ELECTRON MICROSCOPY OF PHAGE *E. COLI* DEVELOPMENT INSIDE THE HOST CELL

by

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The aim of this paper is to summarize some of our results[1-13] on the electron microscopy of phage development. The morphological features of phage multiplication have not yet been sufficiently accounted for. This may be due to several factors, one of which may be that the action of T phages is very rapid, the stages in phage maturation being consequently very difficult to follow. On the other hand, most of the phage multiplication proceeds inside the host cell and it is therefore difficult to find such a bacterial lysis where all the stages would be visible. We tried to avoid the difficulties by using a phage with a long latent period and by making several thousand micrographs in the hope of ascertaining all the stages in phage development with greater probability.

Our results seem to indicate that it is possible to make the observed stages conform to a scheme of phage development which would agree well with data on phage multiplication obtained by other methods.

METHODS

The phage T2 was added to a one-hour-old broth culture of *Escherichia coli* in the ratio of 1:1–50:1; after 60–90 minutes of slow shaking in a water bath at 38° C, when the suspension had begun to clear, the lysed bacteria were washed with saline, resuspended in distilled water and centrifuged, and the usual preparations were made for electron microscopy with chromium shadowing according to WYCKOFF AND WILLIAMS. The long time necessary for the lysis to occur indicated that the phage strain had a long eclipse period. This served our purpose well since we were able to observe the slow formation of the new phage particles.

In a series of experiments we observed the phage formation in the presence of capillary active substances[9,10], terpinhydrate, urea and glycin[6,10]. In these experiments amounts of 0.1 g terpinhydrate, or of 0.1–10 g urea, or 0.1 g of glycine were added to 100 ml broth. As a capillary active substance conc. caprylic alcohol and conc. oleic acid were used. They were added to 100 ml broth in amounts of 0.5–16 drops.

The preparations were observed in an RCA microscope EMU 2a. About 5,000 electron micrographs were made.

RESULTS

The formation of the bacteriophage particle inside the bacterial host may be divided into two stages. During the first stage the phage is adsorbed by the bacterium cell, which afterwards undergoes important structural changes in that the bacterial cell content is totally transformed into uniform globules. Some of these globules stick together and form ringlike structures (some authors call these structures "doughnuts"; we will denote

References p. 11/12.

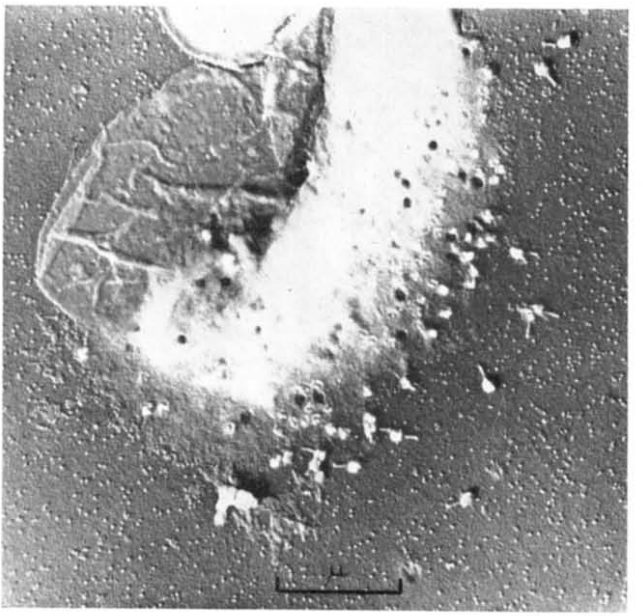

Fig. 1. A bacterial lysis where all the stages of the phage development are visible.

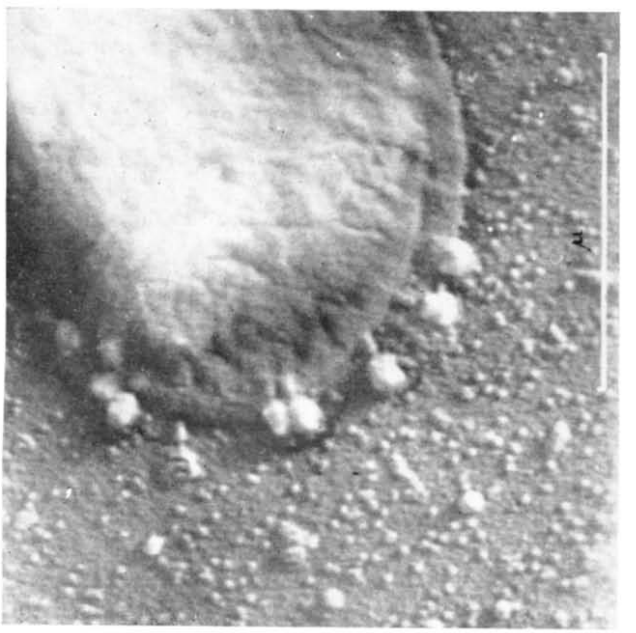

Fig. 2. Adsorption of the phage by the tip of the tail.

them in the following paragraphs as "rings"). The formation of rings mark the completion of the first stage in the phage development. The second stage is characterized by the filling of the ring and by the formation of the phage head together with the tail. This second stage is manifested by a complete disappearance of the uniform globules inside the phage head with the exception of the tail, where the globules remain visible.

Among our micrographs there is a considerable number of those where the above mentioned stages with their different steps are present in the lysed bacteria. Micrographs of this kind allow us to put the different steps in their proper order (Fig. 1).

The first stage: ring formation

The phage is mostly absorbed by the tip of the tail (Fig. 2). In some instances we observed T2 phage absorption by the head, but this may be due to surface forces (ANDERSON[14]). On the other hand the absorption by the tail cannot be regarded as a common phenomenon among phages. We observed for instance that a wild phage from a hay infusion was absorbed by the head (Fig. 3), which was afterwards dissolved in the bacterial host[8].

The adsorption of the phage is followed by important changes in the structure of the phage. The tail thickens (Fig. 4), grows in volume and merges into the bacterial body.

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The same happens with the phage head (Fig. 5). Under normal conditions we did not observe a single case in which the phage head divided into two parts, *i.e.* the inner part of the head entering the bacterial host, the outer part remaining outside the body in the form of a ring. Such instances were observed only when capillary active substances, urea or terpinhydrate, were added to the phage-bacteria system. In such cases the development of the phage was incomplete and remained at the stage of ring formation. The close union of the whole phage with the bacterium is quite understandable if we accept the fact that the phage consists of outer protein shell and an inner structure (HERSHEY[15]) containing mainly DNA. There cannot be any doubt of the outer protein shell playing a more important role in phage action than merely functioning in phage adsorption.

After the adsorption of the phage and its merging into the bacterial host, there are two marked changes in the host. There is mostly a swelling* of the bacterial body (Fig. 6) and a change of its structure in the site of the attachment. This increase in the volume of the bacterial body may be due to the increased incorporation of material during the first half of the latent period (COHEN[17]). But

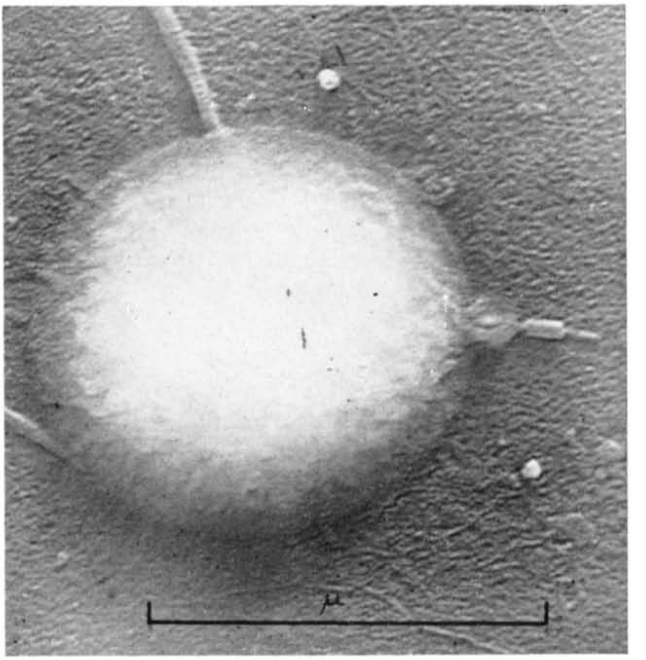

Fig. 3. Adsorption of a wild phage from a hay infusion by the head.

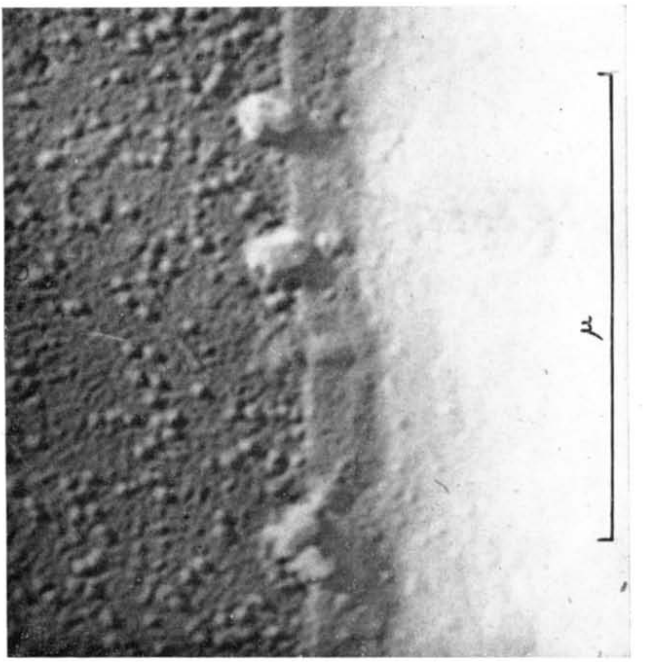

Fig. 4. Thickening of the tail during the adsorption.

* According to DELBRÜCK[16] the incidence of the swelling depends on the number of phage particles added to the bacterial suspension.

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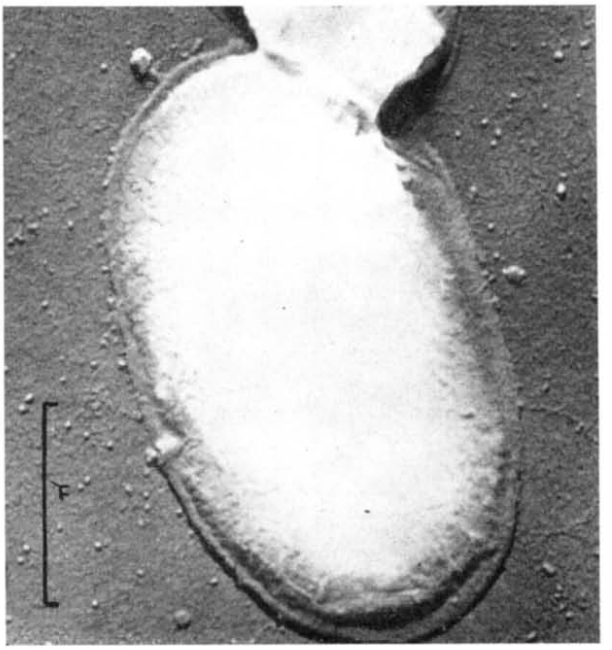

Fig. 5. The head of the phage merges into the bacterial body.

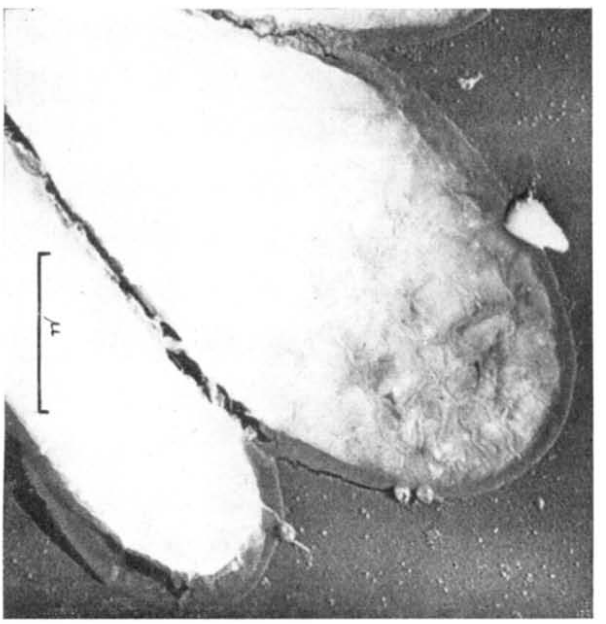

Fig. 6. The swelling of the bacterial body and change of its structure in the site of the attachment.

it cannot be excluded that to a certain degree this increase is due to osmotic forces (BRONFENBRENNER[18]). Such increase in internal osmotic pressure can further be due to the disintegration of the macromolecular structure of the bacterial body into smaller blocks. On micrographs (Fig. 7) of cells during the lysis it may be observed that the whole bacterial body is transformed into a large number of uniform particles (WYCKOFF[19], HERČÍK[7], LEVINTHAL AND FISHER[20], RAETTIG[21]). It seems that this transformation proceeds from the site of the phage attachment, which on the micrographs shows an increased transparency and at the same time a globular structure.

These globules are characterized by the uniformity of their diameter. This can be well seen on the frequency curve, which is considerably monodisperse[11]. The diameter of these globules for *E. coli* is 250 A (the figures given by LEVINTHAL AND FISHER are similar, *i.e.* 200 A).

Uniform globules show a tendency to form ringlike structures in which they adhere to one another and fuse in the form of a ring. This may be seen in many micrographs taken of lysed cells in which the cell content was poured out of the cell membrane (Figs. 8, 9). In such a way fields of uniform globules arose where adhering tendency of the globules can be easily studied.

The rings are formed in a similar manner directly in the bacterial body and in the beginning have the appearance of small

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holes (Figs. 10, 11). These are then enlarged, probably by an enzymic process dissolving the central part of the hole until a certain diameter is arrived at, which corresponds to the diameter of the ring. All these processes are started by the substrate of the uniform globules. During the ring formation the individuality of the globules is lost.

The mean circumference of the rings illustrated in Fig. 12 is 290.3 mμ ± 25.8 mμ. The uniform globules in the ring are considerably flattened, their mean diameter being 12 mμ. The ring therefore contains approximately 22 of these globules. The total number of globules, together with the globules in the phage tail (6–8), is approximately 30. Each of these globules has a molecular weight of about 6,000,000. The total mol. weight consequently is 180,000,000. If we accept that the molecular weight of a phage particle is 300,000,000 a difference of about 120,000,000 remains which may be accounted for by the subsequent growth of the ring as the result of DNA synthesis.

Only in the presence of foreign substances such as terpinhydrate, in the lysates, may rings be observed which undergo only a partial development and in which the inner globular structure may be seen. The globules in the ring are considerably flattened (Fig. 12).

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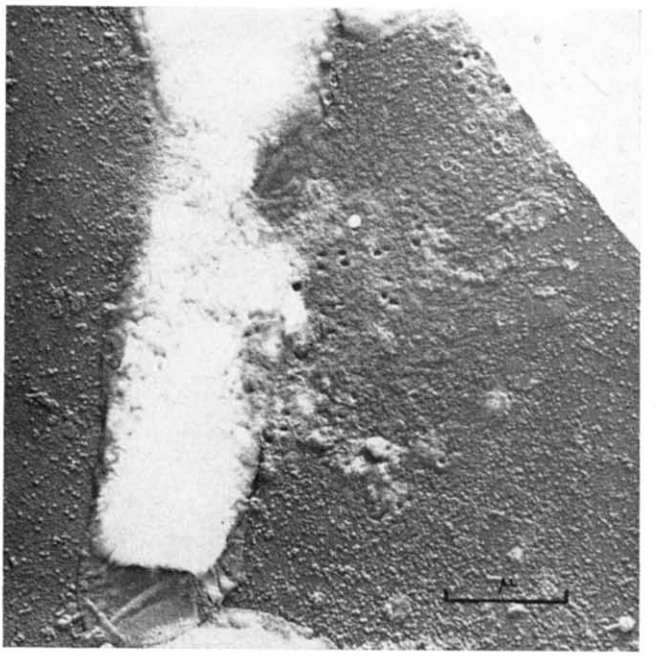

Fig. 7. The transformation of the bacterial body into a large number of uniform particles.

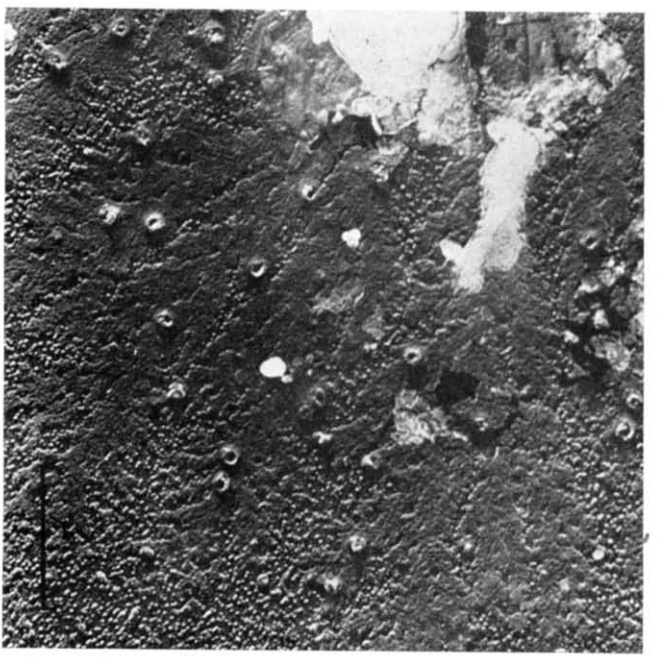

Fig. 8. The transformation of uniform particles into ringlike structures.

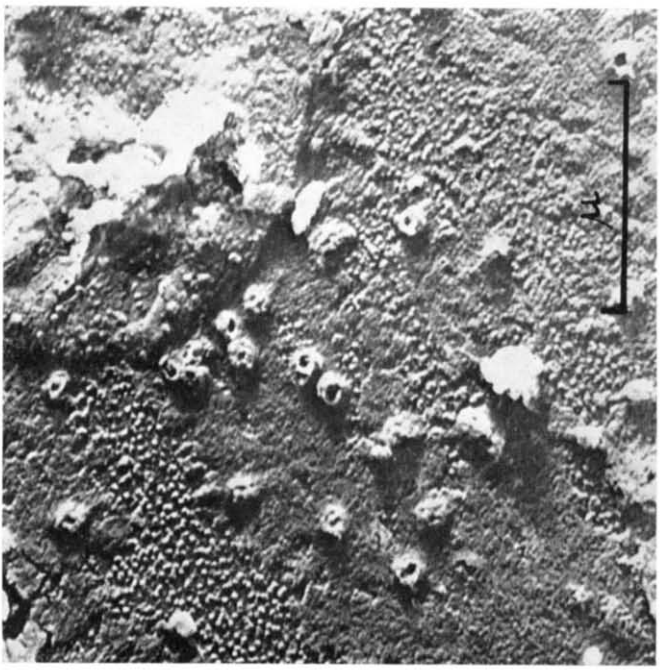

Fig. 9. The adhering tendency of the uniform globules and the formation of rings.

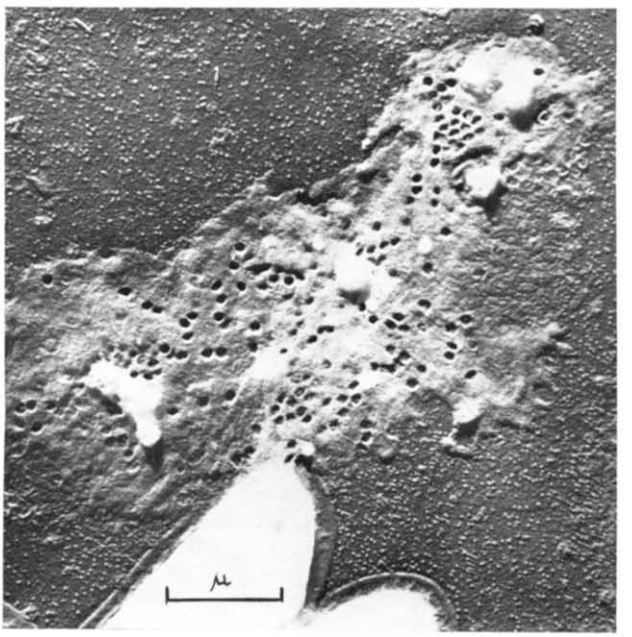

Fig. 10. The formation of the rings inside the bacterial cell.

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In the presence of heterogeneous bacterial strains or under the influence of foreign substances such as caprylic alcohol, glycin, urea *etc.*, the phagolysis of the bacteria is sometimes stopped at the stage of uniform globules without any further phage development (HERČÍK AND HRADEČNÁ, GUELIN[22], PRICE[23]). These phage inhibitors obviously interfere with the mechanism of ring formation (Fig. 13).

Second stage: growth of the ring

As stated above, during the ring formation uniform globules lose the individuality of separate macromolecules, and at the same time a new process takes place which consists in the transformation of the ring. This transformation manifests itself in the growth of a protuberance which fills the cavity of the ring (Fig. 12, 14). The ring itself thickens considerably. An entirely new chain of processes seems to start which may be identical with the synthesis of DNA as has been stated by several authors.

The DNA content of the immature forms of the phage is only a small percentage of that in the intact phage. But the mature form of the phage contains a considerable quantity of DNA, which according to

HERSHEY AND CHASE[24] is situated in the central part of the phage head. It does not seem that the phage is actually separated into two biochemically different parts, but the possibility cannot be excluded that to a certain degree the DNA synthesis is responsible for the growth of the ring and its further transformation, *i.e.* for the growth of the central protuberance and the completion of the phage head.

The formation of the tail probably proceeds simultaneously with the growth of the ring. The tail consists of 6–8 globules having the same diameter as the uniform globules (Fig. 15). The last two globules are slightly thicker. The tail seems to be composed of globules which adhere to one another as may be seen in several micrographs which show globules that have passed out of the lysed cells and become short and rod-like. It is possible that the same process goes on in the cell, where out of the mass of globules several form the tail. It is interesting that the globules of the tail neither lose their globular character nor their individuality. From this fact it may be deduced that the tail remains in some kind of paracrystalline state.

Under the influence of capillary active substances (caprylic alcohol, oleic acid) the growth of the ring is manifestly retarded, and the head of the phage particle seems to disintegrate (Fig. 15) or to form "ghosts" which have been ob-

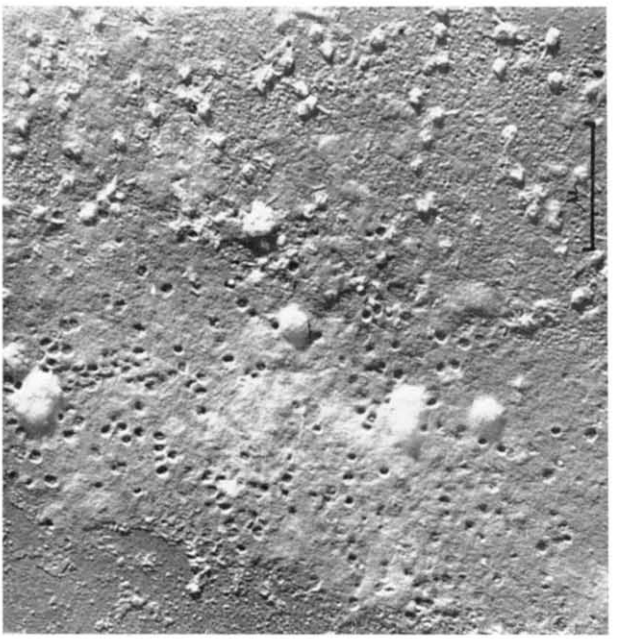

Fig. 11. A further stage in the formation of the rings inside the bacterial cell: growth of central protuberance.

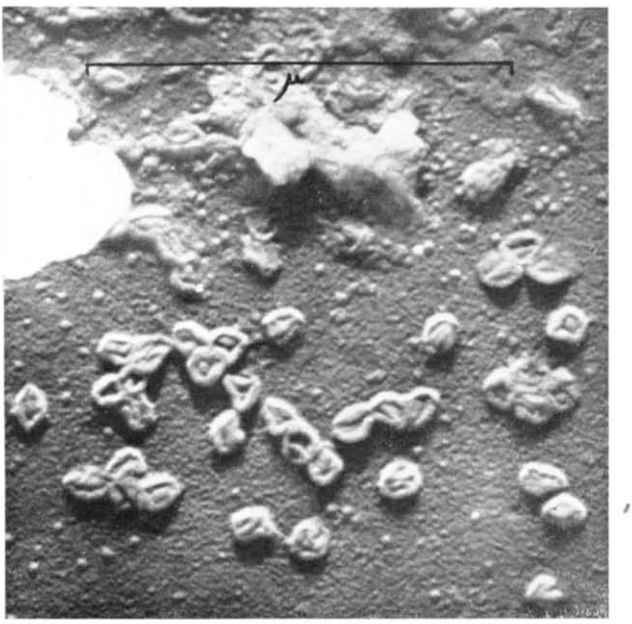

Fig. 12. The formation of the rings in the presence of terpinhydrate.

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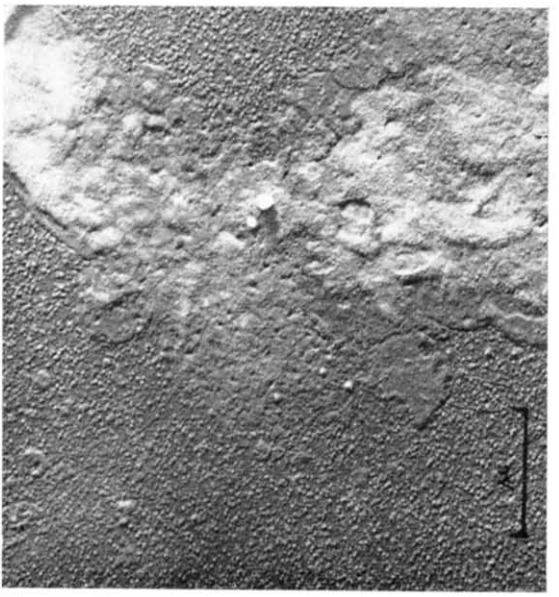

Fig. 13. Globular disintegration of the bacterial cell without ring formation.

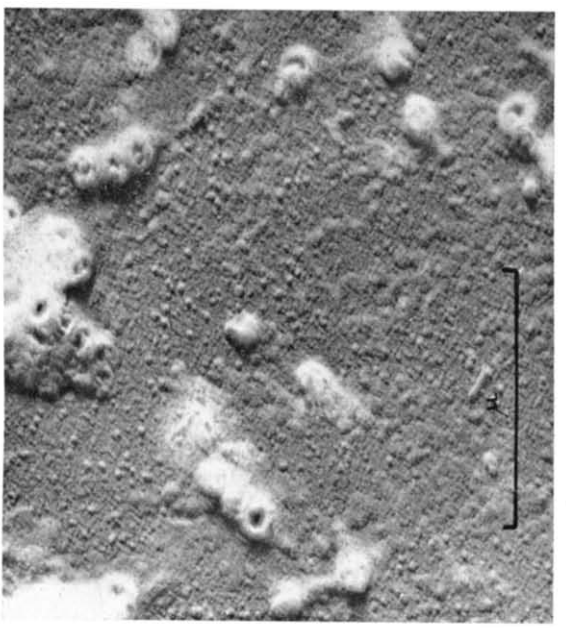

Fig. 14. The growth of central protube rance which fills the cavity of the ring.

served also by other authors (*e.g.* ANDERSON[14]). From this point of view it is important that during the preparation of the phage suspension for electron microscopy no capillary active substances, such as amylacetate, alcohol, *etc.*, should be used.

Similar phenomena were observed when terpinhydrate was added to the phage-bacteria system. The growth of the ring in such instances was more or less checked with the exception of the central protuberance. In Fig. 12 several rings formed in the presence of terpinhydrate may be seen. Some of the particles have a knob-like inner structure, others have a rod-like structure. We suppose that in the latter case we are looking at the ring from above and in the former from below. In some cases the inner protuberance does not develop in the presence of terpinhydrate and the particle takes on the form of a key (Fig. 16).

DISCUSSION

As stated above, all our preparations were made by the simplest method without organic solvents and other capillary active substances and also without the use of high or extremely low temperatures.

Nevertheless the effects of drying and surface forces should be taken into consideration.

The water content of the bacteriophage is not yet exactly known. It may be expected that the phage does not contain large quantities of water and that the amount of water present is firmly held. In support of this opinion we mention the fact that the surface observed on healthy phage particles is very smooth, with-

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out manifest irregularities. Only when the lysis proceeds in the presence of foreign substances, may one observe different structures (as stated above). These structures, however, have a regular character, *i.e.* they are not due to a chance collapse after drying. No one will, for example, maintain that the retracted inner content of bacterial cells which is often observed in an electron microscope is a picture of the real structure of the bacterial cell. In such a case there are too many irregularities. But most of the structures that could be observed on phage particles whose maturing was inhibited may be interpreted as steps in phage development and may consequently be coordinated in time (*i.e.* the formation of the ring, the growth of the central protuberance and the filling of the ring). The reason for this coordination in time is prompted to us by several micrographs where the hypothetical steps are present at the same moment, *i.e.* the process of phage formation begins in a certain part of the bacterial body while in another part fully developed phages leave the cellular detritus. It may be induced from such micrographs that the maturation of phage proceeds by different steps. When such formations are found outside the bacterial cells, where they could be observed with better resolution, then it is justifiable to regard them not as effects of drying

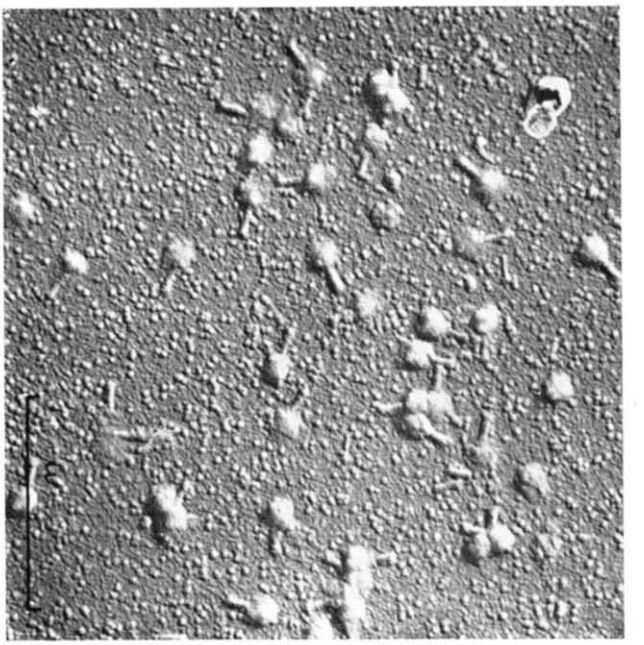

Fig. 15. The disintegration of the phage head under the influence of oleic acid.

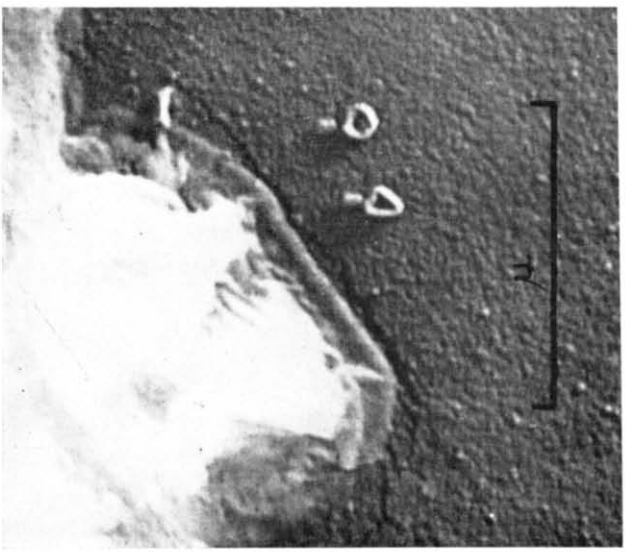

Fig. 16. The phage particles take on a form of a key under the influence of terpinhydrate.

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but as immature particles disengaged during the process of specimen preparation.

Let us now return to the discussion of the important stages in phage formation. Under the influence of the adhering phage the structure of the bacterial cell is totally changed into a mass of uniform globules. These globules generally stick closely together, and only when the content of the cell is poured out during the lysis can we ascertain their globular character and their uniform diameter. In many parts of the bacterial cell little holes appear which are afterwards enlarged and surrounded by the globules. In such a way a ring arises made up of the globules, which lose their individuality. This can be well ascertained on globules which make rings out of the poured-out mass of transformed bacterial plasma. The formation of rings corresponds to the end of the first stage. The second stage is characterized by the growth of the ring by thickening and formation of the central protuberance. During this stage the ring is filled and transformed into the phage head. The tail, which is made up of 6–8 uniform globules, is probably formed at the moment when the central protuberance starts to grow.

There seems to be no doubt that the ring observed in our micrographs is identical with the "doughnuts" observed by WYCKOFF[19], LEVINTHAL AND FISHER[20], ANDERSON[25], PENSO[26] and others. This is in agreement with the fact that the number of rings corresponds to the number of phage heads[23] and that the synthesis of phage proteins begins earlier than the synthesis of DNA[27]. It is also known that it is not possible to detect any phage activity[28] during the first half of the latent period. In some cases the "ghosts" observed by ANDERSON[11] have a central protuberance which is clearly visible in his Fig. 6. From our point of view such ghosts are residual phage structures in which, owing to a sudden change of salt concentration, the protein is detached from the greater part of DNA content. The flattened structures which are described as phage membranes are consequently rings with a poor growth and incomplete DNA content. The proflavine, urea, capillary active substances, terpinhydrate, *etc.* stop the definite association of phage constituents (LURIA[29]) in that only the ring alone or a ring with a slightly developed central protuberance remains.

The existence of uniform globules observed in our micrographs may be associated with several facts. These globules have been postulated by KRISS[30,31] and are regarded by KALINA[32] as ultrafiltrable entities which may change to a phage or to filtrable forms of bacteria. LURIA[28] frequently states that the material for a new phage is first reduced to a non-specific level before the proper synthesis of the phage begins. This means a two-stage process which is in good agreement with our findings. According to LURIA[29] the phage antigens are attached to ultrafiltrable material with a diameter of 200–300 A. This may be the uniform globules. RAETTIG[21] has found also particles which he calls "lysomes" and which in his material (typhoid and paratyphoid phage) have a smaller diameter (100 A) than displayed by the phage T 2. In his view "lysomes" play an important role in phage development.

On the other hand it must be stated that there is a certain disagreement of our findings with the syringe model of phage (HERRIOT[33].) Neither is it possible in our micrograph to find any evidence of the phage membrane (see *e.g.*, Figs. 12, 14). The tail of the phage does not appear to be a hollow tube but a row of globules and this seems to indicate its crystalline or semicrystalline character. The tip of the tail is broadened, which may be interpreted as a kind of growth of the last macromolecule. Such an interpretation would be in agreement with the idea that there is a site of an antigen which is able to react with a neutralizing antibody[34].

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SUMMARY

On the basis of a detailed electron microscopical study of the T2 phage development, a scheme of phage multiplication is put forward which supposes that the induced bacterial cell is disintegrated into globules of uniform diameter. These globules are able to form ringlike structures which are equivalent to the "doughnuts" described by other authors. During this process their individuality is lost. The formation of the rings corresponds to the first stage in phage development. The second stage is characterized by a central protuberance which eventually fills the ring. At the same time the tail is formed of 6–8 uniform globules which maintain an individual spherical character. This scheme of phage multiplication fits in with the facts of phage maturing processes which have been obtained by other methods.

RÉSUMÉ

Une étude détaillée au microscope électronique du développement du phage T_2 permet de proposer pour la multiplication du phage un schéma qui suppose que la cellule bactérienne induite est désintégrée en globules de diamètre uniforme. Ces globules sont susceptibles de former des structures en anneau, équivalentes aux "doughnuts" décrits par d'autres auteurs. Au cours de ce processus, ils perdent leur individualité. La formation des anneaux correspond au premier stade de développement du phage. Le second stade est caractérisé par une protubérance centrale qui, dans certains cas, remplit l'anneau. En même temps, la queue est formée par 6 à 8 globules uniformes qui conservent leur caractère sphérique individuel. Ce schéma de multiplication du phage peut être mis en accord avec les faits de maturation du phage qui ont été observés par d'autres méthodes.

ZUSAMMENFASSUNG

Auf Grund einer eingehenden Untersuchung der Entwicklung des Phagen T2, mit Hilfe des Elektronenmikroskopes, wird ein Schema der Phagenfortpflanzung vorgeschlagen, in welchem angenommen wird, dass die induzierte Bakterienzelle in sphärische Partikeln von gleichem Diameter zersetzt wird. Diese Partikel können ringartige Strukturen bilden, welche mit den von anderen Autoren beschriebenen "doughnut"-Ringen gleichwertig sind. Während dieses Prozesses geht ihre Individualität verloren. Die Ringbildung entspricht dem ersten Stadium der Phagenentwicklung. Während des zweiten Stadiums erscheint ein zentraler Auswuchs, welcher nach und nach den Ring ausfüllt. Gleichzeitig wird der Schweif aus 6–8 gleichen Partikeln gebildet, welche ihren individuellen sphärischen Charakter beibehalten. Dieses Schema der Phagenfortpflanzung stimmt mit den Beobachtungen überein, welche mit Hilfe anderer Methoden an Phagenreifungsprozessen angestellt wurden.

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