CYTOLOGICAL CHANGES IN ESCHERICHIA COLI PRODUCED BY INFECTION WITH PHAGE T2

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The bacteriophages of the "T" system acting on *Escherichia coli* have been studied extensively and have provided a considerable basis for theory of the mechanism and processes of a virus infection. Although methods have been developed for the detailed cytological study of bacterial cells, studies of phage-infected bacteria by such methods have been infrequent. Preliminary experiments by Luria and Palmer (1946) and by Beumer and Quersin (1947) demonstrate the fruitfulness of this line of attack. They showed that each phage produces modifications in the internal structure of the infected cell peculiar to the race of phage and not to the strain of host cells and, further, that these studies "indicate an intimate relation between this process and the behavior of the Feulgen-positive nucleoprotein bodies of the bacterial cell" (Luria and Palmer, 1946). Confirmation and extension of this work is important for two reasons: (1) The correlation of structural changes during phage infection with chemical and metabolic changes (such as those reviewed by Cohen, 1947, 1949) should be of value. (2) Attempts to define the mechanism of reproduction of phage by means of electron microscope studies (e.g., Wyckoff, 1948; Merling, 1949) would be aided by cytological and chemical data as a basis for the control and the interpretation of structures. Studies using phase microscopy (such as those of Boyd, 1949*a*,*b*) should be similarly integrated. A further return may well be a better understanding of the structure or arrangement of the *normal* nucleus, since a great part of the change early in phage infection is reflected in alterations in the nucleus of the bacterial cell.

As an approach to this problem it was decided to study first the sequence of changes to be observed in *E. coli* infected with the T2 phages, since a great volume of published data has accumulated for this particular system. The results are presented in this paper.

MATERIALS AND METHODS

Escherichia coli, strain B, was used as the bacterial host, with coliphages T2r and T2r$^+$. Two media were used: (1) the synthetic medium described by Monod and Wollman (1947) with 0.2 per cent glucose or carbohydrate source, and (2) Difco nutrient broth. The bacteria were maintained on nutrient agar slopes, but were subcultured daily, in the medium used, for at least 48 hours before any experiment.

For each experiment fluid medium was inoculated with *E. coli* B and incubated for 2 to 4 hours. When the cells were in the logarithmic growth phase they were

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spun down and then resuspended in fresh medium. This new suspension was made up to an optical density of approximately 0.025 (approximately 10^8 cells per ml) and divided into two colorimeter tubes for the experiment. To one tube was added a sufficient amount of phage suspension to give the degree of infection desired; the other tube was an uninfected control and received medium only. The total volume in each was 11.5 ml. Usually phage to bacteria ratios of from 2:1 to 4:1 were used. Both tubes were placed in a water bath at 37 C and were shaken mechanically. Specimens for cytological examination were taken every 2 to 3 minutes until lysis occurred. Optical density was followed in the Evelyn colorimeter, which was used also to standardize suspensions. Phage counts were done by the method of Asheshov *et al.* (1933) and cytological preparations were made following the methods described by Robinow (1944).

Giemsa staining of nucleus. Agar blocks 1 cm square were cut out of a well-dried plain agar plate and placed on cover slips for ease of handling. A loopful of the culture to be examined was then spread over the surface of the block. Usually duplicate preparations were made. The groups of blocks were then transferred to a closed vessel for fixation in OsO_4 vapor for 3 minutes. Immediately upon removal, an impression film was taken from each block on cover slips. The films, drying almost instantly, were stored in 70 per cent alcohol. The fixed films were further processed in batches, those from the infected suspension together with the corresponding controls. They were transferred to N HCl at 60 C for 9 minutes, quickly washed, stained in dilute Giemsa (2 drops per ml of M/15 Sørensen buffer pH 7.0) for 10 minutes, and mounted in the staining fluid.

Thionine staining of cytoplasm. A loopful of culture was spread on an agar block as above. After standing for 30 seconds for the absorption of fluid the block was placed, cells down, on a cover slip. The block and cover slip were then immersed in Bouin fixative for fixation through the agar. They were left in the fixative for 1 hour, or until ready, when the block was flicked off the cover slip. The fixed impression film was then washed in water preparatory to staining. The preparation was immersed in 0.01 per cent thionine (Grübler) for 105 seconds, washed, and mounted in water.

Cell wall. Fixed cells were treated with 5 per cent tannic acid for 30 minutes, washed, and stained with 0.02 per cent gentian violet. Preparations were examined by transmitted light using a Leitz N.A 1.32 apochromatic oil immersion objective (90×), an aplanatic condenser N.A. 1.4, and aplanatic 10× eyepieces. Photographs were taken using a Wratten green B58 filter.

OBSERVATIONS

The nuclear structures of *E. coli* from the cultures before the addition of phage and from the control cultures during experiments were substantially as described by others for actively growing cells (Robinow, 1945). In Giemsa preparations the cells showed compact, well-defined chromatinic bodies, varying in number according to the size of the cell (figure 1). Normal cells, fixed with Bouin's fluid and stained with thionine showed the reverse picture (figure 7), the nuclei appearing as unstained patches in a basophilic cytoplasm.

Cytological changes observed in cultures infected with phage T2r. Visible lysis began about 20 to 24 minutes following infection with phage T2r and was soon complete. Three different cytological changes could be seen. During the first 10 minutes a cell type appeared in which the chromatinic material was distributed along the cytoplasmic membrane, usually appearing on both sides of the cell opposite the region where a nuclear body would be expected. Occasionally, however, the chromatin was arranged as a polar cap. The cells showing margination of the chromatin were very different in appearance from uninfected cells (figure 2). During the last 10 minutes of the infection very granular cells appeared and increased in number, whereas the cells showing margination of chromatin were less frequent. In these granular cells the chromatin appeared to be peppered throughout the cytoplasm (figure 3) as granules of assorted sizes, which became larger and more intensely stained as the infection progressed. The granular cell persisted up to the time of lysis and, in fact, seemed to be the cell that lysed. The ghosts of lysed cells (figure 4) were first seen about 16 to 18 minutes after infection and often contained chromatin granules surrounded by an irregular mass of dispersing cytoplasm in which minute dots of faintly basophilic material were visible.

In the marginated stage the cell outline was regular, but irregularities appeared in the granular stage especially toward the end of the process. When cells were stained to show the cell wall, the irregularities were apparent and sometimes small outpouchings were seen or even discontinuity suggestive of a tear in the membrane. These appeared very close to the time of lysis. The irregularities of outline appeared to be related to the position of the granules seen in Giemsa preparations. This was more evident with $T2r^+$ phage.

The progression of events was followed by making differential cell counts on successive preparations. A hundred cells were counted on each slide and the distribution of cell types was plotted graphically (figure 5). It became clear that the cells showing granulation were derived from the cells showing margination of chromatin. When cells of both types were present, their total approximated very closely the peak proportion of marginated cells at 6 to 8 minutes. This relation was also expressed by the symmetrical crossing of the curves for the two cell types between 8 and 20 minutes after infection. The proportion of granular cells was never so high as the proportion of marginated cells counted at their peak. Two explanations for this are advanced: (1) the progression to granular cells may be less regular than the development of margination; and (2) some granular cells may have lysed and disappeared before others had developed. In any case, the proportional figures were subject to error once lysis had begun because of the likelihood that a number of ghost cells would be missed. When the inoculum of phage was small (figure 6) and presumably each cell was infected with only a single phage particle, the picture was the same except for a secondary rise in marginal cells due to infection of normal cells by the released phage. There was no evidence that multiple infection caused any modification of the cytological changes.

The most striking observation in these experiments was the short time interval

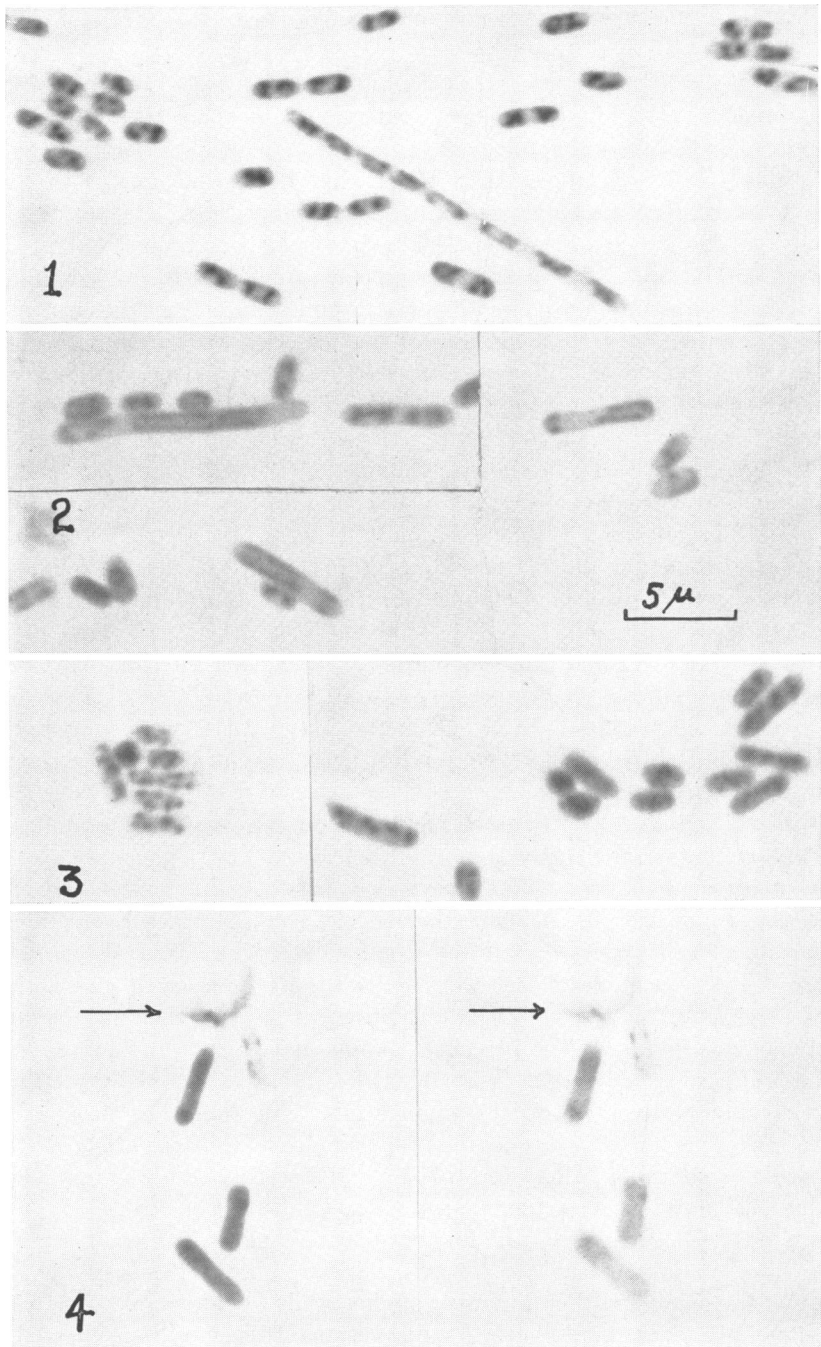


Figure 1. A preparation of normal *E. coli*, hydrolyzed and stained with Giemsa.

Figure 2. Giemsa preparations, 10 minutes after infection with T2r, showing margination of chromatin. Some uninfected cells are present.

Figure 3. Giemsa preparation showing the granulation of chromatin ($T2r^+$).

Figure 4. Giemsa preparation (T2r) showing a lysed cell (arrow). On the left the cells were photographed with green light, and on the right with red light. The intact cells are all infected and their chromatin is very dense.

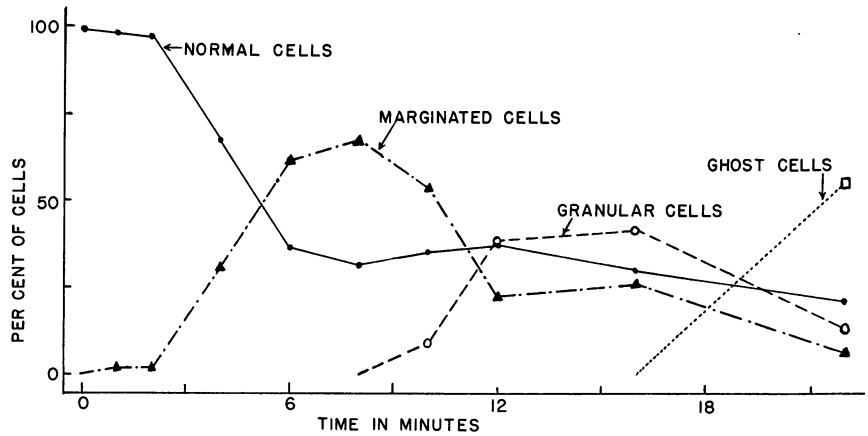
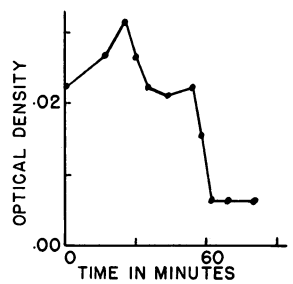


Figure 5. Graph showing the distribution of cell types (Giemsa) during infection with phage T2r.



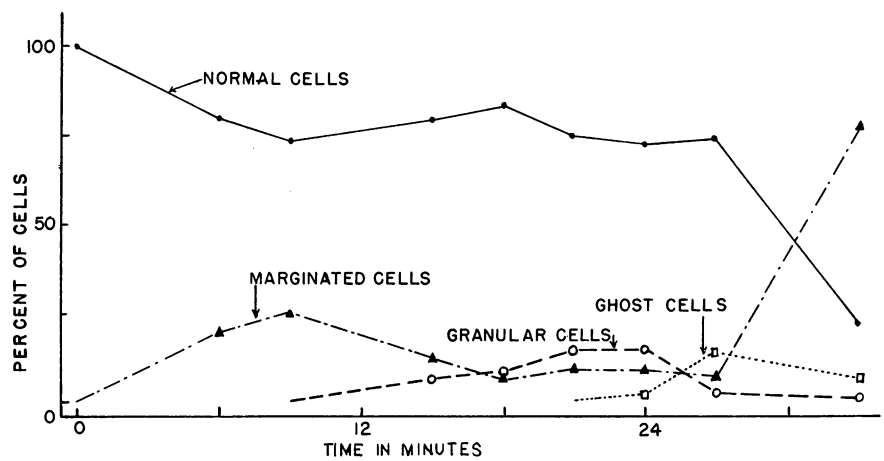


Figure 6. Graph showing the distribution of cell types (Giemsa) in an experiment using an initial concentration of 1×10^8 bacteria per ml and 0.5×10^8 phage T2r per ml. Lysis of the infected cells was followed by infection of the remaining normal cells. The curve for optical density in the experiment is shown above.

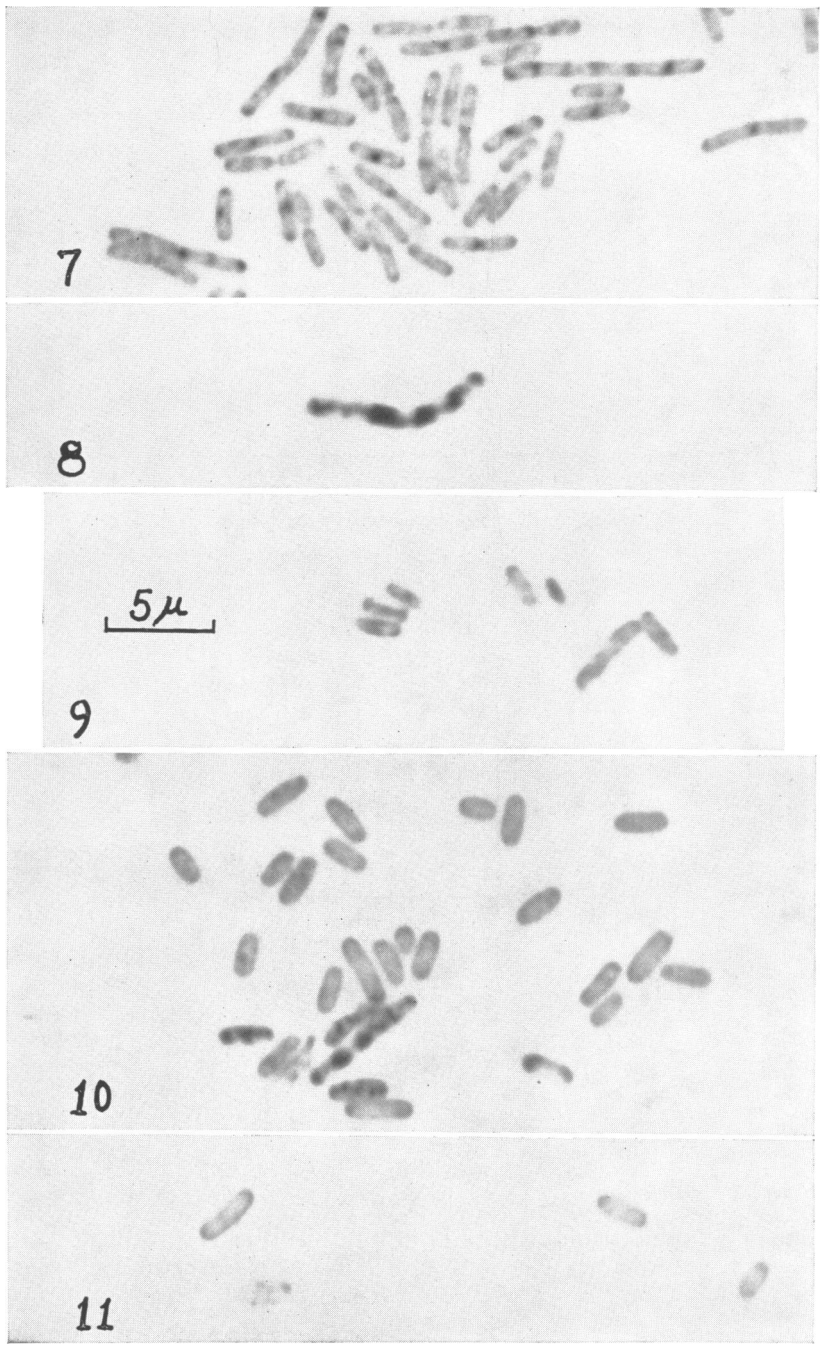


Figure 7. Bouin-fixed normal *E. coli* stained with thionine.

Figure 8. Thionine preparation of cells, 8 minutes after infection with T2r, showing the stage equivalent to margination.

Figure 9. Thionine preparation, 13 minutes after infection (T2r), showing partial loss of basophilia.

Figure 10. Thionine preparation, 18 minutes after infection (T2r), showing a high proportion of very pale cells.

Figure 11. Thionine preparation, 23 minutes after infection (T2r), showing cell ghosts and very pale cells.

between infection and recognizable cytological changes. In most experiments, between 20 and 50 per cent of the cells to show changes had done so within 2 minutes of infection. In occasional experiments, when samples were taken earlier, a few marginated cells were seen 1 minute after infection. This suggests that the

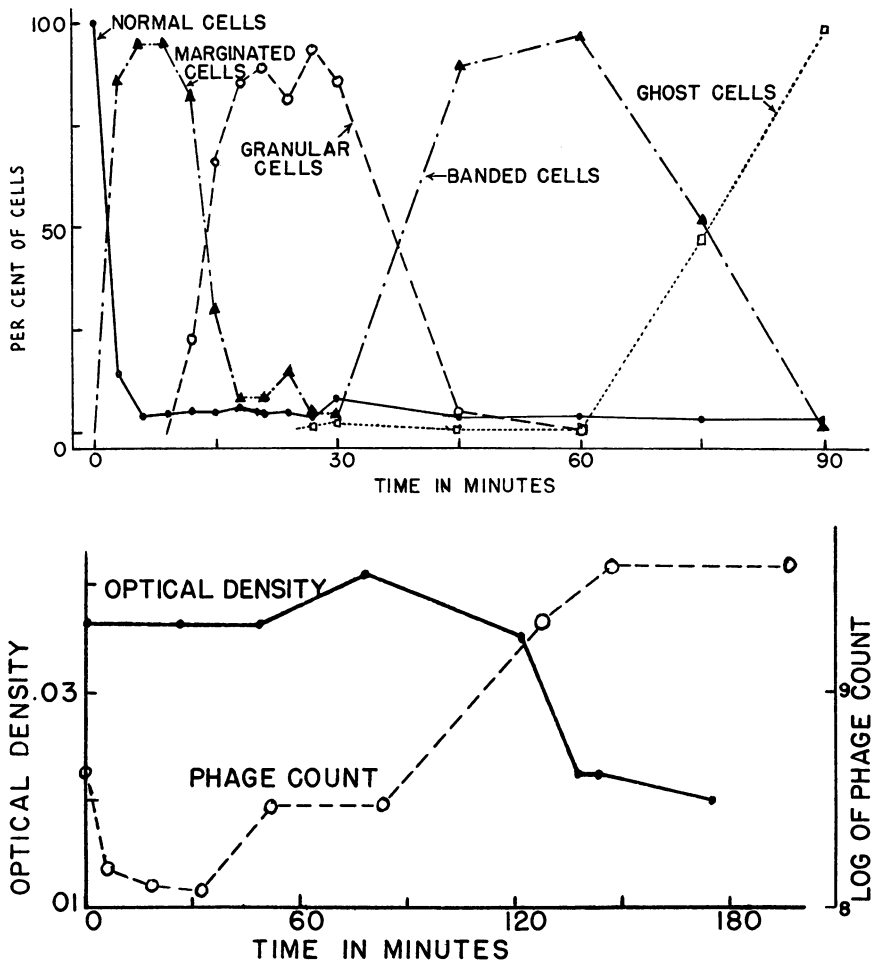


Figure 12. Upper: A graph showing the progression of cell types during infection with T2r+. The curves for marginated and banded cells are continuous because at 24 minutes it was hard to distinguish the two. *Lower:* Data on phage counts and optical density are given.

time required for the nuclear changes following infection may be extremely short and may be determined largely by the time required for the adsorption of the phage particle.

During the first 10 minutes of the infection the observations with thionine staining confirmed those with Giemsa. In the normal cells the nuclei traversed the cell as unstained patches (figure 7) and the outline of the cell was distinct.

Within a few minutes of infection these patches appeared to become applied closely to the surface of the cell and the cytoplasmic membrane in these regions was very hard to see (figure 8). This stage corresponded entirely to the stage of margination observed in Giemsa preparations. Most of these cells also showed a central basophilic core that separated the marginated nuclear material and joined the strongly basophilic patches of cytoplasm. The cytoplasmic basophilia in normal cells and in infected cells in the first 10 minutes seemed roughly equivalent. At 12 to 13 minutes the basophilia decreased and the cytoplasmic membrane became more easily visible (figure 9). This loss was progressive (figures 10 and 11), and near the time of lysis the cells were very pale and hard to see.

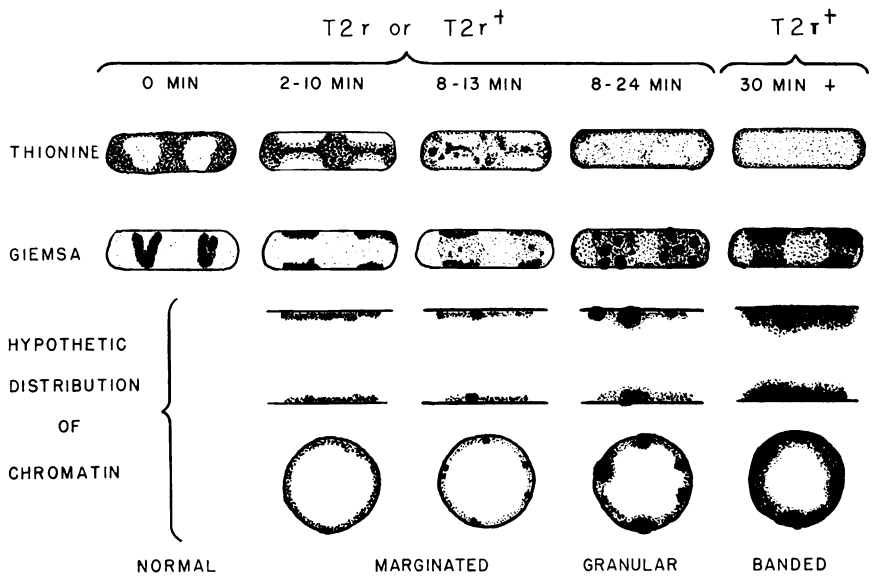


Figure 13. A schema of the changes in *E. coli* infected with T2 phage. The upper two rows represent the changes observed in thionine and Giemsa preparations. The lower two rows represent a concept of longitudinal and cross sections through a chromatin-containing region of the cell.

There was no evidence of cytological differences between infected cells in synthetic medium as compared with nutrient broth.

Cytological changes observed in cells infected with T2r+. Phage T2r+ differs from phage T2r in that it can cause lysis inhibition (Doermann, 1948). Under conditions of single infection and high dilution, lysis of the infected cells occurs at 20 to 21 minutes, as with T2r. However, under the conditions of the experiments reported here, lysis inhibition was the rule and lysis was delayed for from 1 to 8 hours.

During the first 20 minutes the cytological changes observed in cells infected with T2r+ were the same as those observed in the cells infected with T2r. The time relations of the sequence of marginated and granular cells were also similar to those of T2r up to 20 minutes after infection (figure 12). Between 20 and 40

minutes a few ghost (lysed) cells were seen, with a peak of 2 to 30 per cent at about 30 minutes, in experiments in which 90 to 95 per cent of the cells were initially infected. However, at 40 minutes ghost cells were few or absent. This apparent lysis of a small percentage of the cells roughly coincided with an alteration of the remaining infected cells. Cells appeared in which there were dense elongated chromatin bodies against the cytoplasmic membrane, usually paired on opposing sides of the cell. These were reminiscent of the early marginated cell; but they differed in that, joining the paired chromatin bodies, there was a somewhat lighter, yet still intensely staining band. These cells increased in number (figure 12) to the highest level attained by the other cell types, reaching this peak after 50 to 60 minutes. These cells were very swollen compared with normal cells, and during the course of time their chromatin increased in both amount and density of staining. Just prior to lysis some cells seemed to be almost full of densely staining chromatin. Lysis occurred suddenly and without the appearance of any new cell type.

DISCUSSION

The cytological methods employed have advantages for this type of study, because they reflect changes in desoxyribosenucleic acid (DNA) and ribosenucleic acid (RNA). The results can be compared with those of chemical studies of nucleic acids in phage infections.

Staining with Giemsa following hydrolysis showed discrete chromatinic bodies. Corresponding structures are shown by the Feulgen reaction. The Giemsa technique may be considered to indicate the distribution of DNA in the cell (Vendrely and Lipardy, 1946; Tulasne and Vendrely, 1947). We have observed that, following infection, the chromatin abandoned its usual position in the cell and became arranged along the cytoplasmic membrane. This had the appearance of a redistribution, and there was no evidence of a quantitative change. Between 8 and 12 minutes after infection the cells showed patches of more intense staining in some parts of the rearranged chromatin and less intense staining in other parts. The intensely staining patches are interpreted as points of new synthesis. This interpretation is supported by the development of the granular type of cell in which the patches of chromatin increased in density up to the time of lysis. We have the impression that, while there was an increase of DNA in certain areas, existing DNA was lost in other areas. In many respects these observations confirm those of Luria and Palmer (1946). These changes should be correlated with the quantitative DNA changes observed by Cohen (1948) in *E. coli* infected with T2 phage. Cohen found that the DNA increase started at about 7 to 10 minutes after infection, which corresponds almost exactly to the first appearance of the granular type of cell in our experiments.

It is tempting to speculate that the points of chromatin synthesis in the granular cells may be centers of phage reproduction. In support of this idea it may be noted that T2 phage has a very high DNA content (Cohen, 1947). Furthermore, the inactivation studies by Luria and Latarjet (1947) and especially by Latarjet (1948), using ultraviolet and Roentgen irradiation, respectively, indicate that

phage is probably synthesized at multiple loci within the cell after the seventh to ninth minute of infection.

The cells in which lysis was inhibited following T2r$^+$ infection showed a progression of the granular stage. The chromatin became confluent and gave an appearance of concentration at the cytoplasmic membrane with extremely intense staining. This increase in chromatin is consistent with the observation by Cohen (1948) that after the first 10 minutes of T2r$^+$ infection DNA was synthesized at a constant rate throughout both the normal latent period and the period of lysis inhibition. It should be noted that the confluent chromatin often had the appearance of transverse bands in the cell at the approximate positions where margination was noted earlier during the infection. A tentative conclusion is that most of the DNA synthesis takes place within the area originally occupied by the redistributed chromatin.

In preparations fixed with Bouin's fluid and stained with thionine, the cytoplasm was strongly basophilic, but the nucleus was unstained. The basophilia is abolished by hydrolysis, which removes RNA according to Tulasne and Vendrely (1947) and is equivalent to the effect of ribonuclease. It is concluded that this staining procedure probably reflects the RNA distribution in the cell. The first change following infection complemented the change observed in Giemsa preparations: the unstained nucleus was no longer in its normal position, but unstained areas appeared adjacent to the cytoplasmic membrane. The basophilia of the cytoplasm did not appear to change during the first 10 minutes, but subsequently decreased so much that staining could scarcely be detected. If the assumption is true that this staining method reflects RNA content, one could postulate that there is a sudden decrease in RNA beginning about 10 minutes after infection. This interpretation conflicts with chemical data indicating that the RNA content of T2r$^+$-infected cells is constant (Cohen, 1948). Possibly this discrepancy is due to differences in the physiological state of the cells, since our methods differed in important points. Working with phage-infected *Staphylococcus muscae*, Price (1949) found a decrease in RNA, which depended on the physiological state of the cells at the time of infection.

In the scheme shown in figure 13, we have tried to interpret our observations in terms of three-dimensional changes inside the bacterial cell. As already mentioned, there is no disagreement between the pictures seen in Giemsa-stained cells and in thionine-stained cells; they complement each other. We are not prepared to advance an opinion about the structure of the normal nucleus. Following phage infection it appears that the nuclear substance becomes dispersed and takes the form of a sort of cylinder near the cell wall, giving the appearance of margination when viewed under the microscope. At about 8 to 13 minutes the cell seems to be peppered with small granules, which probably are close to the cytoplasmic membrane. Some of the granules seem to disappear, but most of them increase in size and apparent density until lysis finally destroys the cell. If lysis is delayed, because of inhibition by the r$^+$ factor, the granules may become so large that they appear to coalesce at each side of the cell. There appears to be a chromatinic band connecting the opposite marginal structures thus formed, and

so we think that this DNA-containing material still has its cylindrical arrangement. In general, these changes seem to take place in the same cross section of the cell that contained the original nuclear body.

Published electron micrograph studies of phage-infected cells have been disappointing because they have not revealed the course of events during the latent period. In part this appears to be due to the density of the bacterial protoplasm and to difficulty in relating the structures observed to known bacterial constituents. According to Hillier, Mudd, and Smith (1949) the "light areas" in the protoplasm of young, actively growing *E. coli* B correspond to the chromatin bodies or nuclei. Since extensive nuclear changes occur during phage infection, further electron microscope studies are indicated. It seems certain that cytological methods, such as those used here, would be of great value as a basis of control and comparison. Conversely, interpretation of structures seen with the light microscope would be aided by comparison with electron micrographs. For instance, if there are cylindrical structures containing the nucleic acid material in infected cells, as suggested by Giemsa preparations, one wonders whether they might be precursors of the structures in which phage appears to be embedded (*vide* micrographs by Wyckoff, 1948). Such questions can be answered only by examining material from the same source by both methods.

A similar comment applies to phase microscopy. Using this technique, Boyd (1949*a*,*b*) observed the progressive development of areas of lesser density up to the time of lysis. This should be correlated with the general observation that nuclear structures in bacteria appear as light areas under the phase microscope—a characteristic that Tulasne (1949) has used to observe the division of these bodies in growing normal cells. Allowing for the differing techniques employed, the drawings published by Boyd (1949*b*) of *E. coli* B infected with the T phages correspond moderately to the description of nuclear changes by Luria and Palmer (1946) and the observations on T2 reported here.

The rapid rearrangement of nuclear chromatin that takes place in the first few minutes of infection raises questions about the normal bacterial nucleus. Its precise spatial configuration is unknown, which complicates the interpretation of the observed changes. On the other hand, the study of early changes induced by agents, such as phage, that affect the nucleus, may give some information about nuclear structure. Since *E. coli* is a difficult subject for such detailed cytological investigation, experiments on larger cells (such as *Bacillus* sp.) may be more rewarding.

Biochemical evidence (see reviews by Cohen, 1947, 1949) indicates that important changes in the metabolism of the bacterial cell begin immediately following infection by bacteriophage. The cytological studies reported here, along with reports by other authors, show that equally important structural changes occur, and the earliest of these also can be observed very soon after infection. Investigations of nucleic acid metabolism, as well as cytological investigations, make it certain that very striking changes take place in the nuclear material of the infected cell. It would be easy to assume that these are the chief changes in the infected cell, but it should be remembered that techniques have been developed

specifically to investigate nucleic acid material, and fewer data are available about changes in many other cellular components. Nevertheless, it is probable that the fundamental changes in virus infections occur in structures concerned with nucleic acid metabolism.

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SUMMARY

The succession of changes that occur in *Escherichia coli* B, in nutrient broth or synthetic medium, following infection with phage T2r or phage T2r$^+$ have been studied by observing suitably fixed cells stained with either Giemsa or thionine. The two staining methods complement each other.

In Giemsa preparations the first change is the appearance of "marginated' cells in which the chromatin from each nuclear body is distributed along the adjacent cytoplasmic membrane. These are succeeded by "granular" cells in which the chromatin is irregularly distributed. The granules increase in size and density until, with T2r infection, the cells lyse and cell "ghosts" may be observed.

Following infection with T2r$^+$, marginated and granular cells occur as with T2r infection. Instead of lysing, most of the granular cells change to "banded" cells in which the granules seem to coalesce, giving the appearance of thick bands of densely staining chromatin almost filling the cells in the areas originally occupied by the nuclear bodies. The banded cells eventually lyse.

Thionine preparations (in which the cytoplasm but not the nucleus is stained) confirm the initial change described above. Subsequently, the staining capacity of the cytoplasm decreases and is almost lost at the time of lysis.

The interpretation of these observations is discussed. It is suggested that the chromatin has a cylindrical distribution in the cell during the latent period of phage infection.

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