cytoplasmic growth.

One interesting observation of Kellenberger (1953), that there is a separation or fragmentation of chromosomes in many cells in which rejuvenation will occur, is confirmed in our work by two additional staining techniques. The chromosomal units of cells capable of later divisions are reorganized again before orderly nuclear division ensues. These cells become multinucleate by the segregation of normal-appearing nuclei from the chromatinic masses. Later cytokinesis resumes. The resumption first of nuclear and later of cell division are presumed to constitute the recovery process; nuclear fusion appears to be a result of the ultraviolet treatment and seems to precede cellular recovery—not constituting a part of this latter process as suggested by Wyss *et al.*, (1950).

Hayes (1953) has observed that ultraviolet irradiation of $F^+$ donor strains of K12 may enhance their fertility whether such strains are lysogenic or non-lysogenic. It has also been observed that ultraviolet can stimulate chromosomal segregation of K12 cells already in the diploid state (Beckhorn, 1951; Lederberg *et al.*, 1951). The initial daughter cells are of normal length, pinching off the ends of the elongated rods as was noted for ultraviolet-treated *E. coli* by Gates (1933). However, many of the small cells derived from elongated rods of strain K12S contain complex nuclei, are abnormal in appearance in the electron microscope, and are deficient in tetrazolium-reducing activity.

One striking appearance following ultraviolet exposure is the presence near the cell poles, and later along the periphery of the cell, of ribonuclease-sensitive basophilic granules. These granules are present as early as 5 minutes after ultraviolet and increase in size and appear in greater numbers later; they become especially large in those cells destined to die. Our results thus indicate that the intracellular disposition of ribonucleic acid or ribonucleoprotein may be altered. An earlier observation of these granules apparently was made by Delaporte (1949) but interpreted by her to be "of chromatic substance . . . perhaps detached from the central mass of nuclear substance." The development of elongated beaded cells was noted by Gates (1933) in light microscopic observations of *E. coli* cells exposed to lethal doses of ultraviolet. Dense granules can also be seen in electron micrographs and in wet mounts by light microscopy. Whether the granules seen by these methods are the same as the basophilic residues following acid hydrolysis can only be presumed. These granules are to be distinguished from the tetrazolium-reducing granules described above.

## SUMMARY

Lysogenic (strain K12) and non-lysogenic (K12S) cells of *Escherichia coli* from log phase nutrient broth cultures are cytologically indistinguishable. Each cell contains either two or four nuclei, many in divisional stages. Log phase salt-glucose cells present a similar picture.

After ultraviolet treatment the nuclei of viable K12S cells progress through a series of changes characteristic also of other strains of *E. coli*. These include: (1) chromosomal contraction and aggregation, (2) nuclear fusion, (3) chromosomal replication without nuclear or cytoplasmic division, (4) grouping of chromosomes, (5) segregation of chromosomes into haploid nuclei, (6) resumption of cytokinesis. With higher doses of ultraviolet, chromosomal breakdown occurs. Ribonuclease-sensitive polar and peripheral granules are produced in many cells after ultraviolet.

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