THE DEVELOPMENT OF CELLULAR STALKS IN BACTERIA

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ABSTRACT

Extensive stalk elongation in *Caulobacter* and *Asticcacaulis* can be obtained in a defined medium by limiting the concentration of phosphate. *Caulobacter* cells which were initiating stalk formation were labeled with tritiated glucose. After removal of exogenous tritiated material, the cells were subjected to phosphate limitation while stalk elongation occurred. The location of tritiated material in the elongated stalks as detected by radioautographic techniques allowed identification of the site of stalk development. The labeling pattern obtained was consistent with the hypothesis that the materials of the stalk are synthesized at the juncture of the stalk with the cell. Complementary labeling experiments with *Caulobacter* and *Asticcacaulis* confirmed this result. In spheroplasts of *C. crescentus* prepared by treatment with lysozyme, the stalks lost their normal rigid outline after several minutes of exposure to the enzyme, indicating that the rigid layer of the cell wall attacked by lysozyme is present in the stalk. In spheroplasts of growing cells induced with penicillin, the stalks did not appear to be affected, indicating that the stalk wall is a relatively inert, nongrowing structure. The morphogenetic implications of these findings are discussed.

INTRODUCTION

The family Caulobacteraceae consists of rod-shaped or vibrioid bacteria, which can produce filiform extensions of the cell, devoid of reproductive function, known as *stalks*. The vegetative population is a mixture of nonmotile *stalked cells* and motile, nonstalked *swarmers*. Division, which occurs by transverse binary fission of a stalked cell, gives rise to a basal cell which retains the stalk of the parent, and an apical swarmer, which is motile by means of a single polar or subpolar flagellum. After its release, the swarmer develops a stalk at the site of attachment of the flagellum, and, as a rule, simultaneously loses its flagellum and its capacity for movement. These events take place prior to the next division. In the genus *Caulobacter*, stalk and flagellum are polar; in the genus *Asticcacaulis*, they are subpolar (1). An amorphous mass of adhesive material, termed the *holdfast*, is characteristically secreted at the pole of the cell. In *Caulobacter*, the holdfast consequently occurs around the terminal end of the stalk, but in *Asticcacaulis*, where the stalk is subpolar, the two structures are not associated (2). The division cycles and characteristic structural features of *Caulobacter* and *Asticcacaulis* are shown diagrammatically in Fig. 1.

The electron micrographs of Houwink and van Iterson (3) and Houwink (4) showed that the wall of the stalk is continuous with that of the cell, and suggested that the core of the stalk might contain cytoplasmic material. The later ultrastructural study of Poindexter and Cohen-Bazire (5) showed that the stalk is completely enclosed by an extension of the cell wall, which has a multilayered structure similar to that of many other Gram-negative bacteria. The core of the stalk is surrounded

by an extension of the cytoplasmic membrane, and contains a special membrane system which is continuous with an accumulation of membranous material in the cortical cytoplasm immediately adjacent to the stalk. The stalk is consequently devoid of both ribosomes and nucleoplasm. Crossbands (4, 6) frequently traverse the stalk and were found by Poindexter and Cohen-Bazire (5) to be narrow, electron-opaque, annular structures lying within the external layers of the cell wall. The caulobacters typically also contain mesosomes. However, the membranous stalk organelle does not have a typical mesosomal structure as judged from its profile in thin sections (5), and does not constitute an intrusion of the cytoplasmic membrane as do the mesosomes (7).

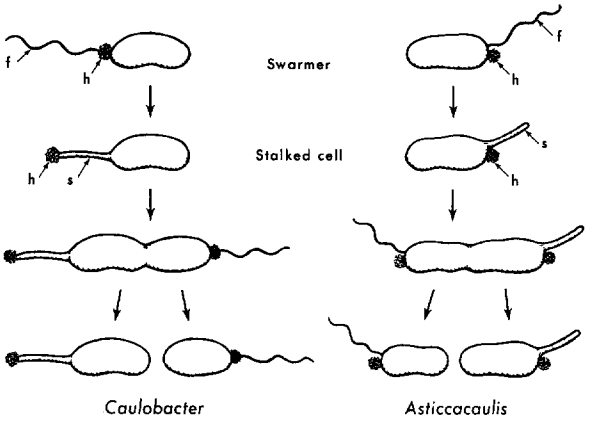


FIGURE 1 A semidiagrammatic representation of cellular differentiation and division in bacteria of the family Caulobacteraceae. Holdfast, *h*; flagellum, *f*; stalk, *s*.

In nature and in the very dilute media used for their enrichment (2), caulobacters frequently possess stalks with a length of 20 μ or more. However, in pure cultures, the stalks are typically short, not exceeding 3 μ in length. Dr. John C. Gerhart (personal communication) observed that stalk elongation can be induced in pure cultures growing in a defined medium by limiting the concentration of inorganic phosphate.

The formation of the caulobacter stalk, an event of cellular growth sharply localized both in time and in space, is a morphogenetic process of considerable interest. We have undertaken its analysis in two different ways: by radioautography experiments, using a tritiated carbon source to study the deposition of stalk material; and by examining the effects of lysozyme and penicillin on the structure of the stalk wall.

MATERIALS AND METHODS

Two strains were used in this work: *Caulobacter crescentus* CB15 and *Asticcacaulis excentricus* AC48, obtained from Dr. J. Stove Poindexter. *C. crescentus* has no growth factor requirements; *A. excentricus* requires biotin.

A complex medium (PYE) consisting of 0.2% Bacto-peptone (Difco), 0.1% yeast extract, 0.02% $MgSO_4$, and tapwater was used for maintenance of cultures. Solid medium was prepared by addition of 1% Difco agar. A synthetic medium (G-I) which contained 0.2% glucose, 0.005 M imidazole·HCl (pH 7), 0.05% NH_4Cl, and 1% Hutner's mineral base (8) in deionized water was used in studies involving phosphate limitation. In experiments with *A. excentricus* this medium was supplemented with 1 μg of biotin per ml. In preparation for radioautography experiments, cultures of *C. crescentus* and *A. excentricus* were grown in G-I medium containing 2×10^{-4} phosphate. For radioautography, D-glucose-6-tritium was purchased from the Nuclear Chicago Corp. Des plaines, Illinois.

Dry weights were determined by placing washed cell suspensions in previously dried and weighed aluminum foil cups, which were again heated to dryness in a 60°C oven. Deoxyribonucleic acid was estimated by the diphenylamine reaction (9). The orcinol reaction (10) and the diphenylamine reaction were used in ribonucleic acid determinations. Standards were purified yeast RNA (Worthington Biochemical Corp., Freehold, New Jersey) and deoxyadenosine (Calbiochem, Los Angeles). Protein was determined by the method of Lowry et al. (11) using bovine plasma albumin (Armour) as the standard. Phospholipids were extracted by the procedure of Clark (12), and phosphate was estimated by the

method of Fiske and Subbarow (13). Poly-β-hydroxybutyrate was estimated gravimetrically (14).

TECHNIQUES OF CELLULAR SEGREGATION: In several of the experiments to be described, it was necessary to obtain relatively homogeneous populations of either stalked or swarmer cells. The method of differential centrifugation described by Stove and Stanier (1) was used to isolate stalked cells of *C. crescentus* for experiments on spheroplast formation with lysozyme. At least four successive centrifugations at a gravitational field of 6000 *g*, using a Servall Superspeed centrifuge, were required to obtain adequate segregation.

Populations of swarmers were isolated by a method suggested by Dr. John Gerhart. A cellulose fiber suspension was prepared by adding 5 g of filter paper (sheets of Whatman No. 1) to 200 ml of distilled water and agitating the mixture overnight on a rotary shaker. A portion of the resulting thick suspension was diluted 1:50, and successive aliquots (10 ml) were placed in a Pyrex Buchner funnel (diameter 30 mm) with a fritted disc (C porosity). After each addition, the liquid was forced through the disc by air pressure. In this way, an evenly packed layer of cellulose about 4 mm thick was built up. A liquid culture of *C. crescentus* was harvested by centrifugation, washed twice with the phosphate-free mineral base of medium G-I, and filtered through the cellulose pad under air pressure. If the pad is of the correct thickness, it effectively retains stalked cells while permitting passage of swarmer cells. Populations of swarmer cells of 95% homogeneity could be rapidly obtained by the filtration method.

RADIOAUTOGRAPHIC METHODS: The methods of Caro and van Tubergen (15) were adapted to the present study. The photographic emulsion employed was Ilford L-4 (Ilford Ltd., Ilford, Essex), in which the diameter of the silver halide grains is slightly in excess of 0.1 μ.

A suspension of tritium-labeled bacterial cells was placed on Formvar-covered electron microscope grids, and a thin layer of carbon was evaporated over the cells. Three grids were attached to a clean glass microscope slide by means of double-sided cellophane tape; ten to twenty slides were prepared for each experiment. The loop method of Caro and van Tubergen (15) was used to cover the grids with the photographic emulsion. The emulsion was melted and cooled nearly to the point of gelation. A film of the emulsion was suspended across a loop of copper wire, which was placed over the grids attached to a glass slide. When the emulsion had been allowed to cool to a proper consistency, the film would break after a few seconds of contact with the slide, depositing a monolayer film. It is important that the emulsion be near the point of gelation in this step, because the intact cells used in these experiments present a rough surface; in order to obtain a monolayer film over such a surface, the emulsion must be ready to solidify at the time of contact with the grids. If the film was applied to the grids in a liquid state, thick regions of emulsion formed at the periphery of the cells, causing artifacts in the arrangement of the exposed grains.

After the emulsion had dried thoroughly, the radioautographs were stored at 4°C in the presence of Drierite for 1 to 2 months. The radioautographs were developed with Microdol-X (Kodak) for 5 min. An acid stopbath and Kodak Rapid Fix were used. The gelatin of the emulsion was partially digested by treatment with 0.05 N NaOH for 10 min. This was necessary in order to visualize the outlines of the cells in subsequent electron micrographs. The radioautographs were examined with a Siemens Elmiskop I electron microscope.

SPHEROPLAST FORMATION: Lysozyme spheroplasts of *C. crescentus* were produced by a modification of the technique of Fraser and Mahler (16). The suspending medium consisted of 0.05 M Tris (Sigma 121), pH 7.8, 8% (w/v) polyethylene glycol (Carbowax 4000, Union Carbide), 10 μg/ml lysozyme (Armour), and 4 μg/ml Versene. Stalked cells, obtained by the centrifugal segregation procedure, were incubated in this medium at 30°C, and the reaction was stopped at various times by addition of 1% $MgCl_2$. Magnesium ions also made the spheroplasts more resistant to lysis, so that they could be washed without damage. After one washing in distilled water, the spheroplast suspensions were prepared for electron microscopy.

Penicillin spheroplasts of *C. crescentus* were produced under conditions of limited growth. A complex medium consisting of 0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, and 7% polyethylene glycol as an osmotic stabilizer was used. Cultures were incubated at 30°C in a gently agitated shaking water bath. Penicillin G (4000 units/ml, Parke, Davis, and Co., Detroit) was added and cultures were allowed to grow for 8 hr. Only about 30% of the cells were converted to spheroplasts in these preparations. $MgCl_2$ (1%) was added to the culture, and the cells were harvested, washed once with distilled water, and prepared for electron microscopy.

ELECTRON MICROSCOPY: Cells were fixed by the method of Ryter and Kellenberger (17) for examination of thin sections. The pH of the veronal-acetate buffer used in the fixation was 6.8. Fixation with osmium tetroxide was carried out for periods of 2 and 4 hr. The embedding medium was Maraglas, polymerized at 60°C for 48 hr. Sections were obtained with a diamond knife on a Porter-Blum ultramicrotome. The sections were poststained with lead hydroxide, using Millonig's method (18). The sectioned preparations were examined with a Siemens Elmiskop I.

For shadowed preparations, washed cells were allowed to settle on Formvar-covered electron microscope grids which had been carbon-stabilized. Uranium metal was then evaporated onto the grids at an angle of less than 20°. The shadowed preparations were examined with a Bendix Tronscope electron microscope.

TABLE I

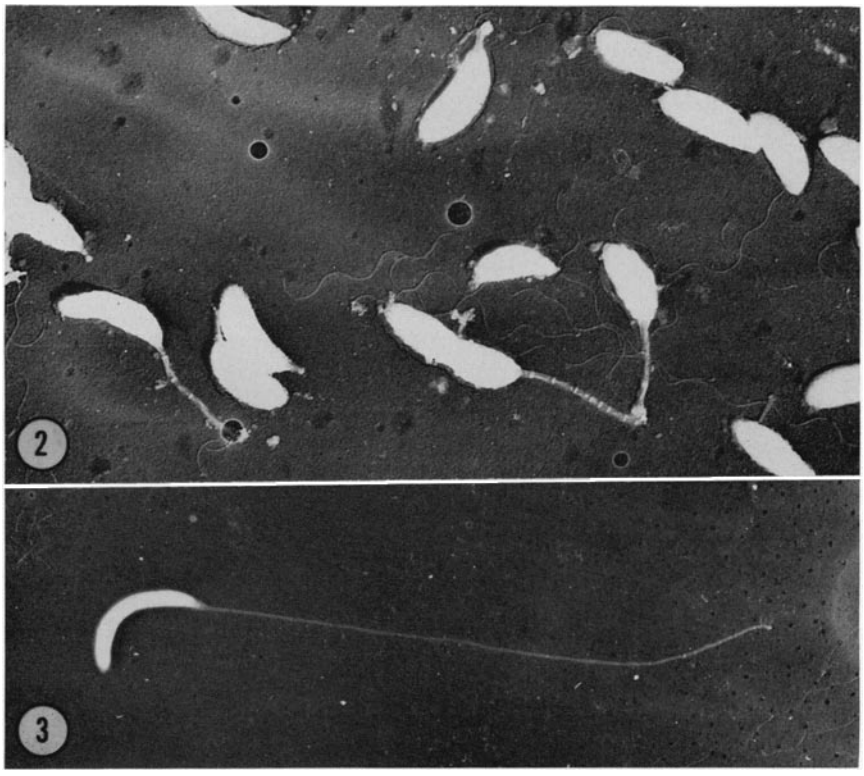
The Effect of Phosphate Limitation on Stalk Length of Caulobacter crescentus Grown to Stationary Phase in G-I Medium

Initial phosphate concentration	Final turbidity	Stalk length
molarity	*Klett units*	μ
2×10^{-4}	261	2 to 3
10^{-4}	260	4 to 6
10^{-5}	66	14 to 18
No added phosphate	16	18 to 20

RESULTS

Effects of Phosphate Limitation

Four cultures of *Caulobacter crescentus* were prepared in medium G-I furnished with different levels of phosphate (Table I) and were incubated until the stationary phase of growth was reached. In the culture containing 2×10^{-4} M phosphate, the length of the stalks is 2 to 3 μ, comparable to that of cells grown in PYE broth. In cultures containing lower phosphate concentrations, the length of the stalks is inversely related to phosphate concentration, reaching a maximum length of approximately 20 μ in the basal medium without added phosphate. Figs. 2 and 3 show electron micrographs of normal and phosphate-limited cells of *C. crescentus*. *Asticcacaulis excentricus* responds similarly to phosphate limitation. Although extreme phosphate limitation causes a 10-fold increase in stalk length, the number of cross-bands



FIGURES 2 and 3 *Caulobacter crescentus* CB15. Uranium-shadowed cells.
FIGURE 2 Grown in G-I medium containing 10^{-3} M phosphate. × 7,500.
FIGURE 3 Grown in G-I medium containing 10^{-5} M phosphate, a limiting concentration. × 4,000.

per stalk does not increase, so that the cross-bands become more widely separated.

Stalk elongation occurs slowly. Cells of *C. crescentus*, washed with distilled water, were resuspended in 20 ml of medium G-I containing 10^{-5} M phosphate, a limiting concentration, and the culture was incubated on a rotary shaker at 30°C. Turbidities and stalk lengths (measured in microscopic preparations) were determined during its growth. Several hours were required for stalks to attain a length of 10 μ (Table II). Not all the cells of such growing, phosphate-limited cultures have stalks of maximum length, since swarmers are continuously produced by division and are continuously initiating stalk formation. The data presented in Table II were all obtained during the course of growth of a phosphate-limited culture. However, when such a culture has entered stationary phase, swarmers are almost unobservable, and the stalked cells have stalks of uniform length. Presumably under these conditions no new swarmers are arising, and all those formed at early divisions have undergone extensive stalk elongation.

Table III shows the macromolecular composition of cells of *C. crescentus* grown in medium G-I with and without phosphate limitation. The content of nucleic acids and phospholipids (expressed in terms of dry weight) is reduced by phosphate limitation, whereas the content of protein is only slightly less than that in normal cells. Phosphate limitation causes a substantial increase in the content of poly-β-hydroxybutyrate, which rises from a value of 10% of the dry weight in normal cells to over 25% in phosphate-limited ones.

TABLE II

The Kinetics of Growth and Stalk Development in a Culture of Caulobacter crescentus Growing in G-I Medium with an Initial Concentration of 10^{-5} M Phosphate

Time	Turbidity	Range of stalk lengths in the cell population
hr	*Klett units*	μ
0	14	2 to 3
0.8	16	2 to 3
4	32	3 to 4
6	54	3 to 6
9.5	82	3 to 8
12.5	105	4 to 10

TABLE III

Macromolecular Composition of Normal and Phosphate-limited Cells of Caulobacter crescentus

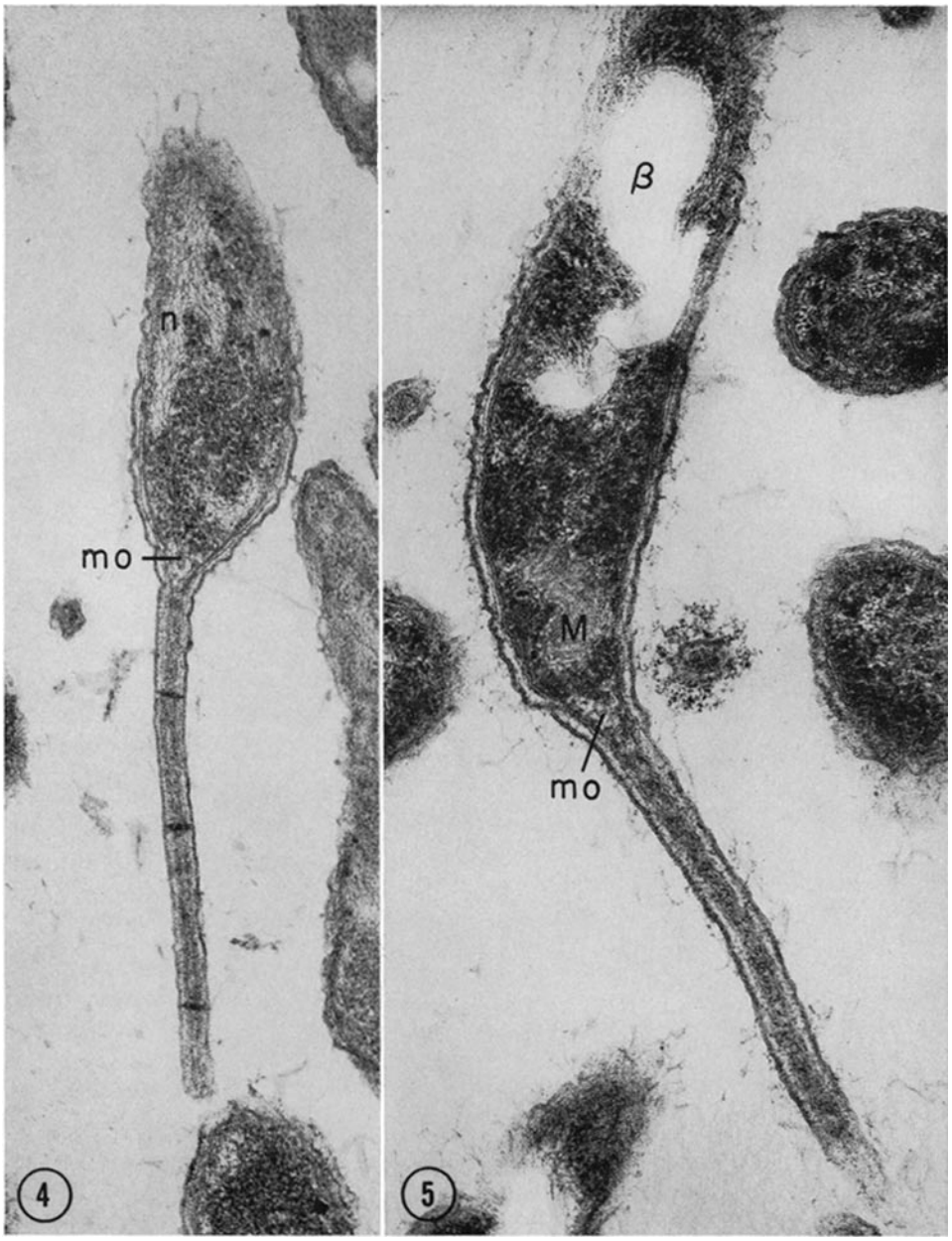
	Cells grown in G-I medium with an initial phosphate concentration of: 10^{-3} M	10^{-5} M
	μg/mg dry wt.	
Protein	590	505
Deoxyribonucleic acid	70	36
Ribonucleic acid	278	92
Phospholipid phosphorus	0.074	<0.01
Poly-β-hydroxybutyrate	98	260

Electron microscopic examination of thin sections of *C. crescentus* grown under these two sets of conditions revealed that phosphate limitation causes two conspicuous structural changes (Figs. 4 and 5). Firstly, there is massive development of internal membranes: although an occasional large mesosome can be seen in sections of normal *C. crescentus* cells (Fig. 5), these membranous structures occur more frequently and are larger in the phosphate-limited cells (Fig. 5). Secondly, much of the cytoplasm is occupied by large, electron-transparent regions. These are not characteristic of normal cells, which do, however, contain a few similar but much smaller areas, interpreted as the sites of poly-β-hydroxybutyrate deposition (2). The much larger electron-transparent areas characteristic of phosphate-limited cells can also be so interpreted, in view of the exceptionally high poly-β-hydroxybutyrate content of these cells.

Despite the modifications of cytoplasmic structure observable in sections of phosphate-limited cells, the structure of the stalks in such cells appears completely normal (Fig. 5). Thus, phosphate limitation seemingly affects the length of the stalks but not their organization, insofar as this can be deduced from their appearance in thin sections.

Radioautographic Studies on the Formation of the Stalk

In an attempt to determine the site of stalk growth, the deposition of tritiated stalk material during stalk elongation was examined. Two distinct experimental designs were employed. In experiments of type A (experiments Ia and II, Table IV), swarmers were exposed to tritiated glucose during the initial period of stalk formation. They were then thoroughly washed to remove



FIGURES 4 and 5 Thin sections of *Caulobacter crescentus* CB15.

FIGURE 4 Grown in G-I medium containing 10^{-3} M phosphate. × 60,000.

FIGURE 5 Grown in G-I medium containing 10^{-5} M phosphate, a limiting concentration. × 84,000.

Abbreviations: n, nucleoplasm; *mo*, site of membranous organelle; *M*, mesosome; *β*, poly-*β*-hydroxybutyrate deposits.

TABLE IV

Summary of Experimental Conditions for Radioautography of Caulobacter crescentus and Asticcacaulis excentricus

Type of exp.	Exp. No.	Strain used	Length of stalk at initiation of labeling	Glucose-6-tritium added to cells	Length of incubation with glucose-6-tritium	Length of incubation after removal of glucose-6-tritium
			μ	μc	*hr*	*hr*
A*	Ia	CB15	<0.5	400	2	10
B*	Ib	CB15	6–8	200	3	0
A*	II	CB15	<0.5	100	2	12
B*	V	CB15	6–8	300	2	0
B*	VII	AC48	8–15	400	3	0
A‡	IV	CB15	<0.5	200	0.16	1.3
A‡	VI	CB15	<0.5	200	0.33	2.3

* Experiments involving extensive labeling and phosphate limitation.
‡ Experiments involving pulse labeling and no phosphate limitation.

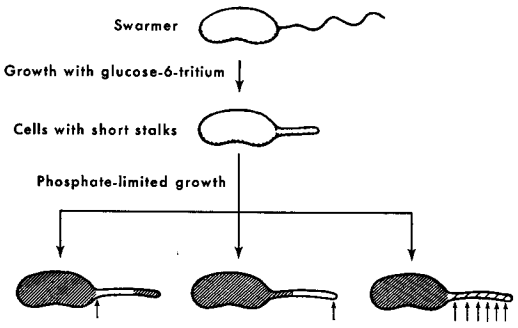


FIGURE 6 A semidiagrammatic representation of the possible patterns of radioactive labeling of stalked caulobacter cells resulting from experiments of type A (see text). The cross-hatching indicates regions of radioactivity within the cell, and the arrows indicate the alternate hypothetical sites of synthesis of stalk material.

extraneous tritiated glucose, and incubated for several hours in phosphate-free medium to induce extensive elongation of the stalks. The cells were then prepared for radioautography.

The experiments of type B were the inverse of type A; experiments Ib, V, and VII followed this design. Populations of cells were grown for several hours in a nonradioactive phosphate-limited medium so that some stalk elongation took place. Glucose-6-tritium was then added, and incubation was continued for 2 to 3 hr. During this period, further elongation of the stalks of some of the cells occurred. The cells were then washed to remove extraneous tritiated glucose, and prepared for radioautography.

There are three possible hypotheses concerning the site of stalk formation in caulobacters. Each predicts a unique pattern of labeling in the radioautography experiments described above. Firstly, if the synthesis of the stalk occurs in a specialized region at its juncture with the cell, an experiment of type A should result in a cell with label in the terminal region of the stalk, whereas an experiment of type B should result in a cell with label in the proximal region of the stalk. Secondly, if the synthesis of the stalk occurs in a specialized region at or near its terminus, experiments of types A and B should produce labeling patterns which are the inverse of those predicted by the first hypothesis. Thirdly, if there are multiple sites of synthesis distributed through the length of the stalk, experiments of both types A and B should yield a more or less even distribution of radioactivity throughout the stalk. The three possible outcomes of experiments of types A and B are shown diagrammatically in Figs. 6 and 7, respectively.

The distinctness of the labeling patterns predicted on the basis of these three alternate hypotheses involved an untested assumption: namely, that extensive turnover and intracellular redistribution of materials does not take place during the elongation of the stalk, and that newly synthesized regions of the stalks are exclusively formed from the exogenous nutrients available to the cell at the time of synthesis. If this assumption is invalid, the localized labeling patterns predicted on the basis of the first and second hypotheses would be obscured by the diffusion of radioactive

biosynthetic precursors, to yield a labeling pattern perhaps indistinguishable from that predicted by the third hypothesis.

In all, two experiments of type A and two experiments of type B were conducted with *C. crescentus* and one experiment of type B with *A. excentricus* (Table IV). We shall first discuss the results of the experiments of type A with *C. crescentus* (experiments Ia and II). As shown in Figs. 8 to 10, radioactivity is localized in the terminal region of the stalk, the pattern of labeling predicted for experiments of this type if stalk synthesis occurs in a localized area at the base of the stalk. The outcome clearly shows that turnover is not sufficiently extensive to obscure the specific labeling pattern. As expected, the experiments of type B with *C. crescentus* (experiments Ib and V) show a complementary labeling pattern (Figs. 11 and 12).

A radioautographic experiment of type A could not be performed with *A. excentricus*, because the cells clumped severely when grown in G-I medium with adequate phosphate, so that a good segregation of swarmers, essential for the performance of a type A experiment, was impossible. Under conditions of phosphate limitation, clumping does not occur, thus permitting the performance of experiments of type B. One experiment of type B was, accordingly, conducted with *A. excentricus*. There was heavy labeling of the cells, and relatively light labeling of the stalks. However, all the label in the stalks was localized in the proximal region (Figs. 13 and 14). These limited observations suggest that, just as in *C. crescentus*, stalk synthesis takes place at the juncture between stalk and cell.

The labeling in experiments Ia and Ib was very heavy, permitting a graphic demonstration of the site of stalk development (Figs. 8 to 12), but preventing a quantitative analysis of the data. In two other experiments, II and V, the labeling was less heavy, and accurate grain counts could be made. Table V summarizes data from these two experiments on the numbers of grains in the proximal and terminal portions of the stalk, and in the cell itself.

Since the stalk is a structure of uniform dimensions, the recorded grain counts in its proximal and distal regions provide an accurate indication of the relative amounts of radioactivity in these two regions. It is uncertain, however, whether the recorded grain counts in the cells are quantitatively comparable with those in the stalks, since the efficiency of impingement on the emulsion of β-emissions occurring in the much thicker cell may be substantially lower. Furthermore, cells which have been labeled during the initial period of stalk development may have undergone one or more divisions prior to radioautography. Each such division would halve the specific activity of the cell, while leaving the specific activity of the stalk unchanged.

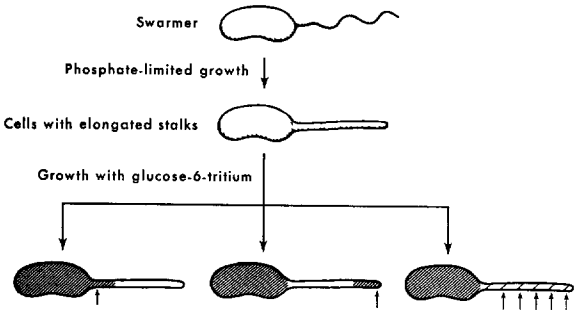
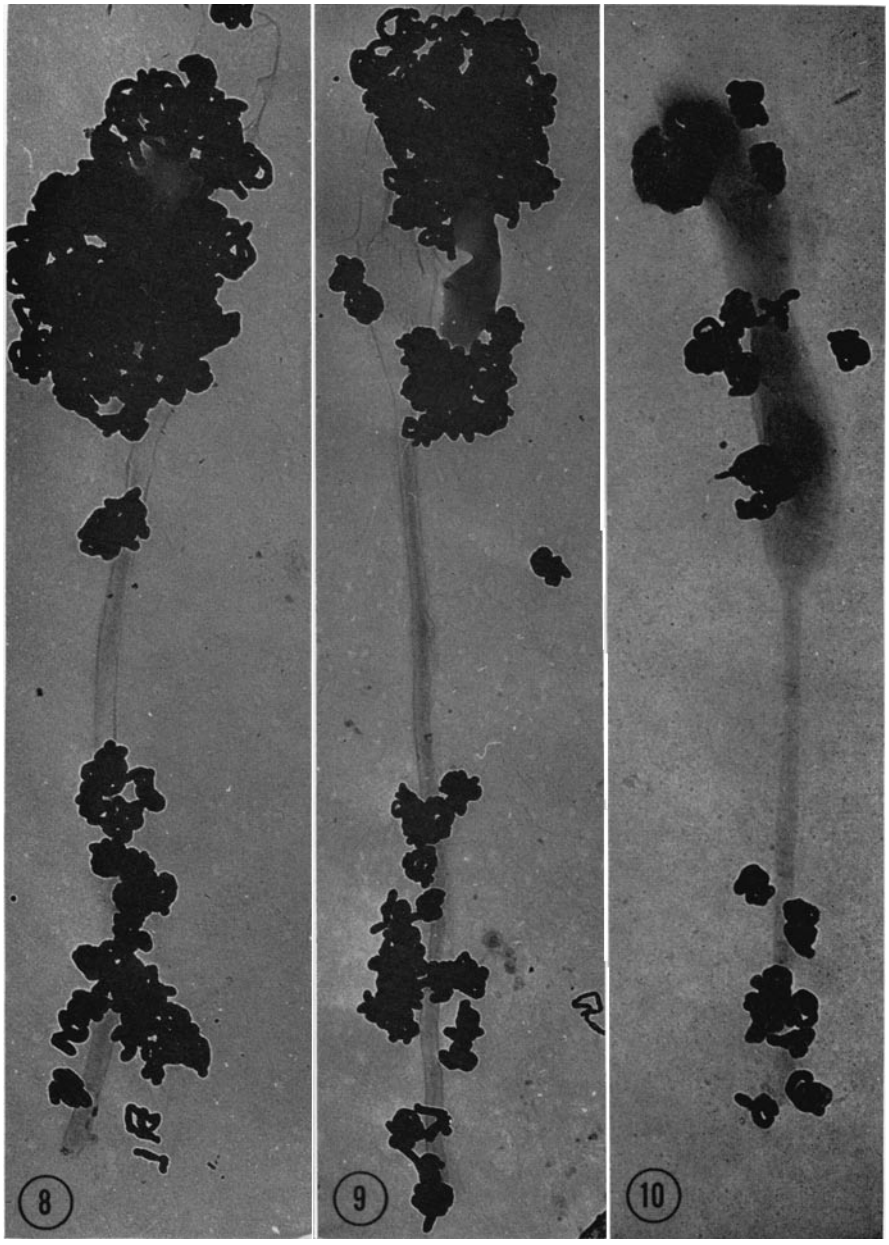


FIGURE 7 A semidiagrammatic representation of the possible patterns of radioactive labeling of stalked caulobacter cells resulting from experiments of type B (see text). The crosshatching indicates regions of radioactivity within the cell, and the arrows indicate the alternate hypothetical sites of synthesis of stalk material.

Control radioautographic experiments, similar in principle to those of type A, but in which the phosphate concentration was not limited during stalk growth, were performed in order to determine whether the site of stalk development is modified by phosphate starvation. A short pulse of tritiated glucose was given swarmers of *C. crescentus* during the initiation of stalk formation in medium G-I furnished with 10^{-3} M phosphate. The cells were then washed and stalk development was allowed to proceed in the same medium without tritiated glucose, until the cells were about to undergo their first division. The cells were then prepared for radioautography. If the site of stalk development occurs at the base of the cell, as indicated by the experiments conducted under conditions of phosphate limitation, a deposition of

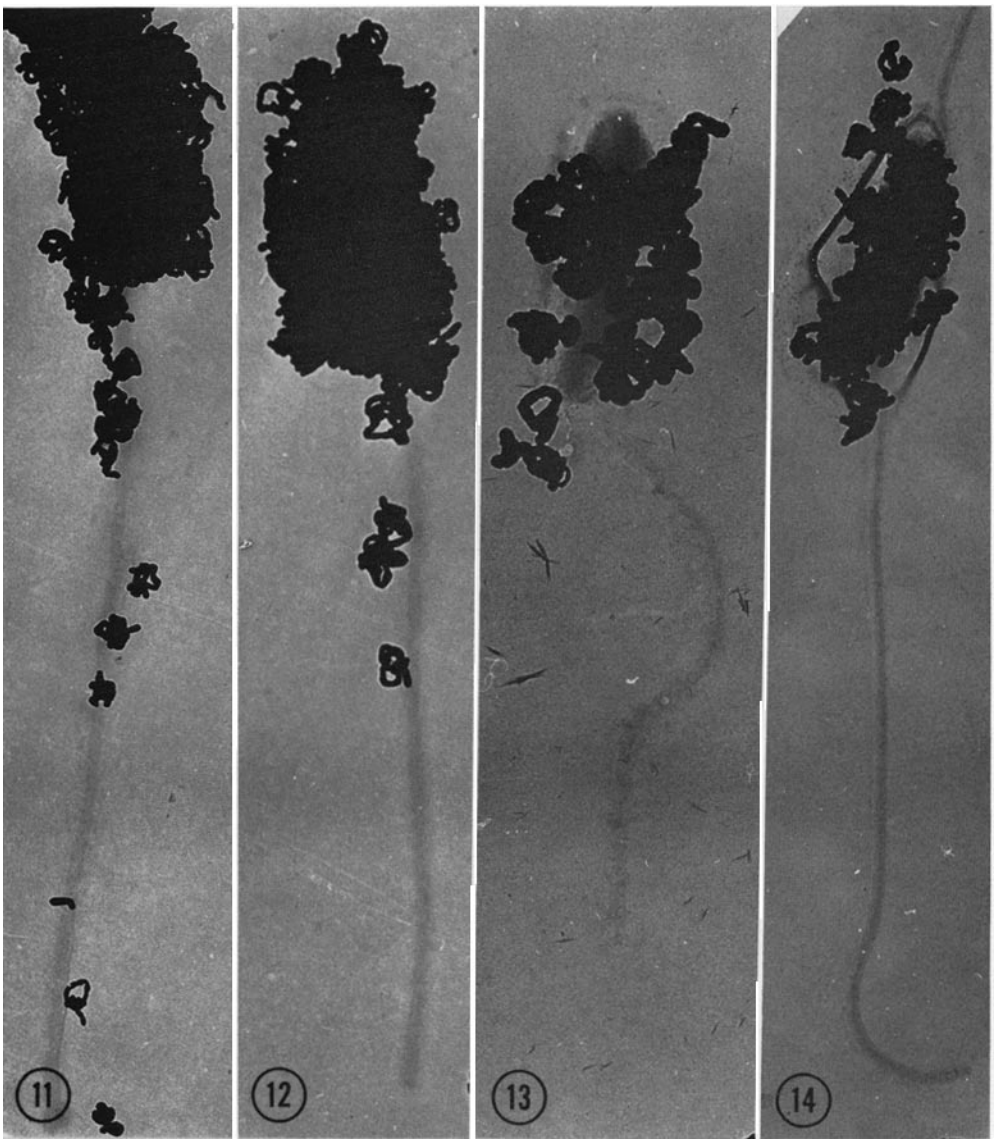


FIGURES 8 to 10 Radioautographs of *Caulobacter crescentus* CB15, obtained from experiments of type A.

FIGURE 8 Experiment Ia. × 20,000.

FIGURE 9 Experiment Ia. × 17,500.

FIGURE 10 Experiment II. × 17,500.



FIGURES 11 and 12 Radioautographs of *Caulobacter crescentus* CB15, obtained from experiments of type B.

FIGURE 11 Experiment Ib. × 15,000.

FIGURE 12 Experiment Ib. × 15,000.

FIGURES 13 and 14 Radioautographs of *Asticcacaulis excentricus*, obtained from an experiment of type B.

FIGURE 13 Experiment VII. × 15,000.

FIGURE 14 Experiment VII. × 12,000.

label in the terminal region of the stalk could be expected to occur in such a control experiment. The two experiments of this nature that were performed (IV and VI, Table IV) confirmed the site of stalk development previously demonstrated by analogous experiments with phosphate-limited cells. Labeling was very light in experiments IV and VI; but where labeling did occur in the stalk, it was localized in the terminal region.

The labeling patterns shown in Figs. 8 to 14 were consistently obtained in each type of experiment. These figures are, accordingly, representative of a much larger number of radioautographed cells actually observed.

TABLE V

Average Grain Distributions over Different Regions of Caulobacter crescentus Cells, Measured on Radioautographs from Experiments of Types A and B

Type of experimental design	Exp. No.	Length of exposure	No. of cells counted	Average No. of grains		
				Cell	Proximal half of stalk	Terminal half of stalk
		wk				
A	II	5	50	6	0.1	1.9
A	II	8	46	11.5	0.3	3.2
B	V	5	12	3.5	1.4	0.1
B	V	7	14	7.2	2.3	0.3

Spheroplast Formation

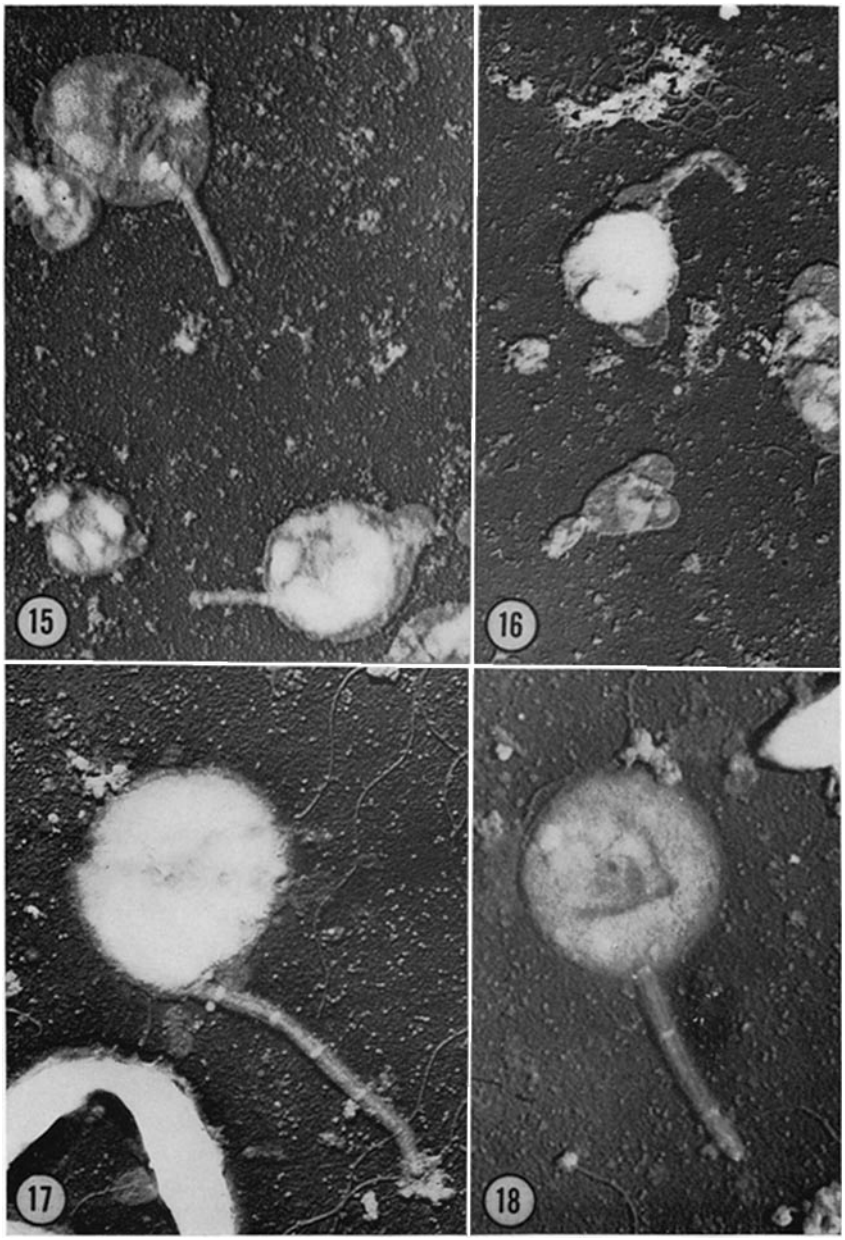
Lysozyme has been widely used to produce spheroplasts of Gram-negative bacteria (19). The chemical site of lysozyme action is the $\beta(1\rightarrow4)$-glycosidic link between *N*-acetylglucosamine and *N*-acetylmuramic acid units of the murein component in the cell wall (20). In *Escherichia coli*, the walls of which have been exhaustively studied from a chemical standpoint (21), the murein component constitutes the innermost wall layer, termed the R layer by Weidel, Frank, and Martin (22). It is overlain by additional layers of different chemical composition, consisting principally of lipoprotein and lipopolysaccharide. This chemical model, largely derived from the work with *E. coli*, appears to be descriptive of the walls of Gram-negative bacteria in general. The R layer can be visualized in thin sections of Gram-negative cells as a thin, electron-opaque line, 20 to 30 A thick. Murray, Steed, and Elson (23) have demonstrated by the electron microscopic study of thin sections that lysozyme does in fact impair the integrity of the R layer, thus confirming the earlier suggestion of Weidel, Frank, and Martin (22) that this layer is responsible for the rigidity of the cell wall.

Grossly speaking, the continuity of the cell wall with the wall of the stalk of caulobacters is firmly established (3, 5); but it is not yet entirely clear that *all* layers of the wall extend around the core of the stalk. In particular, the R layer, difficult to visualize even in thin sections of cells as a result of its extreme thinness, is exceedingly difficult to trace in the stalk region. If the characteristic form of the stalk is, in fact, maintained by its enclosure within a continuous murein layer extending from the cell proper, lysozyme treatment could be expected to produce structural alteration in the stalk, in addition to converting the cell itself to a spheroplast.

At pH 7.8 in the presence of a trace of Versene and an osmotic stabilizer (polyethylene glycol), cells of *C. crescentus* were converted to spheroplasts by lysozyme within 3 min. Examined at this point, almost all the cells had the characteristic form of spheroplasts, but the stalks retained their normal appearance (Fig. 15). A somewhat longer period of lysozyme action was required to produce microscopically observable changes in stalk structure; but after 10 min, the stalks had broadened considerably and lost their rigid outlines (Fig. 16). Accordingly, lysozyme does affect the structure of the stalk, although these effects do not become evident so rapidly as those on the cell proper. Hence, it can be concluded that the stalk contains a rigid murein layer. The longer exposure to lysozyme required to produce evident stalk damage probably reflects the fact that the stalk wall, with its cross-bands, is a stronger structural entity than the cell wall. Furthermore, the membranous core of the stalk may exert less pressure on the weakened wall than do the contents of the cell.

Penicillin is also an agent that can convert Gram-negative bacterial cells to spheroplasts (24). In contrast to lysozyme, it does so not by active destruction of the murein layer, but by inhibition of its continued synthesis in growing cells (20, 23). The action of penicillin on stalked caulobacter cells is, therefore, of particular interest, since it should in principle reveal by its effects on structure the sites (if any) of active murein synthesis in the stalk.

In populations of *C. crescentus* exposed to peni-



FIGURES 15 and 16 Lysozyme spheroplasts of *Caulobacter crescentus*, shadowed with uranium.

FIGURE 15 After exposure to lysozyme for 3 min. × 18,000.

FIGURE 16 After exposure to lysozyme for 10 min. × 15,000.

FIGURES 17 and 18 Penicillin spheroplasts of *Caulobacter crescentus*, shadowed with uranium.

FIGURE 17 × 24,000.

FIGURE 18 × 24,000.

cillin during growth in an osmotically stabilized medium, many stalked spheroplasts were observed, and the structure of the stalk was invariably normal (Figs. 17 and 18). This observation shows that the stalk itself does not contain active sites of murein synthesis, and, therefore, concords with the results of the radioautographic experiments which indicate that new stalk material in its entirety is synthesized at the juncture of the stalk with the cell.

DISCUSSION

Radioautographic studies on strains of *Caulobacter crescentus* and *Asticcacaulis excentricus*, representative of the two principal groups of bacteria capable of forming cellular stalks, have shown that the synthesis of all the components of the stalk takes place in a sharply localized region, at or very close to the juncture of the stalk with the cell. Experiments of a different nature, using lysozyme and penicillin as analytical tools, have shown that the core of the stalk is completely enclosed by an extension of the rigid murein layer of the cell wall, and that the murein layer of the stalk is not a region of murein synthesis. The latter observation confirms, for a particular component of the stalk, the general conclusion that emerged from the radioautographic studies.

These facts show that all the enzymes operative in the polymerization of the various structural components of the stalk—the successive layers of the enclosing cell wall, as well as the membranous core—are concentrated in the cell at the site of stalk outgrowth. Earlier fine structure studies (5) had shown that this site in the caulobacter cell is occupied by a membranous organelle continuous with the membranous core of the stalk. The ribosomal region is accordingly excluded from the site of origin of the stalk by the presence of this organelle. The profile of this organelle in thin sections (5) had already suggested that it does not have the same structure as mesosomes, which also occur in caulobacter cells. The distinctness of these two kinds of procaryotic organelles has now been confirmed by the demonstration that, when phosphotungstate is applied to intact cells, it can readily penetrate into the infoldings of the mesosomes, but cannot penetrate into the stalk organelle (7). This implies that, whereas the mesosome represents a complex infolding of the cytoplasmic membrane, as originally suggested by Fitz-James (25), the stalk organelle does not, and must have a different plan of construction.

The membranous organelle at the base of the stalk has a close topological correspondence with the site of stalk synthesis, inferred from the present radioautographic study. It is, therefore, probably the cellular element which houses the enzymes mediating the ultimate reactions that lead to the synthesis of the stalk.

The synthesis of the cell wall in certain Gram-positive bacteria has been shown by immunofluorescence techniques to occur at the equator of the growing cell; there is no detectable intercalary growth (26). The first such experiments with a Gram-negative bacterium, *Salmonella typhosa*, appeared to show intercalary growth (27), but more recent studies with a closely related organism, *Escherichia coli*, in which slightly modified techniques were employed, revealed that the most active area of wall replication is at the equator of the cell (28). Moreover, the typical "rabbit ears" forms developed by cells of *E. coli* and other Gram-negative bacteria during the course of their conversion to spheroplasts by penicillin (19) suggest that at least the murein layer of the wall is actively laid down in the equatorial plane. If we assume that such is the case in caulobacters, it follows from the cellular developmental cycle characteristic of these bacteria that wall synthesis occurs at two points, separated both in space and in time. In effect, one of the distinctive biological features of a caulobacter as opposed to a simple unicellular true bacterium of similar general cell structure is the existence of an additional site of wall synthesis at the point of development of the stalk. As shown diagrammatically in Fig. 1, full stalk development in a swarmer occurs prior to its division; and the basal cell resulting from this division retains the stalk through all subsequent divisions, without further significant elongation. This implies that in the caulobacter cell two different control mechanisms govern wall synthesis: one operative in stalk formation and one operative in the elongation and eventual binary division of the cell. It is in this context that the morphogenetic consequences of phosphate limitation are of particular interest. Until cells are in extremis as a result of phosphate starvation, and have ceased net synthesis of cell materials, they retain a form indistinguishable from that of cells growing in the absence of phosphate limitation.

However, marked hypertrophy of the stalk occurs at a much earlier point in the growth of a phosphate-limited culture, while cell division is still proceeding in an apparently normal fashion. Evidently, therefore, phosphate limitation derepresses synthesis of wall material at the site of stalk formation, but does not detectably modify the regulation of wall synthesis leading to cell division.

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