LIGHT AND ELECTRON MICROSCOPIC STUDIES OF *ESCHERICHIA COLI*-COLIPHAGE INTERACTIONS*

I. PREPARATIVE METHOD. COMPARATIVE LIGHT AND ELECTRON MICROSCOPIC CYTOLOGY OF THE *E. COLI* B-T2 SYSTEM

by

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INTRODUCTION

Since d'HERELLE's initial microscopic observations in 1921 on bacterial lysis through the action of bacteriophage[1], a number of cytological techniques have been applied to the problem of obtaining from optical observations information concerning the basic processes of bacteriophage multiplication. Early microscopic observations on living cells are briefly summarized by PIJPER[2]. More recent studies have concentrated attention on strain B of *Escherichia coli* and a series of seven phages, T1–T7, to whose action it is sensitive[3]. WEIGLE[4], using dark-field microscopy, and BOYD[5,6] using phase microscopy, have studied these systems, and HEDÉN[7] has described his observations on T2 infection through the use of a number of techniques including phase and ultraviolet microscopy. Nuclear staining techniques have recently revealed striking cytological information on the infectious processes in *E. coli* B infected with the T-series of bacteriophages[8–13]. However, since bacteriophage particles are beyond the limits of resolution (not of visibility) of the light microscope, the finer intricacies of bacteriophage replication cannot be observed by the above techniques.

The morphological characteristics of phage were first demonstrated by RUSKA[14] with the electron microscope. LURIA AND ANDERSON[15] and LURIA, DELBRÜCK AND ANDERSON[16] studied in detail *E. coli* B cells lysed by the action of T1 and T2 phages. The bacteriophage T2 was characterized still further by electron micrographs of purified suspensions of the virus[17]. Other early electron microscopic studies have been well reviewed by RUSKA[18]. More recently, the effects of bacteriophage on bacteria have been studied with the electron microscope by WYCKOFF and colleagues[19–28], BIELIG, KAUSCHE AND HAARDICK[29], HAARDICK[30] MERLING[31], HENNESSEN[32], GRÜN AND HENNESSEN[33],

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HILLIER, MUDD AND SMITH[34,35], HERCIK[36-39], and HEDÉN[7]. BAYLOR AND CLARK[40] studied the progress of infection in cultures infected with a mixture of T1 and T2 viruses.

While these electron microscope studies reveal indications as to the changes undergone by the host cell and perhaps lend suggestions as to the possible processes carried on in infection, several limitations must be placed upon the above-mentioned studies and the various interpretations presented by the authors of those papers for one or more of the following reasons: 1. Observations were made on phage-infected bacteria in which the period of infection was not controlled or upon cells following lysis; 2. preparative techniques used by many workers were probably conducive to the production of artifacts, though this possibility in some cases was overlooked; 3. the shadowing technique, which is valuable but not optimal for the study of *intracellular* structures in intact bacteria, has been used exclusively in many studies; 4. in many cases the knowledge of the bacteria-bacteriophage system gained by other techniques (genetic, biochemical, biophysical) has not been recognized in interpretation of the micrographs.

Certain of the limitations of earlier studies have been inherent in the instrument. The fine structures to be resolved within phage-infected bacteria are superimposed in cells which even after desiccation are many molecular diameters in thickness. Sufficient contrast between the electron images of nuclei, mitochondria, phage particles, etc., must be secured under these difficult conditions to define their several structures and relationships. The problems thus presented are at the very limits of present instrumentation and photographic processing.

Some of the more recent work has been carried out under more carefully controlled conditions. EDWARDS[41], working with T1 phage, NODA AND WYCKOFF[28], and most particularly LEVINTHAL AND FISHER[42], using the T2 system, have examined cells in the electron microscope at successive intervals following infection. LEVINTHAL, with a cleverly devised method for making electron microscope preparations from cells ruptured during the latent period by rapid decompression techniques, has been able to shed new light on the nature of phage precursors and note the time of appearance of these particles in cells by a survey of the contents of the physically lysed cells.

In the present study we have attempted to overcome as many as possible of the limitations mentioned, reconciling conditions necessary for electron microscopy and those required for an accurate study of a biological system. The latent period in the infection of *Escherichia coli*, strain B, with T2 phage has been selected for intensive study, both because it is the period during which the most important changes take place in the host cell and because it has also been intensively studied by a number of other techniques. We have been able to gain further information[43,44] on the changes that take place during phage infection by virtue of greatly improved electron microscopic equipment[45], an improved technique for the preparation of electron microscope specimens, and through the use of parallel light and electron microscope studies carried out under controlled conditions.

PREPARATIVE METHODS

Escherichia coli, strain B, and phages T2r$^+$ and T2r were used (DELBRÜCK[3]). These phages were concentrated and purified by differential centrifugation[46]. A tryptone-yeast-extract-glucose medium (MORTON AND ENGLEY[47]) was employed in all experiments.

Electron microscopy. Electron microscope preparations were made by growing bacteria on collodion overlying agar (HILLIER, KNAYSI AND BAKER[48]). The bacteria were infected by placing a drop of phage suspension on the bacteria growing on the collodion. In order to follow the changes

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in bacterial and phage populations quantitatively under the conditions used for making electron microscope preparations, it was found necessary to keep the micro-colonies on the collodion moist by placing wet filter paper in the lid of the Petri dishes during incubation. Assays were made of the bacteriophage and the bacteria growing on the collodion by the following technique: An area of the collodion that had been seeded with bacteria under standardized conditions, incubated, and infected with phage for an appropriate period, was cut out together with the agar upon which it rested. If there were no signs of leakage through the collodion film, it was floated off onto liquid in a test tube, the film broken up with a platinum loop and the suspension mixed with a pipette. Appropriate dilutions were assayed for either phage or bacteria by the agar layer technique (ADAMS[46]).

The collodion films used for electron microscopy were seeded with bacteria, incubated, and infected in the same way as the films used for assays. Those microcultures on collodion which showed no signs of leakage were selected for further work. A film resting on an agar block was fixed in the vapour of 2% OsO_4 solution for one minute; it was then floated off on distilled water and allowed to dialyze for one hour to remove excess salts and other small molecular substances. Finally, the film was picked up on a copper screen and allowed to dry. Some series of electron microscope preparations were made concomitantly with the growth curves. To insure punching out discs clearly for insertion in the electron microscope and for ease of filing and carrying the preparations, it was found convenient to attach the copper screens by a corner with scotch tape onto appropriately labelled uniform sections of heavy index cards cut to the size of an ordinary microscope slide*.

Light cytology. For light cytological studies, *E. coli* B was grown in broth with aeration. When the culture had grown to a density of about 10^8 cells/ml, it was infected with an appropriate amount of the phage concentrates described above. The multiplicity of infection was determined by assays of phage and bacteria before infection; the usual multiplicity used was close to 8. In some experiments the per cent. infection was checked by assays of bacteria shortly before and several minutes after infection. One-step growth curves of T2r in broth were performed essentially as described by ADAMS[46].

Impression smears were made of normal and infected bacteria by the technique described by MURRAY *et al.*[10]. Nuclear stains were made by one of two techniques. Some preparations were stained by the procedure described by DE LAMATER[49]; other preparations were stained by a modified Feulgen technique. Feulgen preparations were made by a procedure identical with that used for the DeLamater stain except for the substitution of the Schiff reagent for the DeLamater thionin-thionyl chloride stain. The Schiff reagent was prepared by pouring 200 ml boiling distilled water over 1 g Dustless Calco Pararosaniline base** mixed with fresh activated charcoal. After cooling to 56° C the dye was filtered, allowed to cool to room temperature and 1 g $K_2S_2O_5$ added. The pH was then adjusted with 1 *N* HCl to pH 2.3–2.4. Coverslip preparations were rinsed in 0.5% $K_2S_2O_5$ at pH 2.3–2.4.

Light microscopy was carried out with the Bausch and Lomb research microscope CCTB using balcoated lenses, 15 × eye pieces, and a 97 × achromatic oil immersion objective (n.a. = 1.25); critical illumination was provided by a Bausch and Lomb ribbon filament lamp with a daylight blue filter and Wratten filters E22 and B58. Pictures were taken with a Bausch and Lomb L type camera on Ansco isopan film.

EXPERIMENTAL RESULTS

Growth curve studies

A one-step growth curve was performed for T2r phage infecting *E. coli* B grown under the conditions used in preparing specimens for electron microscopy; *i.e.* bacteria were grown and infected on collodion overlying a nutrient agar. The results of this experiment are shown in Fig. 1 together with a one-step growth curve of the same phage infecting bacteria in broth. The rise period of the phage infecting bacteria grown on collodion starts between 19 and 23 minutes, which corresponds to the 22 minute latent period observed in broth. The duration of the one-step growth curve on collodion is (in most cases) somewhat longer, and the burst size is somewhat greater. However, from Fig. 1 it is apparent that the latent periods of the phage infecting bacteria under these two conditions are sufficiently comparable to allow a comparison of the observations made on electron micrographs with results obtained with phage infecting bacteria in broth.

* For this device we are indebted to LOREN C. WINTERSCHEID.

** Obtained from American Cyanamid Company, Calco Chemical Division, Dyestuff Department, Bound Brook, N.J.

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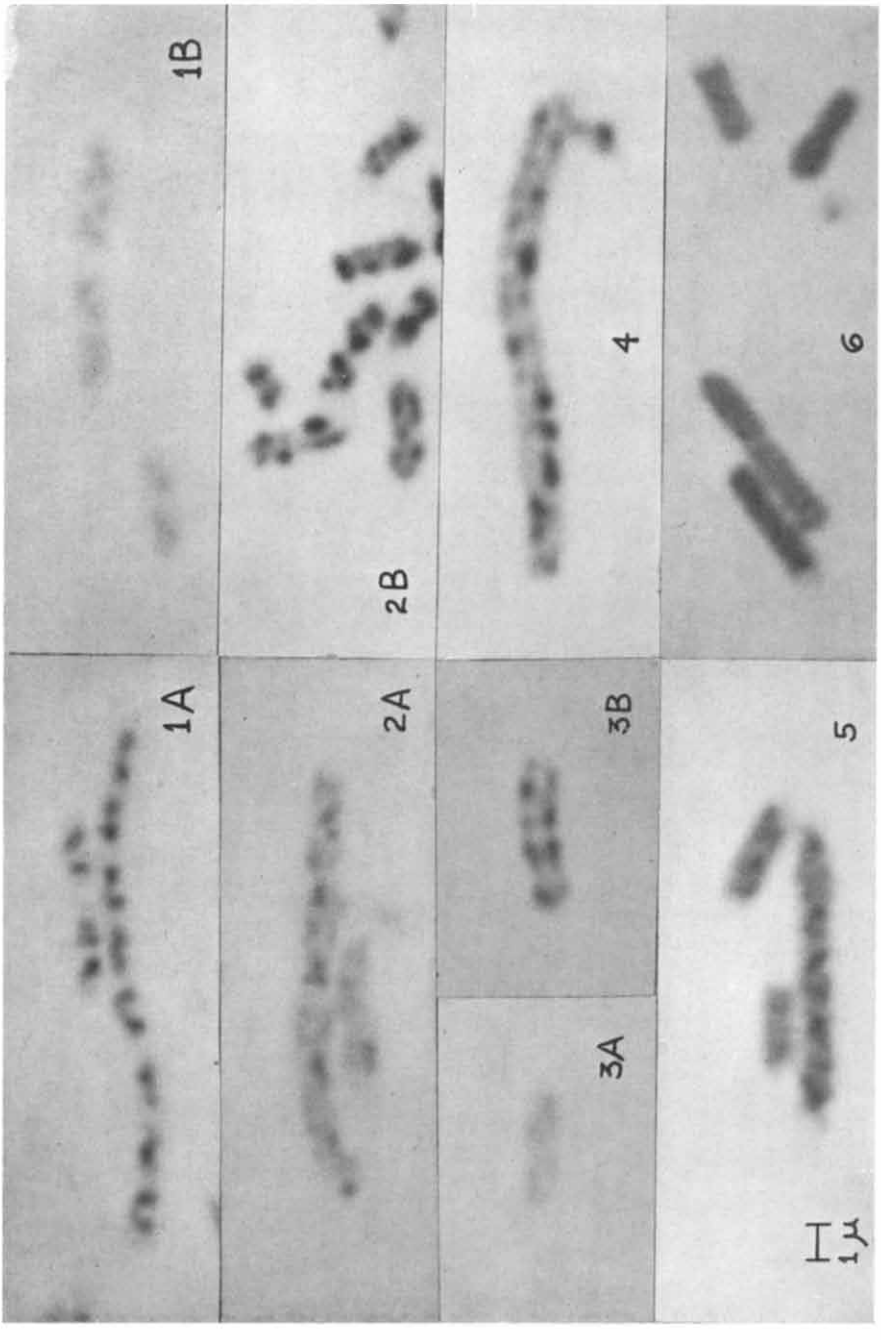

Fig. 1A and 1B. Uninfected *E. coli* B. 1A, thionin-thionyl chloride stain; 1B, Feulgen stain.
Fig. 2A and 2B. *E. coli* B infected 5 minutes, 2A with T2r, and 2B with T2r^{+}. Thionin-thionyl chloride stains.
Fig. 3A and 3B. *E. coli* B infected 10 minutes with T2r^{+}. 3A, Feulgen stain; 3B, thionin-thionyl chloride stain.
Fig. 4. *E. coli* B infected 15 minutes with T2r^{+}. Thionin-thionyl chloride stain.
Fig. 5. *E. coli* B infected 20 minutes with T2r. Thionin-thionyl chloride stain.
Fig. 6. *E. coli* B infected 25 minutes with T2r. Thionin-thionyl chloride stain.

One-step growth curves of T2r+ infected *E. coli* on collodion were somewhat variable; a slight rise was frequently observed at about 22 minutes but the rise period did not end until 80 to 90 minutes after infection. Though the multiplicity of infection cannot be calculated exactly for the cells grown on collodion, phage was observed to be fairly uniformly distributed throughout each set of preparations; conditions were used which provided multiple infection.

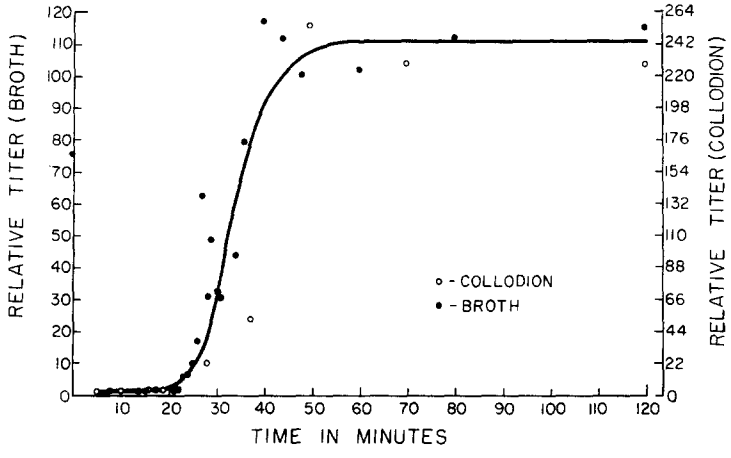

Fig. 1. Comparative one-step growth curves in broth and on collodion of *E. coli* B infected with T2r.

Light cytology of cells infected with T2 phage

Figs. 1A to 6 illustrate the sequence of events following infection of *E. coli* B with T2 phage, as shown by the Feulgen and DeLamater stains. In the systems under consideration the Feulgen and DeLamater procedures give essentially concordant pictures. Since the latter procedure gives better contrast for photography, the majority selected for reproduction were thus prepared.

Figs. 1A and 1B show DeLamater and Feulgen stains of young, uninfected cells of *E. coli* B taken from the logarithmic phase of growth. Two-, four- and eight-nucleate rods are included. The nuclear chromatin presents the pattern familiar in young normal cells. In his most recent work DE LAMATER[50] has presented cogent evidence that this pattern represents mitotic nuclear division.

Figs. 2A and 2B show cells of *E. coli* B five minutes after infection, respectively, with T2r and T2r+ phage. The nuclear chromatin is displaced toward the margins of the cells from its normal axial position, and has already lost the configurations characteristic of normal nuclei.

Figs. 3A and 3B show cells ten minutes after infection with T2r+ stained, respectively, by the Feulgen and DeLamater procedures. The displacement of nuclear chromatin to the margins of the cells has proceeded even further than at five minutes.

Fig. 4 shows cells 15 minutes after infection with T2r+. The chromatin is marginal in position and granular.

Fig. 5 shows cells 20 minutes after infection with T2r. Some fairly coarse granules of chromatin are still detectable, but the cells are filling up with more finely granular material, not removed by the HCl-hydrolysis, which stains with thionin-thionyl chloride and by the Feulgen reaction.

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Fig. 6 shows cells 25 minutes after infection with T2r. These still unlysed cells are filled with much thionin-thionyl chloride-, Feulgen-positive material; the residual granules of chromatin are hardly discernible.

The sequence of cytological changes we have described following infection of *E. coli* B with active T2r$^+$ and T2r phages is essentially similar to and in confirmation of the sequences described by LURIA AND PALMER[8], LURIA AND HUMAN[9] and MURRAY, GILLEN AND HEAGY[10]. We stress particularly the loss of characteristic configuration and position of the nuclear chromatin. These profound structural alterations of the nuclear chromatin are evident within the first minutes after infection and progress during the latent period.

Clear biochemical evidence of the degradation of nuclear desoxyribose nucleic acid (DNA), and of the incorporation of components of the host cell DNA into the virus progeny, has been obtained with *E. coli* B, T-even phage systems[51–55]. According to both groups of investigators most or substantially all of the host nuclear DNA is so transferred. Current data indicate that the transfer of components of host DNA to virus progeny occurs in units at least as small as nucleosides. These building stones of nucleic acids are presumed to be unable to act as genetic determiners.

A second observation deserving emphasis is the filling up of the infected *E. coli* cells with Feulgen-positive material during the second half of the latent period. In the *E. coli* B, T-even systems approximately 20–30% of the N and P of the viral progeny comes from preformed components of the host bacteria and 70–80% from components of the medium assimilated after infection[53,54,56]. In particular COHEN AND ARBOGAST[57] have demonstrated the continuing accumulation of DNA from its time of initiation, in the case of T2 about 7 minutes after infection, throughout the remainder of the latent period. The accumulation of Feulgen-positive material in the latter half of the latent period doubtless reflects synthesis of DNA.

Correlation of light and electron microscopic observations

It now becomes possible to compare the results obtained from the light microscopic study with the electron microscopic observations in view of (1) the controlled period of infection of the electron micrograph preparations, and (2) the comparative one-step growth curves (Fig. 1) correlating the latent period of phage infecting broth-grown and collodion-grown *E. coli*. Striking correlations are observed between the nuclear-stained areas and the vesicular areas seen in electron micrographs.

In normal, actively growing cells, the chromatinic structures extend from side to side (Fig. 1A and 1B). Corresponding to these, the osmium tetraoxide fixed cells seen in the electron microscope (Fig. 1 in the following paper) have vesicular areas extending from side to side in the same parts of the bacilli. This correlation has been carefully described by HILLIER, MUDD AND SMITH[35] and MUDD AND SMITH[58].

Fig. 6 in the following paper is an electron micrograph of 5-minute infected cells. The transverse vesicular areas of normal cells have been replaced by marginal vesicles. These correspond quite closely to the marginal chromatin seen in light micrographs of nuclear stained preparations (Figs. 2A and 2B). Marginal vesicular areas have been seen, not only in electron micrographs of osmic acid fixed cells, but also in unfixed cells.

Figs. 7 and 9 in the following paper are unshadowed and shadowed electron micrographs of cells infected for 10 minutes. The margination of the vesicles has become more pronounced. Likewise, the margination of DNA as seen in Feulgen-stained preparations (Fig. 3A) has become accentuated.

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The vesicular nature of the less electron-opaque areas is apparent from the following paper. The less-dense marginal vesicular areas have collapsed upon drying.

It is not possible to correlate the vesicle formation and the localization of DNA in cells infected for 15 minutes and longer. (See Figs. 4–6 above and the electron micrographs in the following paper.)

DISCUSSION

Past studies of phage-infected bacteria in which the biological state of the system was not clearly defined have contributed but little to knowledge of the changes during the infectious process. In the current study of T2 phage-infected *E. coli* B, techniques were developed that made it possible to control the per cent infection and the period of infection, and to determine the latent period, under the conditions used in the modified HILLIER, KNAYSI AND BAKER[48] technique.

The technique of HILLIER, KNAYSI AND BAKER[48] for preparing specimens for electron microscopy has recently met with some criticism (HENNESSEN, GRÜN AND LINDNER[59]; LIEBERMEISTER[60], KELLENBERGER[61]). The use of rigid standards in the selection of microcultures on collodion at the time of bacterial inoculation, and later at the time of phage infection, eliminated any cultures which leaked through breaks in the collodion membrane. The relatively short maximum period of incubation which we used (4 hours), together with the use of wet filter paper in the lids of the Petri dishes, prevented over-drying and the consequent isolation of bacterial cells from their source of nutrient beneath the membrane. Several preparations shadowed on the reverse (under) side of the collodion revealed no bacteriophage or bacteria to be present between the film and the agar; this observation was confirmed in preparations lightly shadowed on the upper surface whereby either transmission- or shadow-type micrographs could be obtained on the same cell through the control of electron exposure and with special printing processes (HILLIER, unpublished).

The technique of HILLIER, KNAYSI AND BAKER[48] offers a great advantage in that it permits manipulation of the specimens and, at the same time, preserves the intimate relationships existing in both cell structures and infecting virus. The deleterious effects of centrifuging the cells from distilled water suspensions, etc. common to most other techniques, are thus avoided. RAETTIG[62] reports this technique to be superior to suspension, impression or pseudo-replica preparations for studying phage-host cell systems.

The technique has two handicaps in studies of phage infection, namely the difficulty of determining the exact multiplicity of infection and in the failure completely to eliminate residual infecting phage. The latter cannot be avoided in a time-study of lytic systems by this method but of course it would not be present in studies on induced lysogenic cells. The former factor can be partially circumvented by bacterial assays before and after bacteriophage infection.

For light microscopy the nuclear stain of DELAMATER[49] was used in most preparations. Many of the structures (*e.g.* spindles, centrosomes, etc.) associated with mitotic division in this organism (DELAMATER, personal communication) have been hydrolyzed away so that the localization of the nuclear DNA could be followed more accurately in T2 infection. These associated structures, as well as residual mitochondrial granules, do appear, however, in preparations of uninfected cells stained with the DeLamater "double-stain" when *slight under*hydrolysis is used. In neither the light nor electron

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microscopic studies was any difference found between T2r and T2r$^+$ infection other than lysis inhibition and the presence of phage in a mature state only at 25 minutes post-infection in the case of cells infected with T2r. Thus, they have been used interchangeably.

The correlation between Feulgen-positive areas and the electron microscopic vesicles in the case of normal bacteria has been described[35,58,63]. The evidence presented in this paper suggests a similar correlation between the marginal nuclear staining areas and the marginal vesicles seen in the electron microscope in the case of bacteria infected for five minutes. An even more clear-cut demonstration of this phenomenon is seen in cells infected for ten minutes.

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SUMMARY

Preparative methods are described by which young colonies of *E. coli* B on collodion overlying nutrient agar are infected with T2 phage, fixed at selected time intervals following infection, dialyzed for removal of obscuring impurities and studied electron microscopically. One-step growth curves of phage replication under these conditions have been essentially similar to those in broth.

Light cytological preparations, in sequence of time following infection, stained by the Feulgen and DeLamater procedures, have essentially confirmed earlier studies by LURIA AND PALMER[8], LURIA AND HUMAN[9], and MURRAY, GILLEN AND HEAGY[10]. The nuclear chromatin loses its characteristic configuration and position during the first half of the latent period, and is reduced to granular residues along the periphery of the infected cells. During the second half of the latent period the infected cells fill up with finely dispersed Feulgen-positive material. The disintegration of the nucleus is in agreement with biochemical data indicating breakdown of the nuclear DNA and transfer of its components to the phage progeny[51–55]. The filling up of the cells with Feulgen-positive material is in agreement with the synthesis of new DNA by the infected cells, utilizing materials from the external medium[53–57].

RÉSUMÉ

Nous avons décrit des méthodes préparatives à l'aide desquelles de jeunes colonies de *E. coli* B, placées sur un film de collodien à la surface d'agar nutritif, sont infectées par du phage T2, fixées après des intervalles de temps choisis, dialysées pour supprimer des impuretés et étudiées au microscope électronique. Les courbes de croissance à un palier de la multiplication ("réplication") du phage dans ces conditions étaient essentiellement semblables à celles enrégistrées pour la croissance dans du bouillon.

L'étude, au microscope à lumière, de préparations cytologiques, rangées d'après la durée d'infection, colorées d'après la technique de Feulgen et celle de De Lamater, confirme essentiellement les résultats obtenus par LURIA ET PALMER[8], LURIA ET HUMAN[9], et MURRAY, GILLEN ET HEAGY[10]. La chromatine nucléaire perd sa configuration et sa position caractéristiques pendant la première moitié de la période latente et se trouve réduite à des résidus granulaires lelong de la périphérie des cellules infectées. Pendant la deuxième moitié de la période latente, les cellules infectées se remplissent de matière finement dispersée Feulgen-positive. La désintégration du noyau est en accord avec la dégradation du DNA nucléaire et le passage de ses constituants aux phages nouveaux, phénomènes indiqués par les données biochimiques[51–55]. Le remplissage cellules par de la matière Feulgen-positive est en accord avec la synthèse de DNA nouveau par les cellules infectées, synthèse qui utilise de la matière provenant du milieu ambiant[53–57].

ZUSAMMENFASSUNG

Es werden präparative Methoden beschrieben, mit denen junge Kolonien von *E. coli* B auf mit Kollodium überschichtetem Nähragar mit T2-Phage infiziert, nach bestimmten auf die Infektion

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folgenden Zeitabständen fixiert, zur Entfernung trübender Verunreinigungen dialysiert und unter dem Elektronenmikroskop untersucht wurden. Die Einstufenwachstumskurven der spezifischen Phagenvermehrung unter diesen Bedingungen sind im wesentlichen denen in Bouillon ähnlich.

Licht-mikroskopische cytologische Präparate, die, nach der Infektionsdauer geordnet, nach den Feulgen und DeLamaterverfahren gefärbt wurden, bestätigen im wesentlichen die Untersuchungen von LURIA UND PALMER[8], LURIA UND HUMAN[9], und MURRAY, GILLEN UND HEAGY[10]. Das Kernchromatin verliert seine charakteristische Konfiguration und Stellung in der ersten Hälfte der latenten Periode und wird auf körnige Reste in der Peripherie der infizierten Zelle reduziert. In der zweiten Hälfte der latenten Periode wird die infizierte Zelle mit feinzerteiltem Feulgen-positiven Material aufgefüllt. Die Auflösung des Kerns in seine Bestandteile ist in Übereinstimmung mit biochemischen Daten, die den Zusammenbruch der Kern-DNS und die Übertragung seiner Bestandteile an die neugebildeten Phagen anzeigen[51–55]. Das Auffüllen der Zellen mit Feulgen-positivem Material ist in Übereinstimmung mit der Synthese neuer DNS durch die infizierte Zelle, wobei Material des äusseren Mediums benützt wird[53–57].

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