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Electron Microscope Study of Septum Formation in *Escherichia coli* Strains B and B/r During Synchronous Growth

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The formation of cell wall septa was monitored in *Escherichia coli* B and B/r during synchronous growth in glucose media at 37 C by means of electron microscopy. The visible events of septation comprised the following sequence, starting at about 30 min of incubation: (a) bleb formation of the outer membrane; (b) invagination of mucopeptide and cytoplasmic membrane (with associated mesosomes); the outer membrane is excluded from the septum; (c) formation of a cross-wall; (d) ingrowth of the outer membrane during cell separation. The septum is composed of a fold of cytoplasmic membrane plus mucopeptide, and the latter is a double structure, composed of two opposed lamellae separated by an electron-transparent gap. Experiments with chloramphenicol and nalidixic acid suggested that division could occur in the presence of these inhibitors once a round of deoxyribonucleic acid replication is completed. The initial stages of septation, as estimated by the potential of the cells to produce bulges in the presence of ampicillin, may involve the modification of mucopeptide by hydrolases at the end of the C period. Assembly of the septum may occur during the first half of the D period by means of precursors synthesized during the preceding C period.

Much of our basic knowledge of the bacterial cell cycle has been derived from studies of *Escherichia coli* (7, 8, 12, 16, 18, 19, 34, 35, 40). The temporal events of the cycle have been explored in some detail by means of synchronous cultures. For cells with doubling times of 25 to 60 min, one round of chromosome replication occupies about 40 min (the C period), and a definite interval of 20 to 22 min (the D period) elapses between the end of a round and the subsequent division of the cells (8, 12, 18, 34). In rich media, and the circumstances of most rapid growth, rounds of deoxyribonucleic acid (DNA) replication may be initiated before the completion of those already in progress (8, 16, 18). The completion of a round of DNA replication, as well as prior synthesis of ribonucleic acid (RNA) and protein, is a necessary requirement for the stages leading to cell cleavage and separation (7, 8, 11, 12, 28).

Physiological studies suggest that a definite series of events are involved, and that the D period is essentially a portion of the cycle in which cells become compartmentalized and later separated, a process that has been called septation (7). Only in gram-positive bacteria, however, have the structural aspects of septation and cell wall extension been followed in detail (13, 22). In *Bacillus megaterium*, for example, the nascent septum is associated with mesosomes throughout the subsequent stages of invagination, wall thickening, and cleavage (13); in *Streptococcus faecalis* the mesosomes appear to be associated with the initial stages of septation (22). Unfortunately, little is yet known of the quantitative parameters (C + D, above) relating septation to chromosome replication in these organisms.

The formation of cell wall septa is not uncommon in the wide range of gram-negative bacteria and has been described for Neisseriaceae (38) and Beggiatoaceae (29) to take extreme examples. However, septation has seldom been observed in *E. coli* and the Enterobacteriaceae; this is also true for other major groups such as the pseudomonads, spirillums, and vibrios. The cells merely seem to constrict at sites of incipient divisions, and all wall layers, including the cytoplasmic membrane, invaginate together (46). In a previous paper (5) we have shown that the circumstances of fixation dictate the appearance of definite cell wall septa (an unlikely artifact), and we have indicated some of the possible reasons why septa are not easily visualized in *E. coli*, but have argued that a true septum, composed of mucopeptide and cytoplasmic membrane, is likely to be present.

Because *E. coli* is one of the few organisms in

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which the basic parameters of the cell cycle have been analyzed by synchronization techniques of proven reproducibility, it is important to determine some of the essential steps involved in septation. We therefore posed the following questions. (i) Is there a specific morphogenetic event which occurs during the D period? (ii) At what stage of the cell cycle is septum formation initiated? (iii) What are the stages of septum formation and can this sequence be timed? (iv) Can the events of the D period be correlated with other events of the cell cycle, such as chromosome replication?

The object of this study is to provide a structural account of the temporal events involved in septation of *E. coli* B and B/r during synchronous growth.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* B (University of Western Ontario [U.W.O.] collection no. 301) and *E. coli* B/r (ATCC 12407, U.W.O. collection no. 828, obtained from C. E. Helmstetter) were used throughout.

E. coli B was cultured in the M9 glucose medium of Nagata (33), whilst *E. coli* B/r was grown in the glucose minimal medium described by Cooper and Helmstetter (8). The composition of these media are given in a previous paper (5). All cultures were diluted from stocks, initially isolated as single colonies on nutrient agar, and maintained in a reciprocating shaker water bath at 37 C. Flasks with a volume five times that of the culture were used in all experiments.

Synchronous cultures. (i) Some of the results reported here, using *E. coli* B, were obtained by a modification of the density gradient centrifugation technique described by Mitchison and Vincent (31). The original technique was modified in two respects because: (i) we experienced some difficulty in obtaining rapid resumption of growth once the cells had been exposed to sucrose; the use of Dextran T500 or Ficol (Pharmacia Fine Chemicals, Inc. (Canada), Montreal, Que.) in the gradient overcame this problem; (ii) all steps in the synchronization procedure were carried out in a room maintained at 37 C.

Exponentially growing cells (about 10^8 to 2×10^8 cells/ml) in about 200 ml of M9 medium were harvested at 37 C by centrifugation at $2,800 \times g$ for 10 min in a Sorvall SS-1 centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The pellets were pooled and resuspended by pipette in a small volume (1 to 2 ml) of prewarmed M9 medium and then layered onto gradients containing Dextran or Ficol (2 to 12% in M9 medium) made up in glass tubes (150 by 10 mm). The gradients, about 14 ml per tube total volume, were usually prepared the previous day and refrigerated overnight. About 30 min before the start of an experiment the gradients were placed at 37 C. After centrifugation at $1,800 \times g$ for 5 to 10 min in a centrifuge (Christ, Modern Labs., Toronto, Ont.) fitted with a swing-out head, the topmost fraction of cells, amounting to about 5% of the total number layered onto the gradients, was removed by a sterile hypodermic syringe and immediately inoculated into about 100 ml of fresh, prewarmed M9 medium. In general, the cells were found to move one-half to two-thirds of the distance down the gradient and, in some experiments, a small fraction of the cells pelleted at the bottom of the tubes.

Samples (1.0 ml) for counting cell numbers were removed from the culture at 5-min intervals and pipetted into an equal volume of 0.9% NaCl containing 0.5% formaldehyde (previously filtered through a 0.22-μm pore size membrane filter, Millipore Corp.). The fixed cells were diluted into filtered 0.9% NaCl, and cell numbers were estimated with a model A Coulter counter (Coulter Electronics, Hialeah, Fla.) using a probe fitted with a 30-μm aperture.

(ii) The selection procedure outlined above yielded useful results but we obtained greater reproducibility and convenience, for ultrastructural purposes, using the membrane elution technique devised by Helmstetter and Cummings (20) and described in detail by Helmstetter (16, 17).

Cultures of *E. coli* B or B/r (100 ml in a 500-ml flask) in M9 or glucose minimal medium, respectively, were grown at 37 C to a density of about 10^8 cells/ml. The entire culture was then poured onto the surface of a 142-mm diameter type GS filter of 0.22-μm pore size (Millipore Corp., (Canada), Montreal, Que.) clamped to a type PA 15 porcelain funnel (Schleicher and Schuell Inc., Keene, N.H.) exactly as described by Helmstetter (17). This and the subsequent manipulations were performed in an incubator maintained at 37 C. The cells were drawn onto the filter by connecting the funnel stem, via a Buchner flask, to a vacuum line. When most of the medium had passed through, the small volume of culture remaining was poured off, and the filter was inverted and then connected to a second funnel portion of a type PA 15 holder. The top chamber was filled with fresh, prewarmed medium and connected to a pump (type RRP, G-150, Fluid Metering Inc., Oyster Bay, N.Y.). Loosely bound cells were washed out for 2 min at a flow rate of 15 to 20 ml of medium/min, and then the elution rate was reduced to about 2 ml/min. When samples for electron microscopy were prepared, portions of eluted cells (about 10 ml) were obtained every 5 min in 50-ml flasks and incubated for the desired cell age. Cell numbers were monitored at the start and end of the incubation period. Where the effects of inhibitors were being studied, a similar 10-ml portion was removed and incubated at 37 C, and portions (usually 1.6 to 1.8 ml) of this culture were diluted into the appropriate antibiotic or inhibitor (see below). In both types of experiment, samples were usually collected after 35 to 45 min of elution. Cell numbers were estimated with a model B Coulter counter, using a 30-μm aperture, as described above.

Electron microscopy. Two prefixation methods were used: (a) for *E. coli* B synchronized by method (i), samples (5 to 10 ml) were removed at 5-min intervals and placed in tubes containing an equal volume of fixative (10% acrolein, 0.1 M sodium

cacodylate buffer, pH 7.5). This step was performed in a room maintained at 37 C. After 3 to 5 h at room temperature (20 to 22 C), the samples were centrifuged at 5,000 × *g* for 5 min, washed three times in 0.5 M cacodylate buffer, enrobed in 2% agar, chopped into small pellets, and washed overnight in buffer. (b) Later studies, using the membrane elution technique (ii), involved prefixation of the shaking cultures at 37 C with one-tenth volume of stock fixative (5% acrolein, 0.05 M cacodylate buffer, pH 7.5, with or without 0.25% glutaraldehyde). After 2 to 3 min at 37 C, during which time the cultures continued to be shaken, samples were centrifuged and resuspended in 1 ml of stock fixative and then treated as described above. The inclusion of 0.25% glutaraldehyde was found to be necessary to preserve structural detail in *E. coli* B/r (see below), but good results were obtained with *E. coli* B simply by using acrolein.

Details of the postfixation in osmium, washing, dehydration, and embedding are given in a previous paper (5). Uranyl- and lead-stained sections were viewed in a Philips EM 200 operated at 60 kV and photographs were taken on 35-mm Kodak fine grain positive film (see ref. 5).

Inhibitors and antibiotics. Chloramphenicol was obtained from Calbiochem (San Diego, Calif.) or from Sigma Chemical Co. (St. Louis, Mo.), and stock solutions (1 mg/ml) in water were freshly prepared. Nalidixic acid was obtained as a gift from Winthrop Labs., Aurora, Ont., and solutions (1 mg/ml in 0.1 N NaOH) were also prepared just before use. Ampicillin (Penbritin-1000, ampicillin sodium) was obtained as a gift from Ayerst Labs., Montreal, Que. Stock solutions (1 mg/ml in water) were routinely used.

RESULTS

Characteristics of synchrony. Although the density-gradient technique for obtaining synchronous cultures of *E. coli* B was fairly reproducible, the actual duration of divisions, as monitored by estimation of cell numbers with a Coulter counter, was found to be variable. An increase in cell numbers started at about 35 to 40 min of incubation at 37 C, and division of the cells proceeded for a further 15 to 25 min (the doubling time in batch cultures, determined by increase in optical density at 660 nm, was 45 to 48 min). The degree of synchrony obtained was not markedly improved by selecting the cells on gradients run at 0 C (possibly enhancing synchrony through a temperature shock) or by growing the cells in "conditioned medium" (17). Our best results (Fig. 1), on which the ultrastructure of dividing cells is based, were obtained when the entire synchronization procedure was performed at 37 C.

Variations in the duration of division, presumably due to variations in the interdivisional times of individual cells, were also noted using the membrane elution technique. In both *E. coli* B (Fig. 2) and B/r (Fig. 3), the production of

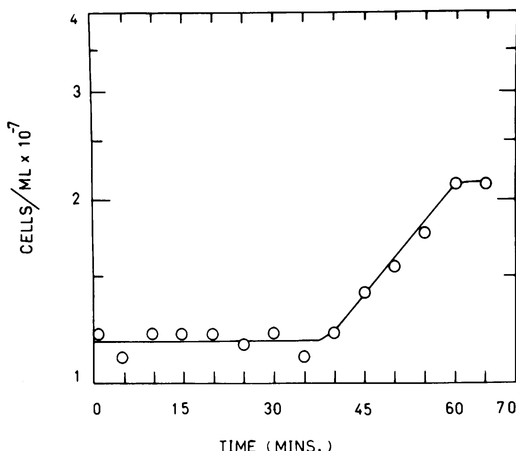


FIG. 1. *Graph showing synchronous growth of E. coli B obtained by selection of cells (about 5% of the total number layered) on a Dextran gradient (2 to 12%) in M9 glucose medium at 37 C.*

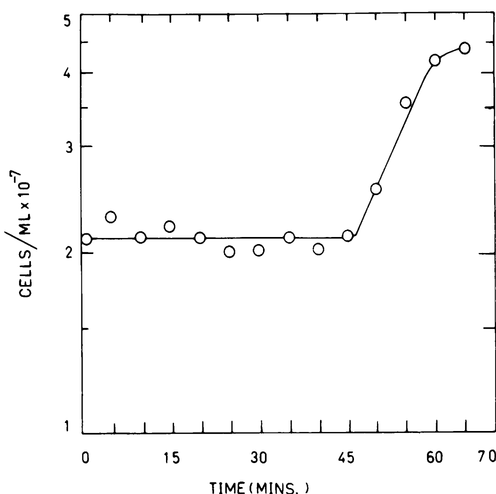


FIG. 2. *Synchrony obtained by eluting cells of E. coli B from a membrane filter, using M9 medium. Cells were collected after 40 min of elution, and samples (10 ml) were removed every 5 min and incubated to obtain cells of the desired age (0 to 65 min).*

new cells occupied some 10 to 20 min. The degree of reproducibility was found to be high, and most of the morphological observations, as well as studies of the effects of inhibitors and antibiotics, were based on the elution technique. The micrographs were drawn from a total of seven experiments using *E. coli* B and four for *E. coli* B/r.

The following points should be emphasized: (i) compartmentalization of the cells started some 15 min earlier than an increase in cell numbers could be detected, and this was seen

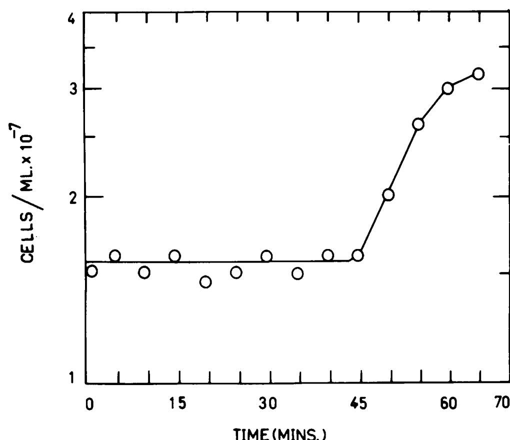


FIG. 3. *E. coli Br synchronized by the membrane elution technique in glucose minimal medium. Samples (10 ml) were collected after 37 min of elution, removed at 5-min intervals, and incubated for the desired cell age (0 to 65 min).*

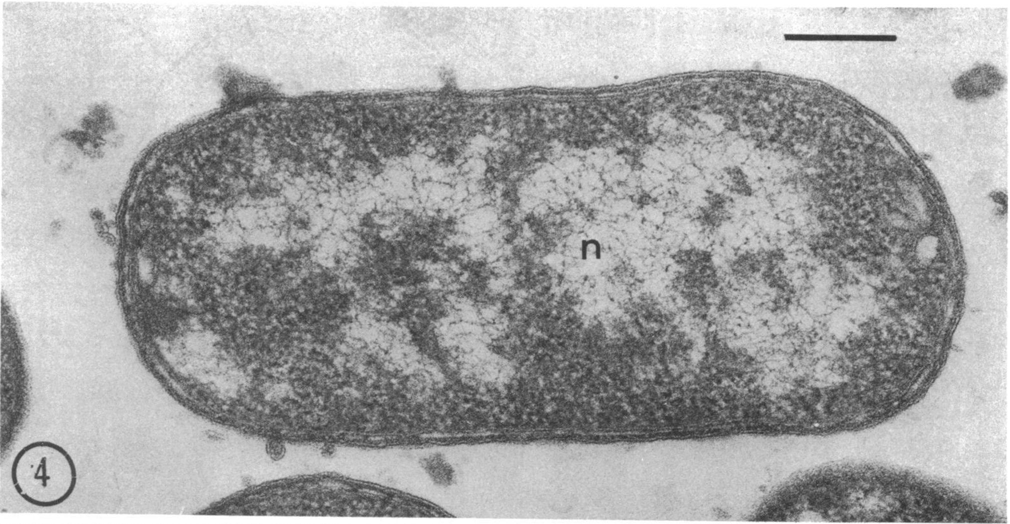
using Nigrosin smears for light microscopy, which showed a progressive alteration in the shape of the cells as a furrow was found at the site of septation (unpublished data); (ii) even though Nigrosin smears provided information on the percentage of dividing cells in any one sample, a septum or alteration in the outer membrane (see below) could not be detected by this technique; (iii) counting procedures applied to thin sections formed only a guide to the approximate time at which an event was first seen or an estimate of the duration of that event. In general, a full statistical analysis of the division cycle could only be made by ensuring that every cell is sectioned longitudinally. We attempted to do this by filtering the fixed cells onto small membranes (Millipore Corp.) and then enrobing the sample with agar. This attempt was later abandoned because the small size of the samples provided difficulties in handling at this and subsequent stages of the fixation and embedding procedures.

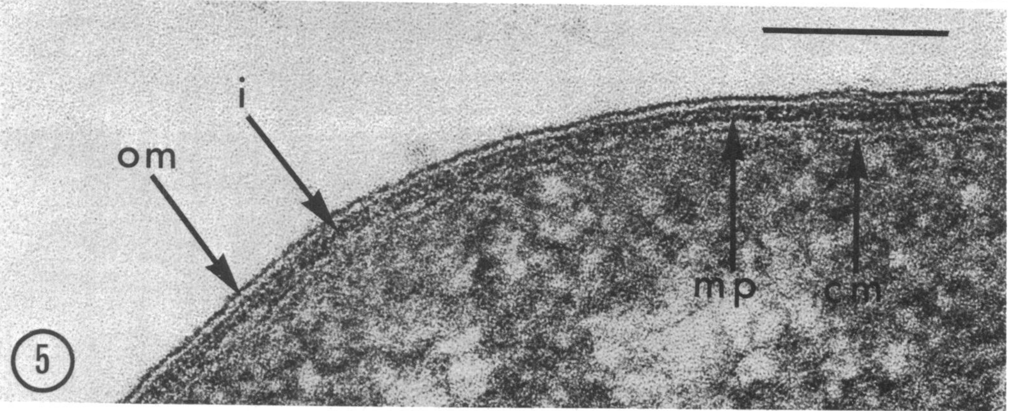
Fine structure of cells prior to septation. The structure of *E. coli* B cells at 15 min of incubation after elution from the membrane is shown in Fig. 4. The appearance of the cell wall and cytoplasmic membrane was similar to that reported by other workers (10, 32). After acrolein or acrolein/glutaraldehyde fixation, the outer membrane (Fig. 5) was relatively smooth in appearance, about 7.5 nm thick and closely applied to a dense layer 2.5 to 3.0 nm thick, interpreted to be the mucopeptide (peptidoglycan, murein) layer (cf. ref. 10, 14, 32). Between the outer membrane and mucopeptide was a more diffuse zone, some 8.0 to 9.0 nm wide, in which fine structure was only barely discernible (Fig. 5), but suggestions of connecting "bridges" could be seen on suitably oriented profiles. The cytoplasmic membrane, about 7.3 to 9.7 nm wide, appeared to be asymmetric, in that its outer face was more prominently stained than the inner, cytoplasmic side. This may have resulted from a masking of the inner edge by the cytoplasm. Retraction of the outer face of this membrane, away from the inner edge of the mucopeptide layer, could also be observed occasionally. The resultant gap or periplasmic space was particularly pronounced in specimens fixed solely by OsO_4 (see ref. 5). The appearance of the nucleoid (Fig. 4) was much more "dispersed" than the image encountered after fixation with the standard Ryter-Kellenberger osmium procedure (see ref. 5, 43). Amorphous zones displacing the closely packed ribosomes of most of the cytoplasm were also seen, especially near the poles of the cell. The above description also applied to cells sampled at 0 min to 25 to 30 min of incubation (elution technique) and also to cells removed from gradients.

E. coli B/r had a very similar structure, except that the outer membrane had frequently wrinkled and small blebs were found along the periphery of the cells (cf. Fig. 24). This apparent disruption of the outer membrane was particularly severe when the cells were prefixed solely with acrolein. Although the inclusion of glutaraldehyde moderated this effect (Fig. 23), it was necessary to include acrolein in the recipe if septa (see below) were to be detected. Prefixation of the cells at 37 C for 1 to 2 min was found to result in satisfactory preservation, although extending the time of prefixation to 5 min (or more) was undesirable.

The initiation of septation. The first visible sign of an alteration in cell structure of *E. coli* B occurred at about 30 min (range 26 to 32 min) when a fold or bleb, formed by the outer membrane, was observed near the middle of the cells (Fig. 6). In many cases this occurred before any indication of a septum could be detected. This observation was obscured in the case of *E. coli* B/r, because, as noted above, the outer membrane tended to vesiculate at many points around the cell. However, this apparent folding of the outer membrane was found to persist well into the septation process (see below). By this stage (22 to 30 min) the nucleoid appeared constricted into roughly two halves (Fig. 6, 23).

At 30 to 35 min (Fig. 7–9) there was a pronounced U- or V-shaped notch produced by an infolding of the cytoplasmic membrane and mucopeptide portion of the wall. Blebbing of





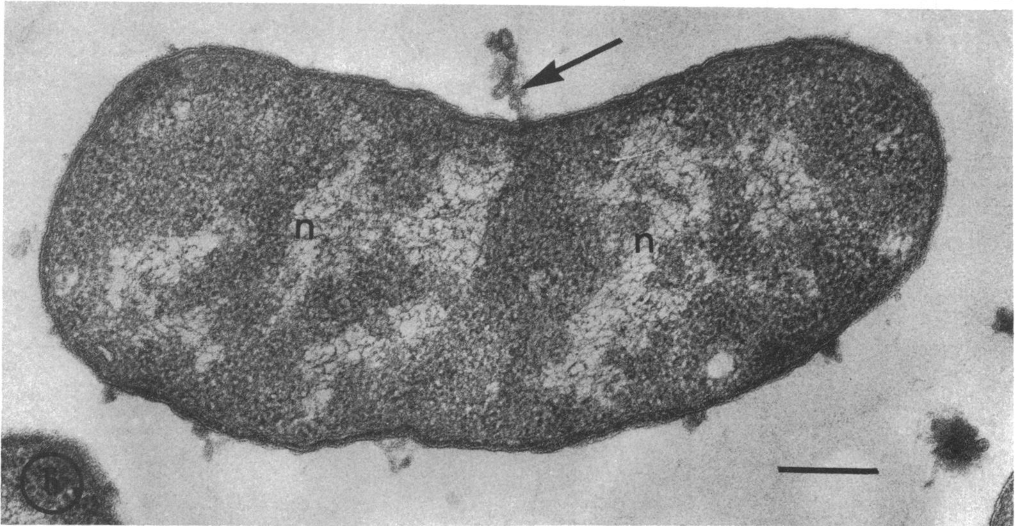


FIG. 4–6. *Sections of E. coli B synchronized by membrane elution and fixed with 5% acrolein. Fig. 4: cell at 15 min of incubation; note nucleoid (n) filling much of the cell. Fig. 5: portion of cell envelope showing outer membrane (om), mucopeptide (mp), cytoplasmic membrane (cm), and a layer (i) intermediate between om and mp. Fig. 6: cell at 30 min of incubation; note bleb of outer membrane (arrow) and nucleoid (n) partitioned into roughly each half of the cell. Magnification bar equals 0.1 μm (Fig. 5) or 0.2 μm (Fig. 4, 6); unless otherwise stated, all bars represent 0.1 μm.*

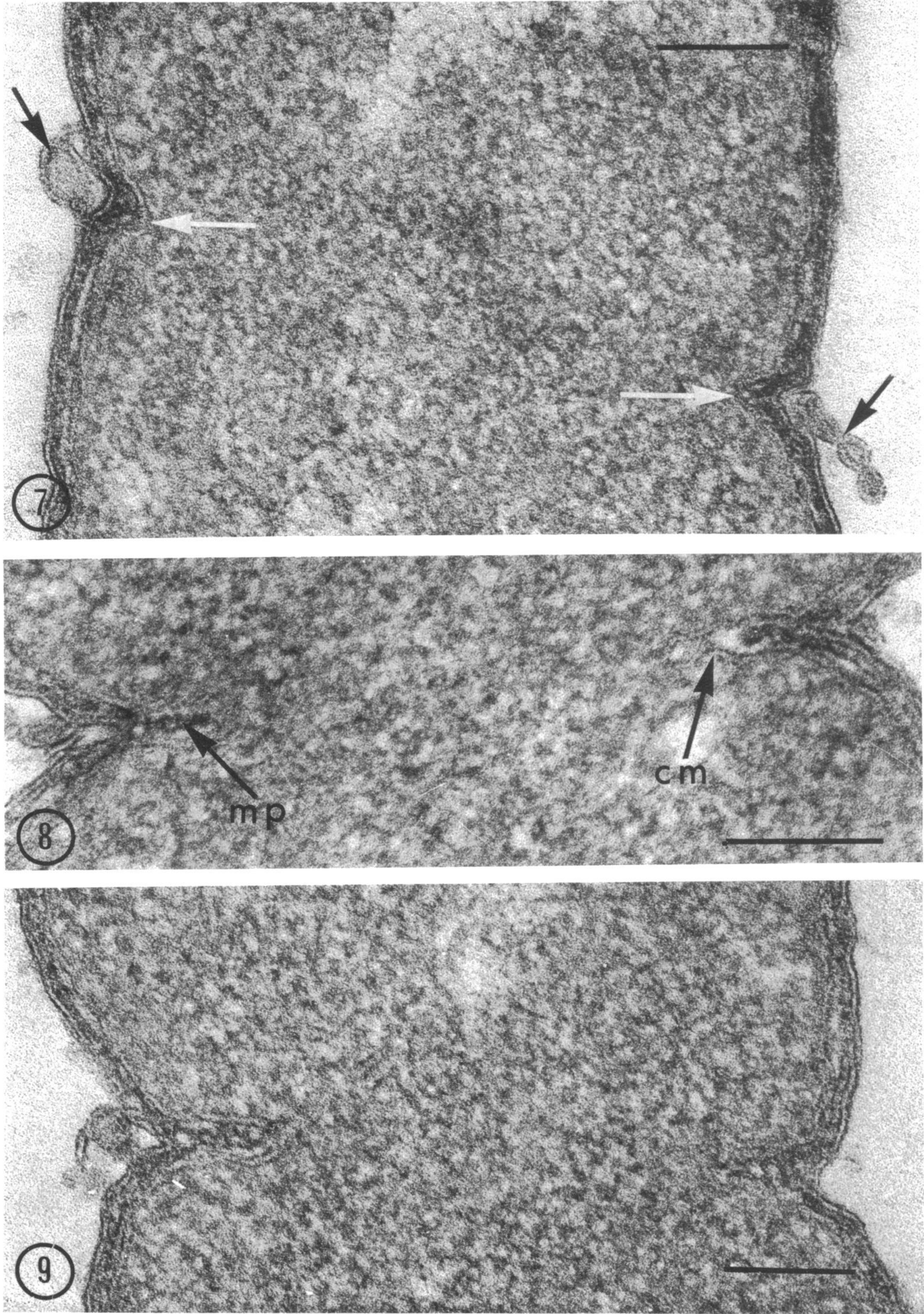


FIG. 7-9. *Sections of E. coli B synchronized by membrane elution (Fig. 7, 9) or density gradient centrifugation (Fig. 8), at 30 (Fig. 7) or 35 min of incubation (Fig. 8, 9). Note vesicles of outer membrane (black arrows, Fig. 7) and ingrowth of mucopeptide (mp) and cytoplasmic membrane (cm)—also white arrows in Fig. 7.*

the outer membrane was also seen at this stage, yet there was continuity of the membrane across the initial of the septum. In samples of *E. coli* B (and also in some cells of *E. coli* B/r) obtained by either method of synchronization, this initial stage of septation was often accompanied by the appearance of mesosomes (Fig. 10–13). Because mesosomes were also observed without any apparent connection to the septum (Fig. 13), it is likely that only one or two points of attachment of mesosomes to the cytoplasmic membrane were present. The mesosomes were composed of stacks of lamellar membranes housed within an apparent infolding of the cytoplasmic membrane (Fig. 10, 12). As shown in Fig. 10–12, the bulk of the mesosome lay well in advance of the invaginating mucopeptide portion of the septum; at this stage the peripheral wall appeared to become more rounded as septation progressed (Fig. 10). The myelinic appearance of the mesosomal membranes may be the result of inadequate fixation, but it is important to note that mesosomes were only seen when techniques involving acrolein/glutaraldehyde were used to stabilize the septum. Where mesosomes were seen (Fig. 10), traces of the nucleoid were also present, even though the bulk of the DNA appeared to be effectively compartmentalized to each half of the cell.

These initial stages of septation were found to be the most difficult to preserve. Using the acrolein prefixation schedule (with or without glutaraldehyde), we estimated that as many as 80% of dividing cells of *E. coli* B contained septa, the remaining 20% being of the constrictive type (ref. 5). *E. coli* B/r was found to be a much more difficult organism to fix, and, in a good experiment, the number of septate cells was about 40 to 50%. If samples of *E. coli* B or B/r, taken from synchronous cultures, were fixed with OsO_4 or with glutaraldehyde, only constrictive divisions were seen.

Septation and cell separation. Following this stage of initial ingrowth, the septum, formed by the fold of the cytoplasmic membrane and mucopeptide, was seen to extend into the cells (Fig. 14–19). In every dividing cell of *E. coli* B or B/r, even at the earliest stages, the mucopeptide portion of the septum was seen to be a double structure (Fig. 15, 16). The two arms of mucopeptide, each about 2.5 to 3.0 nm thick, were separated by an electron-transparent "gap," some 3.0 to 6.0 nm wide. These stages, involving the ingrowth of the septum, were noted in cells harvested at 35 to 45 min. At this stage the outer membrane was confined to an area outside the ingrowing septum (Fig. 16, 18, 19). The fate of the mesosomes was not clear from our experiments, and we have not seen an association of these organelles with the completed septum.

The closure of the septum, involving the joining of the mucopeptide portion and cytoplasmic membrane into what was presumably an annulus, is shown in Fig. 20 and 21. As in the initial stages, the mucopeptide was a double structure, and attempts to stain material in this "gap" or to fill it with an electron-dense extracellular tracer have been unsuccessful. Even at this stage, the outer membrane was apparently excluded from the septum (Fig. 20, 21); the nuclear material was fully divided between the cells (Fig. 24, 25).

The final stages involved the gradual cleavage of the cells and this event, at 45 to 50 min, was accompanied by the ingrowth of the outer membrane (Fig. 22, 24). During this process the poles of the cell, immediately adjacent to the septum, appeared to become more rounded, all wall layers became closely apposed (Fig. 22), and the "gap" between the lamellae of mucopeptide showed continuity with the layer associated with the lipoprotein component (3, 5, 30).

Effect of inhibitors on septation. The aim of these experiments was to see if the sequence of septation could be inhibited by treatments which block DNA and protein synthesis. A 10-ml sample of *E. coli* B/r was collected after 40 min of elution from the membrane, and portions of this culture (1.6 to 1.8 ml) were diluted into the appropriate inhibitor at intervals.

When a portion of the culture incubated for 20 min or less was diluted into nalidixic acid at a concentration (20 μg/ml, final concentration) reported to inhibit DNA synthesis in *E. coli* B/r (21), no division of the cells was observed in the Coulter counter (Fig. 26A). Only if the cells were added to the solution of nalidixic acid after more than 20 min of incubation was there an emergence of newly divided cells (Fig. 26A). The proportion of divided cells in most experiments was approximately half that of the control. We interpreted this to mean that there is variation of the interdivision times of individual cells and only cells already fully committed to division can achieve completion.

Results with chloramphenicol (40 μg/ml, final concentration) produced similar data (Fig. 26B).

Effect of ampicillin on septation. When ampicillin (2.5 μg/ml, final concentration) was added to portions of a synchronous culture of *E. coli* B/r obtained after 40 min of elution, no division was observed in any sample (Fig. 26C). Indeed, the cessation of division in asynchro-

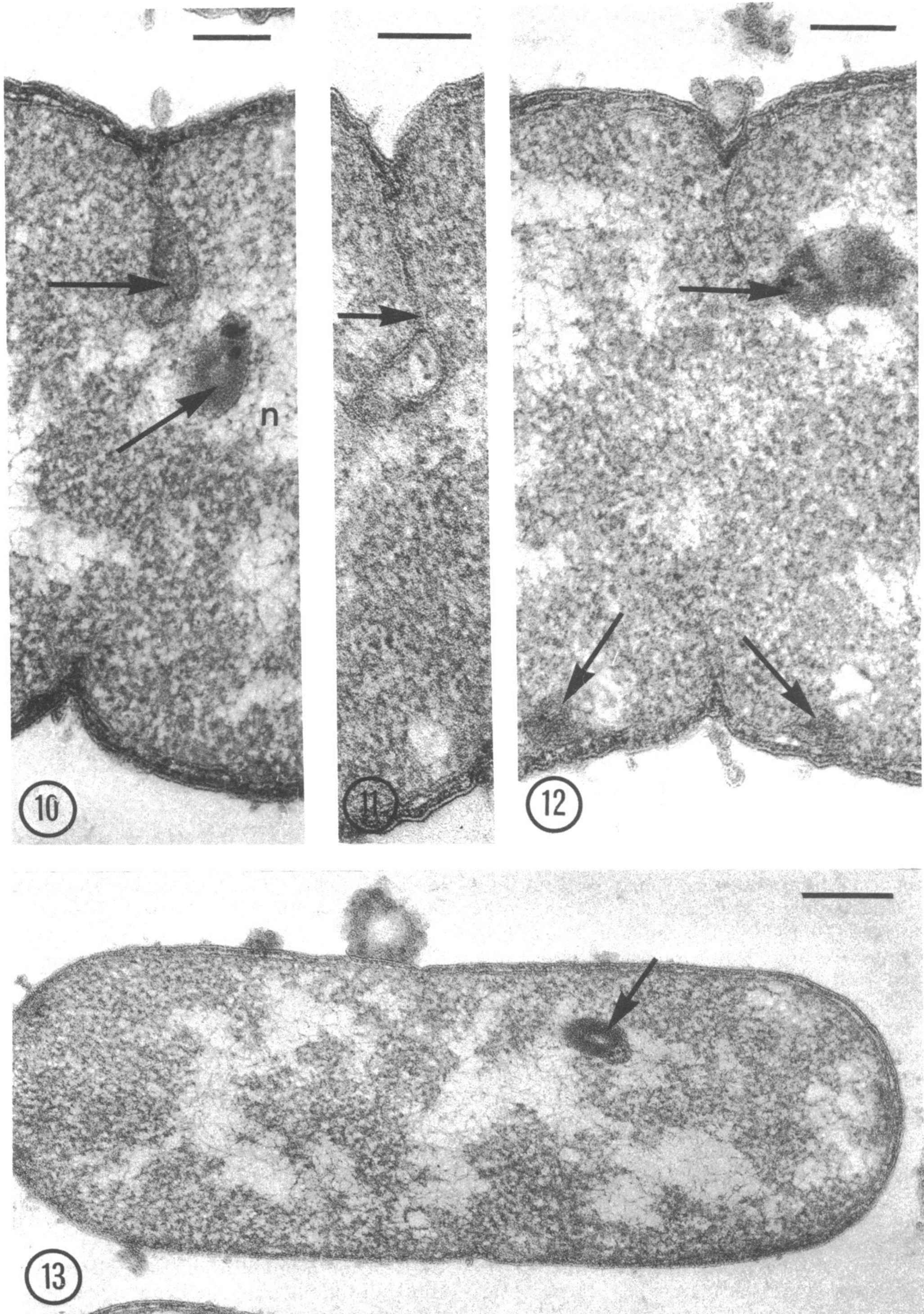


FIG. 10–13. *Sections of E. coli B synchronized by density gradient centrifugation (45 min of incubation, Fig. 10) or membrane elution (Fig. 11–13) at 35 (Fig. 11, 12) or 30 min (Fig. 13). Mesosomes (arrows) are associated with the initial ingrowth of the septum (Fig. 10–12) or at a site remote from the septum (Fig. 13). Bar in Fig. 13 equals 0.2 μm.*

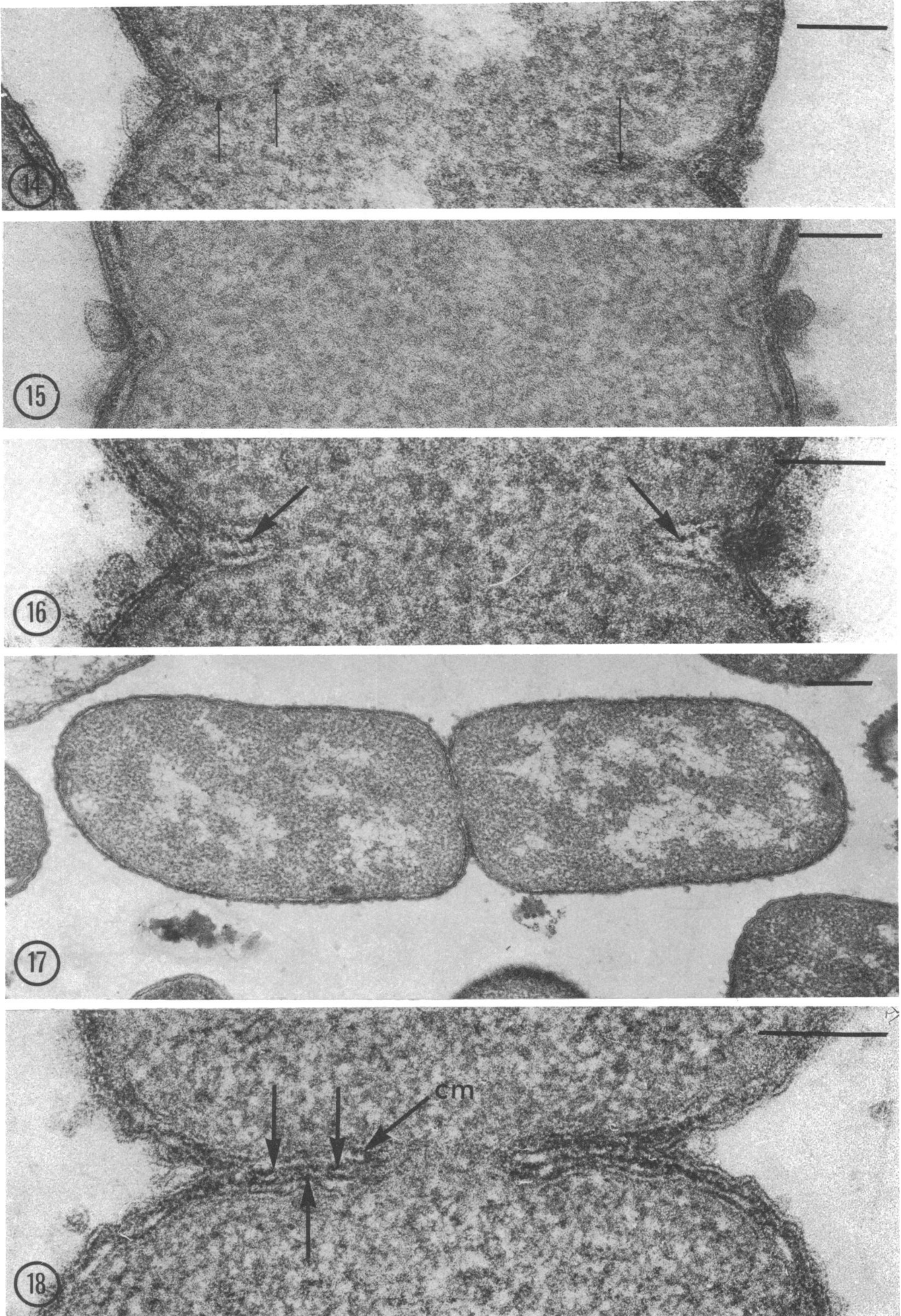
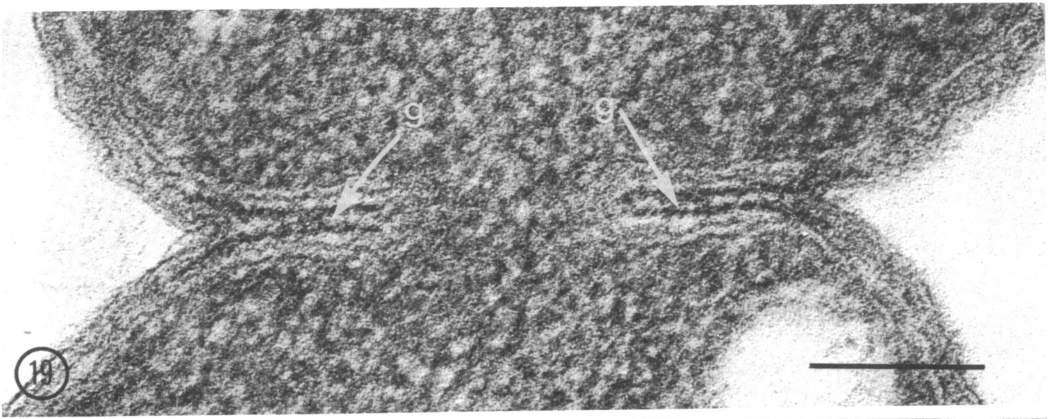
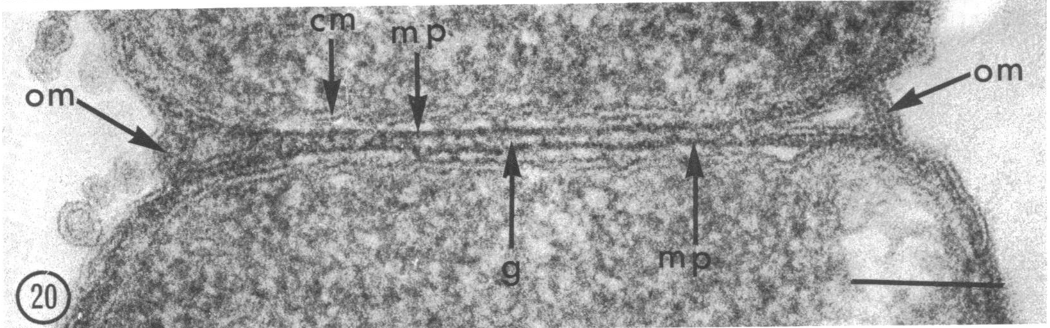
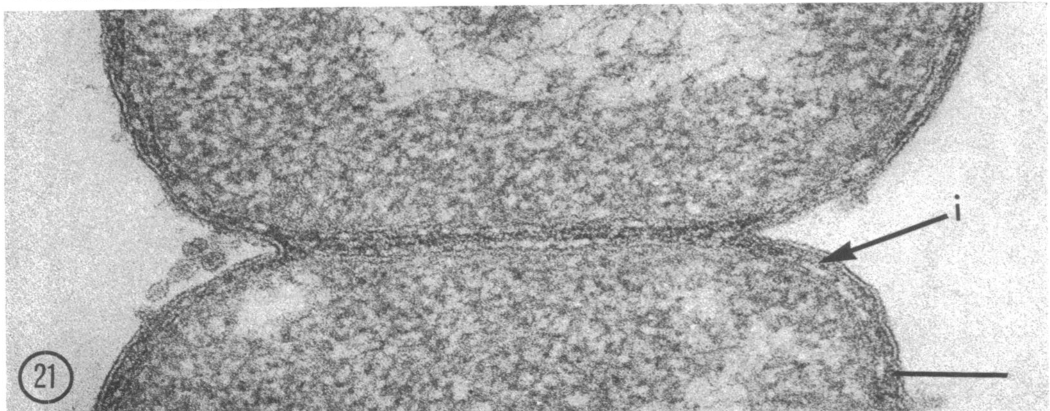


FIG. 14–18. *Sections of E. coli B/r synchronized by membrane elution, at 35 (Fig. 14, 16) and 30 min (Fig. 15), and E. coli B synchronized by density gradient centrifugation (at 45 min of incubation, Fig. 17, 18). Note membrane profiles (arrows, Fig. 14) and double lamellae of mucopeptide in septum (arrows, Fig. 16, 18); cytoplasmic membrane (cm). Fig. 14–15: acrolein-glutaraldehyde fixation. Bar in Fig. 17 equals 0.2 μm.*







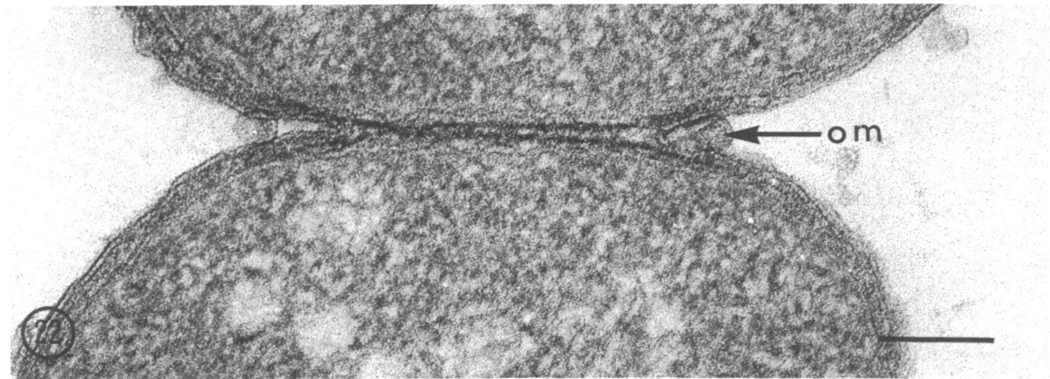


FIG. 19–22. *Sections of E. coli B synchronized by membrane elution at 40 (Fig. 19, 21) and 45 min of incubation (Fig. 20, 22). Note electron-transparent gap (g, Fig. 19, 20) between double lamellae of mucopeptide. The septum is formed by the cytoplasmic membrane (cm) and mucopeptide (mp); the outer membrane (om) and the intermediate layer (i, Fig. 21) are excluded. Separation of cells is shown in Fig. 22.*

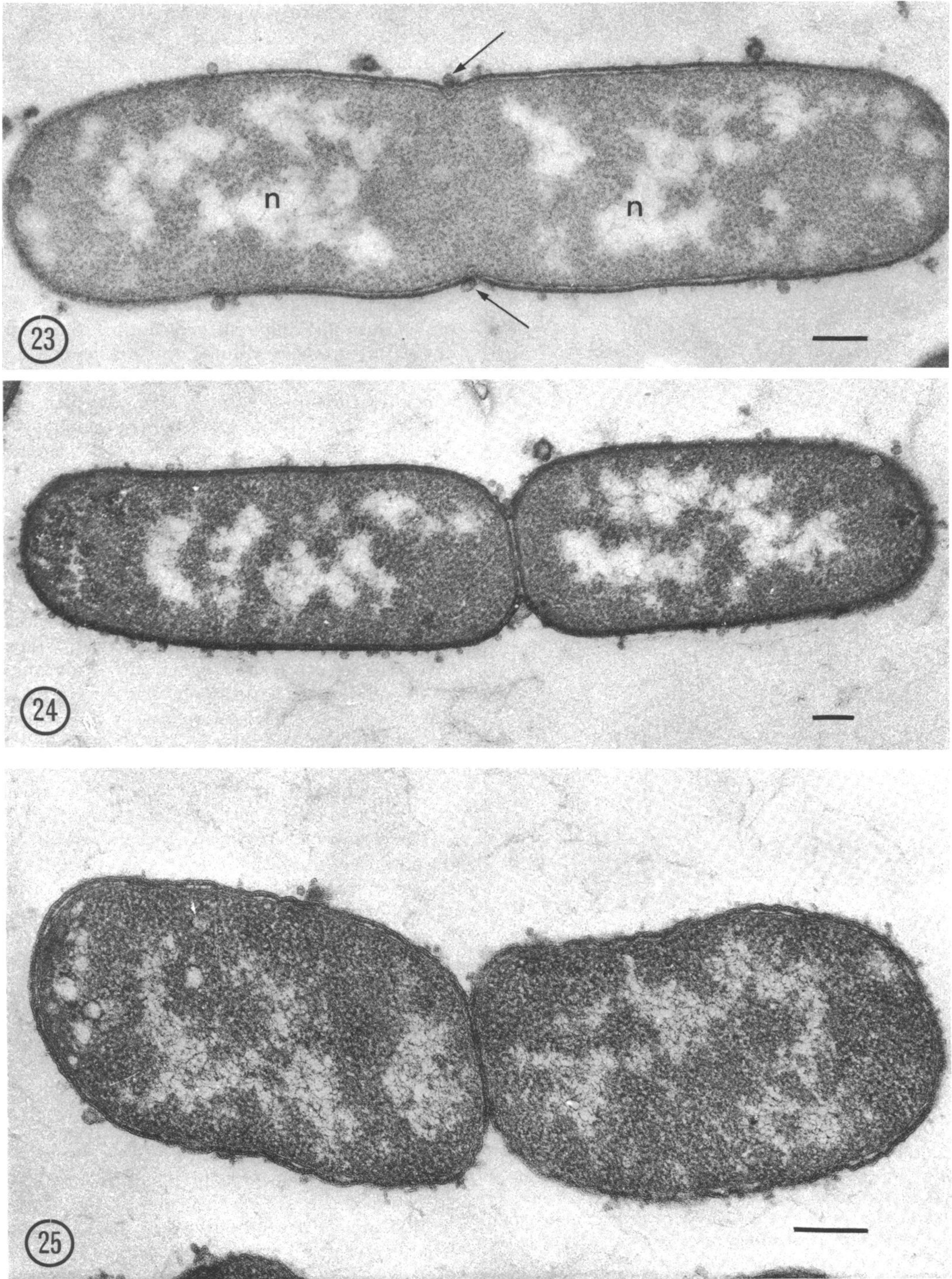


FIG. 23-25. *Sections of E. coli B/r synchronized by membrane elution at 30 (Fig. 23) and 45 min. (Fig. 24), and E. coli B (Fig. 25) at 40 min, membrane elution; fixed with acrolein-glutaraldehyde. Note blebs of outer membrane (arrows, Fig. 23) and nucleoid (n) partitioned to each half of the cell. Fig. 24 and 25 show the completed septum. Bars equal 0.2 μm.*

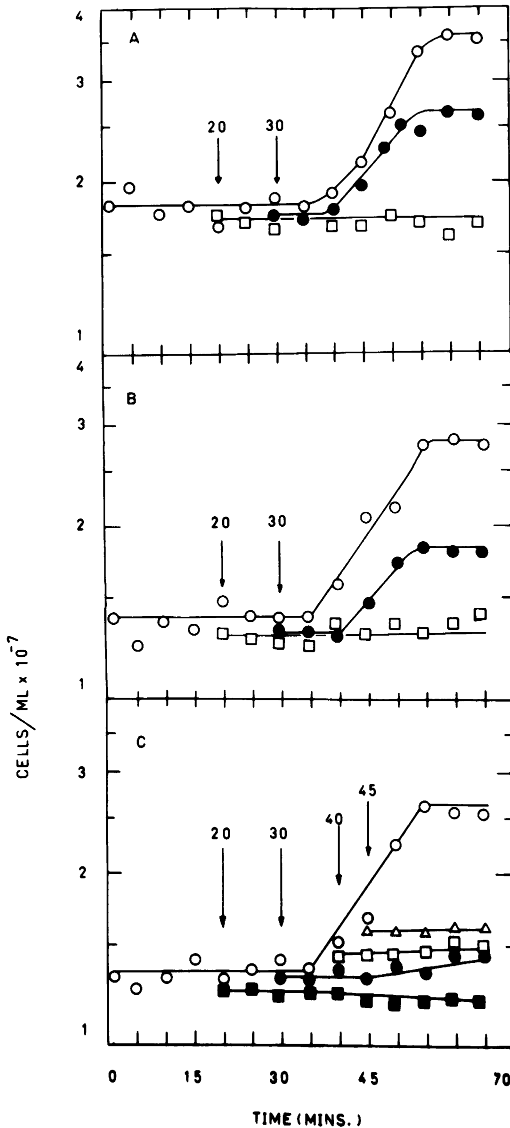


FIG. 26. *Graphs showing effect of nalidixic acid (20 µg/ml, final concentration; Fig. 26A), chloramphenicol (to 40 µg/ml; Fig. 26B), and ampicillin (to 2.5 µg/ml; Fig. 26C) on cell division in synchronous cultures of E. coli B/r. Portions of the culture were diluted into the appropriate antibiotic at different cell age. No division is observed if nalidixic acid or chloramphenicol is added at (or before) 20 min of incubation, but a proportion of the cells divided when the inhibitors were added after 30 min (Fig. 26A, B). No division was observed in any sample to which ampicillin was added (Fig. 26C). Inhibitors were added at 20 (■), 30 (●), 40 (□), and 45 (△) min.*

nous cultures, after addition of ampicillin, was equally abrupt (*unpublished observations*).

Both penicillin and ampicillin (5, 45) block septation in *E. coli* if added in low concentrations and cause a characteristic bulge to be formed in the cells, usually at the site where initiation of division might be expected. We have confirmed that this bulge formation was prevented by the addition of chloramphenicol (5). We also have attempted to determine if bulge formation occurs during a discrete portion of the cell cycle in *E. coli* B/r. Samples (10 ml) were removed every 5 min from the membrane (after an initial elution period of 40 min) and incubated for the desired cell age. At the start and finish of a period of incubation, a small portion of the culture was removed for estimates of cell numbers. Thereafter, samples (8 to 9 ml) of the culture were diluted into flasks containing nalidixic acid and ampicillin, yielding final concentrations of 20 and 5 µg/ml, respectively. The aim was to block DNA synthesis in cells at definite stages of the cell cycle and then to monitor bulge formation by further incubation (60 to 90 min) in the presence of ampicillin. When bulge formation was detected by examination of the cells under phase-contrast optics, the cultures were prefixed with acrolein/glutaraldehyde (see Materials and Methods), and 1.0-ml portions of each sample were removed for counting the number of cells containing bulges. This was accomplished by mixing a loopful of fixed cells with a drop of Nigrosin (0.6% in water) on a cover slip, allowing the preparation to dry, and then sealing it to a glass slide with nail varnish (C. F. Robinow, personal communication). The remaining portion of the culture was processed for electron microscopy.

The results of two experiments, which are based on counts of 150 to 200 cells/sample, are shown in Fig. 27. The majority of cells formed bulges at 20 to 25 and 60 to 65 min of incubation. Electron microscopic examination of cells treated at age 20 min showed that bulges were indeed formed, and, in most cases, these bulges were located at or near the center of the cells (Fig. 28, 29). In contrast, cells of age 35 min appeared cylindrical without obvious bulges (Fig. 30); cells in the process of division, at 40 to 45 min, had no visible septa. The DNA in all samples was composed of fine fibrils, much dispersed and barely visible in the cytoplasm; vacuoles were also present in many cells, irrespective of the age of the cells at the start of incubation with ampicillin and nalidixic acid. Membrane profiles were also seen to be associated with the cells harvested at 20 to 30 min, suggesting that lysis of some cells was occurring; the ampicillin concentration used (5 µg/ml) did not, however, promote extensive lysis, suggesting that elongation of the cells was not affected.

Similar experiments, designed to test the

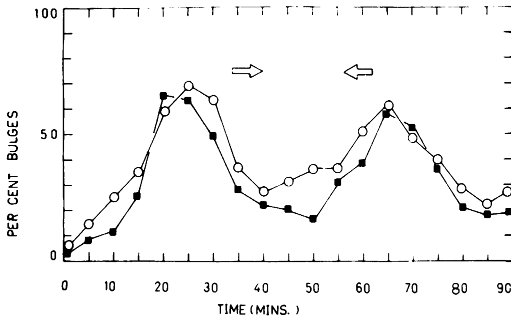


FIG. 27. *Estimate of percentage of cells of E. coli B/r forming a bulge induced by ampicillin (5 μg/ml, final concentration) in relation to cell age. Portions of eluted cells (10 ml) were incubated to obtain the desired cell age and then diluted into nalidixic acid (20 μg/ml, final concentration) and ampicillin and incubated for a further 60 to 90 min at 37 C (see text). Bulge formation was monitored by phase-contrast microscopy. The arrows show the period when cell division was detected in control cultures. The results of two separate experiments are shown.*

susceptibility of the cells to cephalosporins, which appear to block elongation and septation (see ref. 5), were unsuccessful. Lysis of the cells usually occurred, and the presence of sucrose appeared to interfere with the normal growth of the cells.

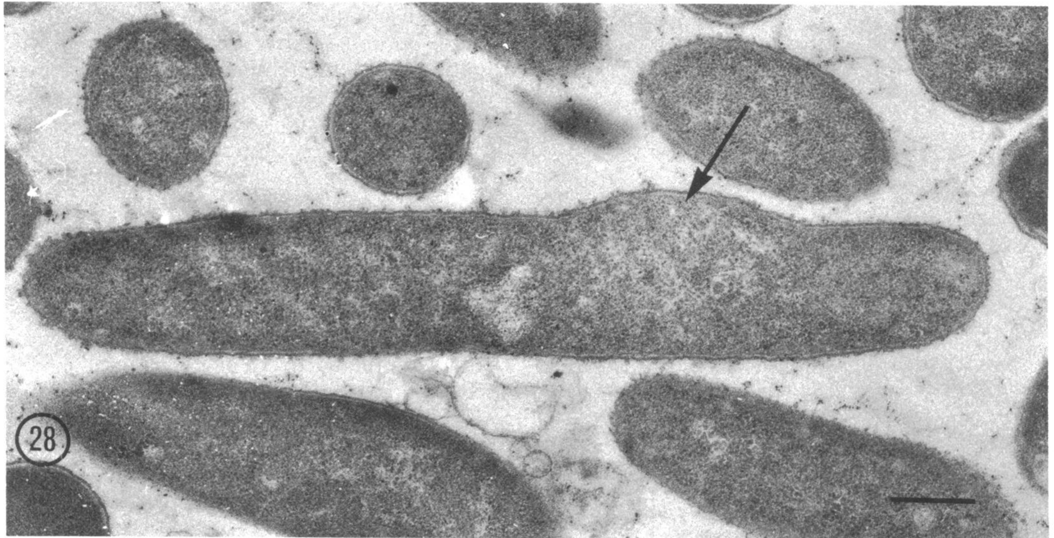
DISCUSSION

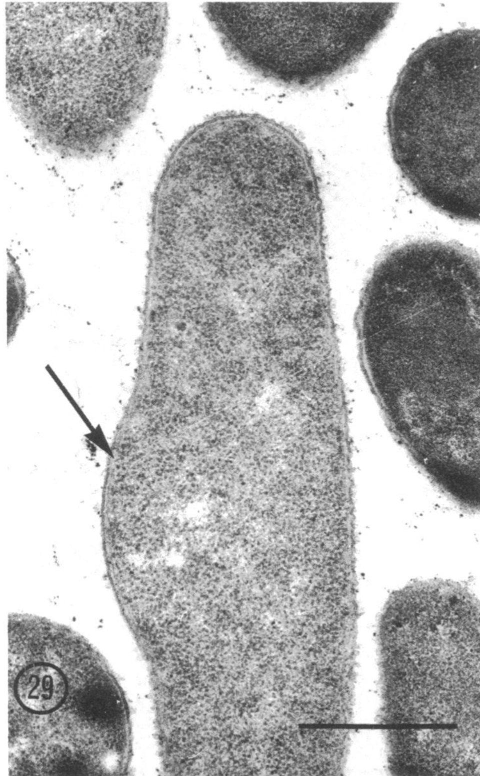
The synchronization procedures utilized, density gradient centrifugation and membrane elution, are both "selective" methods (17) in that a fraction of cells are withdrawn from an asynchronous population and used to initiate a synchronous culture. In the case of the elution technique (16, 17), selection is by cell age, and newborn cells are collected from an eluate of cells growing and dividing on a membrane filter. A full assessment of this method has been published by Helmstetter (16, 17). Although similar results were obtained by means of the Mitchison-Vincent technique (31), it cannot be rigorously proven that a population of small cells is necessarily newborn or at the same stage of the cell cycle.

It is important to identify some of the known physiological properties of cells grown in glucose minimal medium, as they apply to *E. coli* B/r (7, 8, 16, 18, 19, 35). (i) DNA synthesis is continuous and there are no gaps between rounds; (ii) the chromosome of the newborn cell is half way through replication which is completed after a further incubation of 20 min, marking the end of the C period; (iii) a new round of replication is then immediately initiated so that each cell contains two partially replicated chromosomes; (iv) a further 20 min (the D period) elapses between the end of a round of DNA replication and the subsequent division of the cells (see Fig. 31). In essence, therefore, the events of the division cycle are "phased out," such that the end of a round of DNA replication does not coincide with, or overlap, the end of the D period.

This paper has presented evidence that septa are formed in *E. coli* B and B/r during a discrete portion of the cell cycle. Our current interpretation of the sequence of events is shown in Fig. 31 and 32 (stages 1-7). Our micrographs resemble those of completed septa of *E. coli* published by Steed and Murray (46), but the general fixation has been much improved by the use of appropriate aldehydes in prefixation. In general terms our model for septation is similar to that proposed by Clark (7) for *E. coli* B/r/1 in glucose minimal medium, but is based on quite different evidence. The main difference lies in the fact that mesosomes were observed by us in *E. coli* B and B/r, and we have not seen an initial stage where the cytoplasmic membrane divides the cell prior to the ingrowth of the mucopeptide. Such membrane partitions have been seen (5) in *E. coli* B/r cultured in glucose medium supplemented with Casamino Acids.

The visible events, as detected by electron microscopy, appear to start about 10 min after a time coincident with the end of a round of DNA replication (Fig. 31). Earlier events in the cycle defining the beginning of the D period may not be detectable by available microscopic techniques. However, septation may occur in two stages: (i) assembly of the "precursors" of septation; and (ii) initiation and growth of the septum. Our results and the more extensive data of Dix and Helmstetter (11) suggest that all necessary materials needed for septation (in terms of net synthesis of DNA, RNA, and protein) are probably synthesized during the C period. In the case of certain thermosensitive mutants of *E. coli* (5, 37) blocked in septation, a proportion of the cells can divide despite the addition of chloramphenicol to the cultures at the time of transfer back to the permissive temperature. Even in these mutants there is a delay (some 15 to 20 min; ref. 5, 37) before the cells actually begin to divide. These results suggest that assembly of the septum can take place even in the presence of chloramphenicol, providing that the preceding round of chromosome replication has been successfully terminated. However, we must emphasize that the essential structural components of the septum are the cytoplasmic membrane and the invaginated mucopeptide. It is the growth and modification of these components that comprise





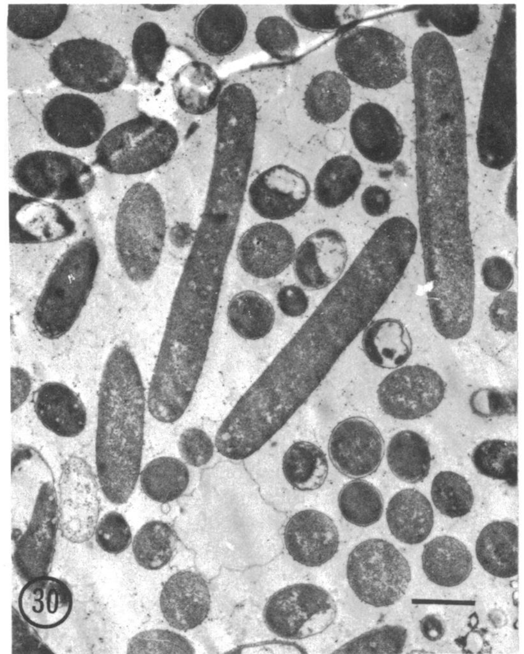


FIG. 28-30. *Sections of E. coli B/r fixed with acrolein-glutaraldehyde after incubation with nalidixic acid and ampicillin (see Fig. 27 and text). Note bulges (arrows, Fig. 28, 29) in cells of age 20 min but absence of bulges in cells of age 35 min (Fig. 30). Bars equal 0.5 (Fig. 28, 29) and 1.0 (Fig. 30) μm.*

the major events. It is useful, therefore, to categorize the subsequent discussion concerning septation into three phases: (i) modification of the wall prior to septation; (ii) assembly of septum "precursors"; and (iii) organization of the components involved in septation (Fig. 33).

In synchronous cultures of *E. coli* B/r treated with nalidixic acid and ampicillin, the maximal

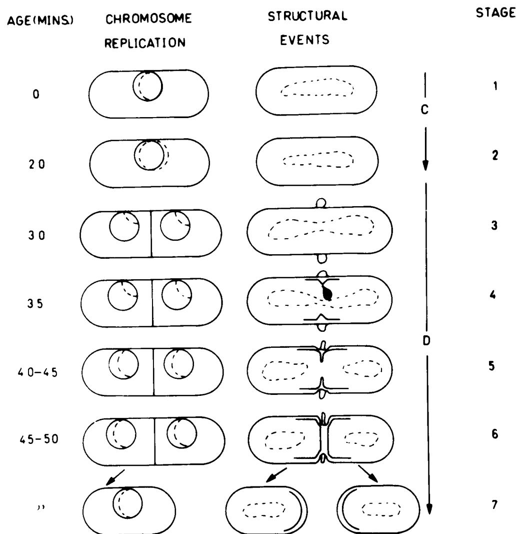


FIG. 31. *Diagram of basic events during the cell cycle of E. coli in glucose minimal medium. The diagram is based on data derived from ref. 7, 8, and 18 and shows that DNA replication is completed at 20 min and a new round of replication is then immediately initiated; separation of the nucleoids (shown by a vertical line in middle of cells) also occurs at about this time. The structural events, as seen in thin sections of E. coli B and B/r, are shown on the right (stages 1–7), and the approximate timing of events (in min) is shown on the left. These stages are shown more completely in Fig. 32.*

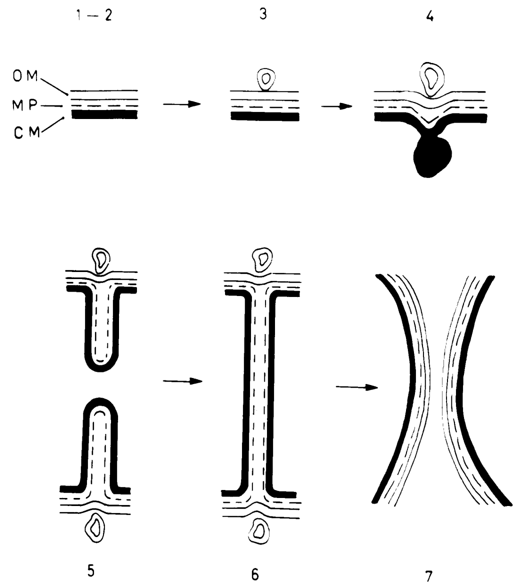


FIG. 32. *Sequence of septation (stages 1–7) in synchronous cultures of E. coli B and B/r. The outer membrane (om) forms a bleb (stages 3–6) but only enters the septum at cell separation (stage 7); the intermediate layer (not shown, see Fig. 5) between om and mucopeptide (mp) may also be excluded from the septum. The septum is composed of the cytoplasmic membrane (cm) and mucopeptide; mesosomes, linked to cm, are found during the early stages of septum formation (stage 4).*

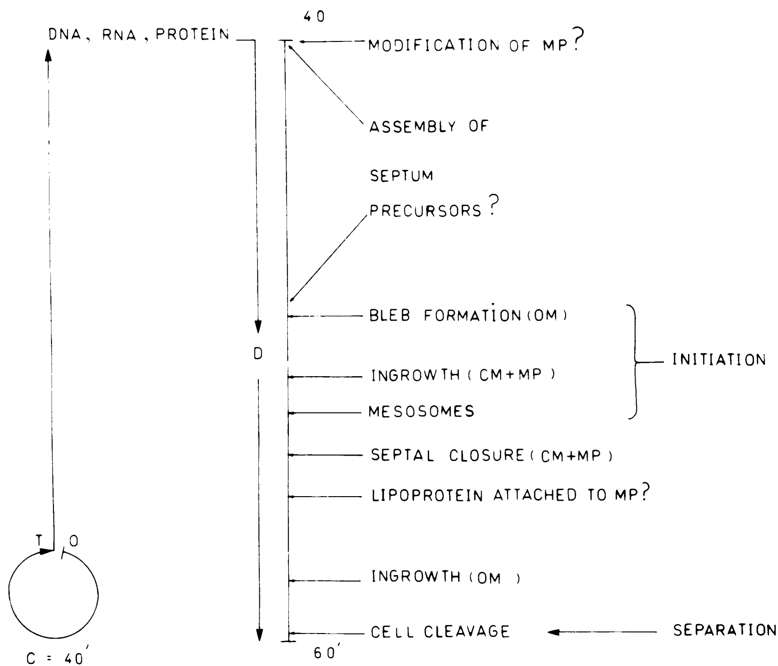


FIG. 33. *Diagram summarizing the possible sequence of structural events during the D period. All the necessary precursors of septation, or conditions necessary to initiate septation (DNA, RNA, and protein), are synthesized during the preceding C period (40 min). During the D period (40 to 60 min), there may occur a modification of mucopeptide (mp) by autolysins and assembly of the septum. The visible events of septation, bleb formation by the outer membrane (om), and ingrowth of cytoplasmic membrane (cm) and mesosomes are associated with the initial ingrowth of the septum. During the terminal stages, prior to ingrowth of the om, the lipoprotein covalently bound to mp could become attached to the mp portion of the septum (see text).*

number of bulges were found in cells after 20 to 25 min of incubation and again at about 60 min (Fig. 27). In general the bulges were located at or near the middle of the cells. The data on bulge formation induced by ampicillin is in agreement with the results of Hoffmann et al. (25) using penicillin. Both these antibiotics inhibit the glycopeptide transpeptidase and the D-alanine carboxypeptidase in *E. coli* (26, 47). There is some evidence suggesting that it is the transpeptidase which is involved in septum formation and which can be blocked by low doses of penicillin (15). If bulge formation is due to the continued synthesis and activity of mucopeptide hydrolases after inhibition of the transpeptidase with penicillin (cf. ref. 5, 45), then the mucopeptide in the zone committed to division can be damaged prior to the synthesis or assembly of the septum. Bulge formation is blocked if chloramphenicol is added at the same time as ampicillin (5). The potential maximal time for bulge formation, therefore, coincides with a time at or about the end of a round of DNA replication and some 20 min before the cells actually divide. It is known that the rate of mucopeptide synthesis probably varies during the cell cycle of *E. coli* (25), and there is a rapid increase in the rate at or about the end of the C period, coinciding with the observable construction of the septum. Although the properties of wall-associated autolysins have been studied in gram-positive bacteria, where they may play a role in cell separation and localized remodelling of the wall, very little is known of the functions of such enzymes in *E. coli* (see ref. 5). Because the mucopeptide sacculus is essentially a closed network of covalently linked polysaccharide chains cross-linked by short peptide bridges (2, 44), mechanisms must exist for opening the wall to allow for insertion of new units during growth.

Penicillin-induced bulges may indicate the site for division but they are many orders of magnitude greater than the width of the septum. This may indicate an extensive zone of hydrolytic activity once the transpeptidase is blocked. Our results and other observations (5, 45, 46) suggest that the septal area is a site where wall structure is highly labile. The probable reason for this now appears clear. By means of autoradiography Ryter et al. (41) showed that *E. coli* W7, growing in glucose or succinate media, probably possesses a single growth site. Pulse-labeling the mucopeptide with [^{3}H]diaminopimelic showed that the grains were localized in a strip at the center of the cells; pulse and chase experiments showed that the label was distributed over the entire surface of the cells. At each division the growth site is apparently relocated from a pole to the middle of the cell. It would be of great interest to apply these techniques to synchronous cultures. The extreme lability of the septal area must presumably indicate that the enzymes concerned in its synthesis are located at or very close to the substrate of mucopeptide, either in the wall structure itself or on the cytoplasmic membrane.

The cessation of division of cells of *E. coli* B/r at all stages of the cycle (Fig. 26C) may imply that penicillin- or ampicillin-sensitive enzymes are operative throughout the cell cycle. However, preliminary experiments involving pulsed treatment of *E. coli* B/r with penicillin terminated by penicillinase suggest that recovery from penicillin damage is age dependent (cf. ref. 25), because cells early in the cycle take longer to complete division than cells already committed to septation. Cells of a chain-forming division of mutant of *Salmonella typhimurium* grown in yeast extract can also separate in the presence of penicillin (1), suggesting that the cells become insensitive to the antibiotic after completion of the septum. There are likely to be a number of enzymes involved in septation, and some of them seem to be directly sensitive to penicillin. Those not sensitive to penicillin may be equally important to the septation process.

An intriguing problem raised by this study is the relation between the requirements of wall synthesis leading to septation and elongation. How separate are these processes? One indication that they are, to some extent, separate processes comes from data obtained by treating *E. coli* with varying levels of penicillin (45) or ampicillin (5). Bulges produced by these antibiotics, when used in low doses, trigger a response in the cells at a fairly well-defined point in the cycle, i.e., at or about the end of the C period. On the other hand, blocking DNA synthesis can still permit elongation to continue (7, 45; unpublished observations). Studies of *E. coli* by time-lapse photomicrography (23) suggest that elongation of the cells ceases during the septation process and that wall synthesis is diverted into construction of the cross wall. Electron microscopy suggests that bleb formation of the outer membrane at the site of division starts at about 30 min of incubation and persists throughout septation until the poles of the cell become rounded at cell cleavage (see below). This observation implies that either (i) synthesis of the outer membrane accelerates as the cells, which may still be elongating, approach septation, leasing to folding of the membrane as synthesis continues, or (ii) cell elongation ceases

when septal initiation starts, and that outer membrane continues to be synthesized at a normal rate, but accumulates at the periphery of the cell as the mucopeptide and cytoplasmic membrane invaginate. Whether the bleb produced by the outer membrane is an artifact of preparation is not clear. Conceivably, it may reflect a loosening of the outer membrane as the cells prepare to septate. Whatever the reason, it is clear that the outer membrane is excluded from the septum until the final stages (Fig. 22). It is probable that the nucleoid is segregated at this stage also and, if as predicted by the replicon hypothesis (26) it moves apart by membrane growth, the cells must initiate septation soon after chromosome segregation (8). As suggested by our studies, separation of the nucleoid into two is apparent in cells fixed after 22 to 30 min of incubation. Chromosome segregation may therefore occur during a brief period of the cell cycle, implying that segregation may not proceed gradually and continuously during replication.

Mesosomes accompany the initial stages of septation, particularly in *E. coli* B, and seem to be closely associated with the ingrowth of the septum. However, it cannot be excluded that mesosomes are also found at stages earlier in the cycle though rarely preserved. Pontefract et al. (36) and Ryter and Jacob (42) also observed mesosomes in *E. coli*, and their micrographs suggest delicate infoldings of the cytoplasmic membrane. In our micrographs (e.g., Fig. 10-12) the septal mesosomes appear to be saclike extensions of the membrane, resembling those observed in *Pseudomonas* (24). In *E. coli* B and B/r, mesosomes were only seen at the initial stages of septation and their ultimate fate is not clear. It is possible that the membrane of the mesosome is gradually contributed to the developing septum (cf. ref. 49). But in some preparations we have also noted mesosome-like membranes at the poles of the cells (unpublished observations) which may represent relics of former divisions (cf. ref. 6). The exact role of these organelles, even in gram-positive bacteria, is still a matter of speculation (for reviews, see ref. 4, 22, 39, 44). It should be noted that Daniels (9) has detected an increase in the incorporation of [^{3}H]glycerol into lipids at about the time of septum formation in cultures of *E. coli* synchronized by a starvation technique. This apparently newly synthesized lipid, as Daniels points out, could be used to construct the septum, although the labeling technique does not distinguish the outer membrane of cytoplasmic membrane or label a defined molecular species.

At some terminal stage of septation the lipoprotein (3) covalently bound to mucopeptide must enter the septum, and it is possible that at least part of the "gap" between the mucopeptide lamellae (Fig. 18, 19) contains the lipoprotein. In isolated sacculi, negatively stained with phosphotungstate, the lipoprotein particles appear to be about 10 nm wide (30); these may represent aggregates of much smaller molecules (the molecular weight of the lipoprotein is about 8,000 to 10,000; ref. 3). The biological role of the lipoprotein is unknown, but may also serve an anchoring function between the outer membrane and mucopeptide (30) or allow it to accomplish separation. Whether there is additional material, such as enzymes, in this space is not known.

In summary, therefore, we envisage the events of the D period (Fig. 33) as follows: (i) modification of the mucopeptide by hydrolases at the end of the C period (this may occur just prior to an increase in rate of mucopeptide synthesis [ref. 25]); (ii) assembly of enzymes involved in septation, which may involve the attainment of a specific membrane configuration (ref. 48); (iii) initiation of wall and membrane synthesis to form a septum with the possible assistance of mesosomes; and (iv) ingrowth of the outer membrane followed by separation of the cells.

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