MICROBIOLOGY AND IMMUNITY

THE GROWTH AND DEVELOPMENT OF BACTERIA IN CONNECTION WITH THE PHENOMENON OF BACTERIOPHAGE

COMMUNICATION VI. INVESTIGATION OF THE NUCLEAR STRUCTURES OF BACTERIA BY MEANS OF THE ELECTRON MICROSCOPE AND MICROCHEMICAL ANALYSIS

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In research on various aspects of the problem of the growth and development of bacteria in connection with the phenomenon of bacteriophage, the study of the influence of phage on the nuclear structures of bacteria is of fundamental importance.

As we know, the foundations of the study of the relationships between phage and the chromatin elements was laid as long ago as 1934 by N. N. Zhukov-Verezhnikov and V. A. Friauf [2]. In 1943-1946, their findings on the loss of basophilia of the protoplasm by bacteria under the influence of phage, together with a sharper delineation of the nuclei, were confirmed by M. A. Peshkov [5], Luria and Palmer [8], and subsequently by the observations of other workers.

In recent years papers have been published in which are given the results of electron microscopic investigations of the nuclear apparatus of bacteria under normal conditions and affected by phage. Since these investigations were carried out on a limited number of bacteria, and since the conclusions derived show certain contradictions, we considered it desirable to continue work in this direction by studying other species of microorganisms both under normal conditions and when affected by phage.

EXPERIMENTAL METHOD

The nuclear structures were studied by means of electron microscopy of cells from 18-20 hour broth cultures of Bact. coli aerogenes 1321 and of sections of these bacteria of varying thickness. The thicknesses of section mainly used were 250, 500, 750, 1000 and 1250 A, prepared with a Cambridge microtome.

In order to obtain ultrathin sections we used fixation and mounting of the bacteria in methacrylic resins according to the Birch-Andersen, Maale and Sjöstrand formula [7]. For this purpose, to 15 ml of an 18-20 hour broth culture of B. coli, diluted in the standard manner to $25 \cdot 10^4$ cells per ml, was added 1.5 ml of 2% OsO_4 in a buffer (a sterile solution of KH_2PO_4 1.45 g, $Na_2HPO_4 \cdot 2H_2O$ 6 g and NaCl 4.8 g in 1000 ml of distilled water). The mixture thus obtained was centrifuged for 5-6 minutes at a speed of 6000 rpm, after which the precipitate was resuspended in 1 ml of buffer and again mixed with 1 ml of 2% osmium tetroxide (OsO_4). The new mixture was incubated at 37° for 2 hours, during which time fixation was completed. After fixation, the bacteria were washed in 15 ml of buffer, and after centrifugation for a second time, they were resuspended in 70° ethyl alcohol, in which they were allowed to stand for 2 hours at room temperature (at 4° at night). The bacteria were then passed through 96° and absolute alcohol, and allowed to stand in each for 2 hours. After dehydration, the bacteria were transferred to 2 ml of a mixture of n-butylmethacrylate and methylmethacrylate (9:1) with 1% of benzoyl peroxide (as a

catalyst). The replacement of ethyl alcohol by the metacrylates enabled 4 successive transfers of the cells to be made at intervals of not less than 1 hour by means of centrifugation for 5-10 minutes at a speed of 6000 rpm. The final (fourth) volume of methacrylates, together with the suspended bacteria, was poured by means of a Pasteur pipette into dry No.2 gelatin capsules, which were incubated at 55-60° for polymerization, which was usually complete after 24-48 hours.

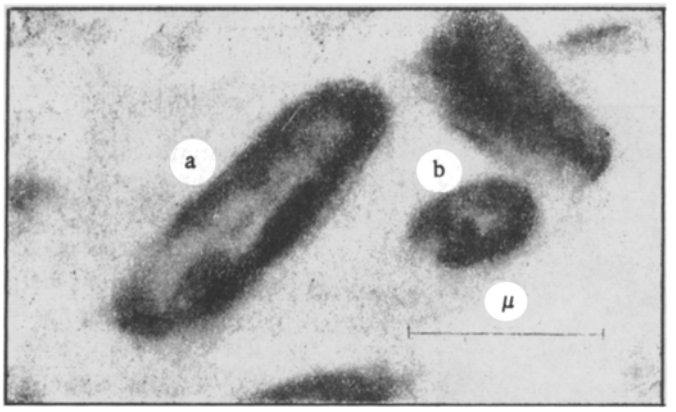

Fig. 1. Sections of Bact. coli aerogenes
a) Longitudinal section; in the center of the cell is a slender, broken up nuclear band; b) transverse section; in the center of the cell is the section of a round, nuclear band. Magnification 20,000 ×.

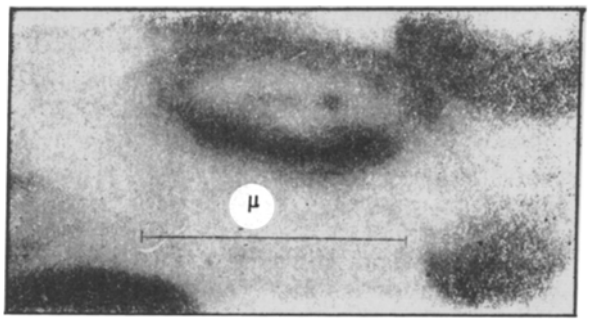

Fig. 2. Longitudinal section of Bact. coli aerogenes showing an elongated nuclear band. Magnification 20,000 ×.

The prepared blocks were cut into sections by means of glass knives. The sections were assembled on copper gauze, coated with a collodion film, and the preparations were subsequently dried in air.

Electron-microscopic examinations of the intact bacteria and of their sections were supplemented by optic studies of stained preparations. For this purpose sections were transferred from the back of the knife to a drop of 20% alcohol on a cover glass. The sections were dried on the glass and then stained by the Romanowsky-Giemsa and Feulgen methods.

Each series of experiments thus consisted of an electron-microscopic study of the intact bacteria and of sections of these bacteria of varying thickness, supplemented by microchemical analysis of stained preparations.

EXPERIMENTAL RESULTS

Intact cells. On examination of intact cells of Bact. coli aerogenes under the electron microscope, no special features could be distinguished on the basis of which some idea could have been obtained on inclusions of

nuclear type in the protoplasm. The protoplasm of bacteria cultivated in meat-peptone broth and on meat-peptone agar was characterized in every case by its homogeneity, in the sense of its density in relation to a stream of electrons. Nor could light zones in the protoplasm, usually taken to be nuclei [6], be detected either during examination of preparations of bacteria which had been grown on a collodion film, i.e. in conditions which most favored the preservation of their natural form. In several experiments preparations of bacteria were examined from cultures after growth in broth for periods longer than 48 hours. The protoplasm of such bacteria was characterized by shrinkage and by general translucency, i.e. by a reduction of electron-optic density. Under these circumstances at the poles of many of the bacteria and sometimes in other areas too, electron-optically dense granules were found in the protoplasm, but these were not nuclei.

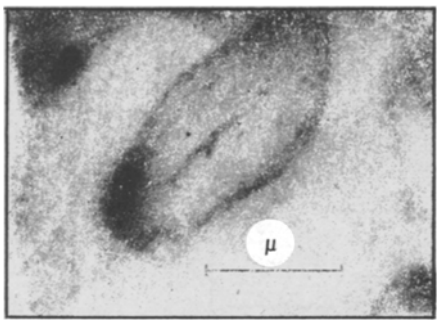

Fig. 3. Longitudinal section of Bact. coli aerogenes showing the nucleus and "polar cap." Magnification 20,000 x.

A study of the intact cells, stained as described above, by examination under the ordinary microscope, showed that certain formations were nevertheless present in the bacteria which, by their microscopic properties might be regarded as nuclear. In bacterial preparations stained by the Romanowsky-Giemsa method, these formations were distinguishable against the pale pink background of the protoplasm by virtue of their intense reddish violet staining. The majority of these had the appearance of structures stretched along the length of the cell. Some of them were characteristically round or spherical in shape, and in such cases 2-3 formations were present. In individual cells, at the poles, at the same time granules were found, which stained intensively a violet color, giving the appearance of polar caps and which were evidently identical with the electron-optically dense granules found under the electron microscope.

In preparations of bacteria stained by Feulgen's method, after fixation with Carnoy's fluid an analogous picture was observed. The field of vision of these preparations showed feebly stained bacilli of a lilac color, with nuclear elements buried in their cytoplasm in the form of reddish violet inclusions. These inclusions resembled spheres or short rods, lying longitudinally, transversely or obliquely in the cells. Under these circumstances a small number of cells were observed in which no reddish violet nuclear structures were present (the bacterial cadavers of M. A. Peshkov). On electron microscopic examination of sections of bacteria, usually in the field of vision were seen bacterial sections whose external form was determined by the direction of the section, i.e. by a mainly longitudinal, oblique or transverse direction.

The internal contents could be examined most completely in sections of bacteria in the longitudinal direction (Figs. 1, 2). In this case, if the anatomy of the bacteria was studied by examining the sections from the periphery toward the center, the following characteristic features could be made out. After the electron-optically dense bacterial wall came a layer of a less dense substance in which were seen some extremely fine granules and tubules between them. In the center of the section of the bacteria was a layer which was almost without density in relation to electrons. On photographic impressions it resembled a characteristic vacuole. Nuclear structures were included within this vacuole and had the appearance of an electron-optically dense band, stretched longitudinally along almost the whole length of the cell. In sections of many of the cells, besides nuclear structures it was possible to see also sections of polar cytoplasmic granules (Fig. 3). Nuclear structures were absent from some sections. Cells which were cut across in a stage of division usually had two bands, situated one in each half of the dividing cell.

On examination of the transverse sections of the bacteria the nuclear structures appeared as small, round formations, resembling bands transversely (see Fig. 1).

In oblique sections the nuclear structures had the appearance of short, round bands with margins cut across at an angle. Comparison of the external appearance of the nuclear structures in the sections in various directions showed that they were dense, round bands included in the zone of the substance which possessed almost no density in relation to electrons.

The external appearance and the character of the arrangement of the nuclear structures in the bacterial sections, as shown by the electron microscope, were analogous to the appearance and character of the nuclei revealed by microchemical analysis of the stained films.

The most suitable sections for staining by the Romanowsky-Giemsa method were those with a thickness of 1000 A. In longitudinal sections it was also easy to distinguish the cell wall, staining a violet color, and the cytoplasm, staining a barely perceptible rose-pink color, and against this background could be seen the outlines of the reddish violet nucleus, in the form of rods, with sometimes the presence of violet polar caps. In the more highly elongated cells, 2 nuclear inclusions were also found. In transverse sections of bacteria, stained by the Romanowsky-Giemsa method, nuclear formations were seen in the form of punctate violet inclusions.

As regards the sections stained by the Feulgen method, we were unable to produce satisfactory ones which showed the individual details with sufficient clarity. This was evidently due to damage sustained by the sections of the bacteria during the performance of the Feulgen reaction.

Certain authors are known to believe that the nuclear substance does not exist in bacteria in a discrete state, and workers who describe these nuclei usually have mistakenly interpreted the so-called polar caps [1, 3, 4].

Strong criticism of these views was expressed some time ago by M. A. Peshkov [6], who showed experimentally that nuclear substance is found in the form of aggregates in the cytoplasm of bacteria independently of the polar caps. Under these circumstances he emphasized that if the nucleoids were spherical in appearance, then polar caps were absent, and conversely, polar caps were always accompanied by rod-like nucleoids.

Our results also showed that the nuclear elements were present in the cytoplasm in a discrete form, independently of the presence of polar caps, which we called cytoplasmic granules, in accordance with the generally accepted terminology, since they might be more than two in number. In contrast to M. A. Peshkov, however, working with the optic microscope, on the basis of our electron microscopic investigations on a large number of sections, we came to the conclusion that the nuclear structures always had the appearance of longitudinal bands, corresponding to the rod-like nuclear elements observed during examination of the bacteria under the optic microscope. So far as the spherical nuclear structures described by many authors, including M. A. Peshkov, are concerned, we considered that their formation was associated with the different directions of the cells on the cover glasses when the films were fixed. In a cell fixed "square" on the glass, for example, the nucleoid was always spherical, just as took place in transverse sections of bacteria. Reports of the different shapes of the nucleoids are, evidently, the result of examinations of bacteria which were orientated differently in the plane of the film and, it appeared to us, do not reflect the true shape of the nuclear structures.

In conjunction with the data in the literature, our findings enable the general structure of the nuclear apparatus of bacteria to be judged to some extent. It may evidently be regarded as vacuoles, situated in the central part of the internal contents of bacterial cells, and consisting of material easily penetrated by electrons, in which are included dense bands, elongated in a longitudinal direction. These bands are possibly identical with the chromosomes.

In the structure of the nucleoids of bacteria further evidence may therefore be seen of their resemblance to the nuclei of the higher forms of cells, which agrees with the opinion of Piekarski and Giesbrecht [9] on the possibility of substituting the idea of "nucleus" for that of "nucleoid", and which indicates the chromosomal nature of the nuclear bands.

SUMMARY

The electron-microscopic and microchemical study of the ultrathin sections of B. coli has established the presence of discrete nuclear formation in the form of elongated oval bands. The nuclear formations being independent elements are not related to cytoplasmic granules ("polar caps") and do not change their morphology in the presence of the latter.

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