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BIOLOGICAL PROPERTIES AND CLASSIFICATION OF THE *CAULOBACTER* GROUP[1]

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INTRODUCTION

The genus *Caulobacter* was proposed in 1935 by Henrici and Johnson (26) as one of the constituent groups of a new order of stalked bacteria, Caulobacteriales. Henrici and Johnson discovered many different types of stalked bacteria attached to microscope slides which had been submerged in a fresh-water lake. The slides were rinsed with water, and the bacteria remaining firmly attached to the slides were observed in wet mounts or after the slides had been dried and stained. *Caulobacter*, as defined by Henrici and Johnson, comprises gram-negative unicellular stalked bacteria, the stalks of which arise from one pole of the cell. The cells are vibrioid, rod-shaped, or fusiform, and multiply solely by transverse binary fission. Fission in some instances is symmetrical, with each of the sibling cells bearing a stalk on the older pole. Certain cells were seen to divide asymmetrically, with only one of the siblings having a stalk. A small swelling was observed at the distal end of the stalk and was interpreted as a holdfast.

Henrici and Johnson did not succeed in isolating caulobacters. However, they could maintain these organisms as components of the microflora in crude liquid cultures containing cellulose or chitin, inoculated with lake water.

Although recognition of *Caulobacter* dates from the work of Henrici and Johnson in 1935, there had been two earlier descriptions of bacteria which Henrici and Johnson considered to be clearly assignable to this group. In 1905, Jones (35) reported the isolation of a peculiar motile vibrioid bacterium. It possessed a "flagellum" which was readily stained with ordinary dyes, and the cells frequently occurred in "chrysanthemum-like clusters," or rosettes. On the basis of Henrici and Johnson's observations on the structure of *Caulobacter*, Jones' rosettes can be interpreted as clusters of stalked cells attached to each other in groups by means of their holdfasts. Omeliansky (54) also observed rosettes in an isolated colony of another vibrioid *Caulobacter* obtained from river water. Although he did not observe motility, he described the appendage as a large flagellum; for this reason he gave the organism the name *Bacillus flagellatus*.

The failure of Henrici and Johnson's attempts to isolate stalked bacteria appears to have discouraged further work on these organisms; in any event, 15 years elapsed before the next publication on *Caulobacter* appeared. In 1950, Houwink and van Iterson (32) reported the fortuitous discovery of stalked bacteria in electron microscopic preparations. They traced the source of these stalked bacteria to the water which had been used to prepare specimens for electron microscopy, and Houwink (29, 31) succeeded in isolating several strains from tap water and also from canal water.

Houwink was the first to study the structure of these bacteria by electron microscopy, and he made several important discoveries. He observed that the nonstalked cell arising by the division of a stalked cell possesses a single flagellum at the older pole. This cell grows into a typical stalked cell by developing a stalk at the flagellated pole of the cell, as evidenced by the occasional presence of a flagellum at the distal end of the stalk. This observation suggested to Houwink that the core of material present in the stalk, which is readily observable in electron micrographs of shadowed specimens, and the surrounding wall are part of the bacterial cell. Rosettes occurred in his pure culture, which supports Henrici and Johnson's presumptive identification of Jones' and Omeliansky's organisms as *Caulobacter*. The appearance of rosettes as seen by electron microscopy justifies the interpretation of Jones' rosettes presented above.

Bowers et al. (2) isolated a stalked bacterium (from well water) which they identified as *C. vibrioides* Henrici and Johnson. Nutritional studies (23) revealed the organism to be a strict

aerobe which required riboflavine for growth in a glucose-ammonium salts medium. Electron microscopy of this isolate confirmed the findings of Houwink regarding the mode of flagellation of the nonstalked cell and the probable cytoplasmic nature of the core of the stalk.

Shortly after the work of Bowers et al., Hund and Kandler (33) published a nutritional study of one of Houwink's isolates. They could not grow this strain in a defined medium and were unable to show a requirement for riboflavine.

The ability of caulobacters to attach to other microorganisms has been observed by several workers (29, 33, 36, 77). Houwink (29, 31) suggested that such attachment allows the caulobacters to parasitize the cells to which they are attached, but he did not report convincing evidence for this contention. An investigation of parasitism by Hund and Kandler (33) provided suggestive evidence that *Bacillus* cells are affected by the attachment to them of caulobacters; however, these workers were not able to demonstrate that such attachment confers on the stalked bacteria any particular nutritional advantage.

The investigations of pure cultures of *Caulobacter* since 1951 have provided only a small amount of information about caulobacters as a group. Only one of the three morphological types described by Henrici and Johnson, viz., the vibrioid type, had been isolated. The limited physiological studies on this type revealed that sugars and amino acids can serve as carbon sources, that nitrate may be reduced to nitrite, and that organic growth factors may be required. Evidence had been presented suggesting that one of the isolates is capable of parasitizing other bacteria.

The electron microscopic studies revealed that the stalk is an outgrowth of the cell, and that the swarmer cell is motile by means of a single polar flagellum. Beyond the observations that cell division is asymmetrical, one swarmer and one stalked cell arising at each division, and that the stalk arises at the site of extrusion of the flagellum, the alternation between the two forms of *Caulobacter* cells had not been investigated.

During the present studies, several morphological types of caulobacters were isolated from a variety of sources, although the large majority of the isolates are from fresh water. These isolates and several strains provided by other workers have been used for a study of the group, with particular regard to gross morphology and fine structure, the life cycle, the property of adhesiveness, and physiological properties. On the basis of these observations, the descriptions of Caulobacteraceae and *Caulobacter* are revised, and a new genus of bacteria with cellular stalks is proposed.

These studies certainly have not answered all the outstanding questions concerning caulobacters. However, it is hoped that they will at least serve as background for further investigations of these bacteria, whose peculiar structure and dimorphism provide a valuable system in which the developmental processes of microbial cells may be studied, and whose physiological properties present an interesting problem in microbial ecology.

OCCURRENCE OF CAULOBACTERS

The organisms isolated in pure culture and identified as *Caulobacter* were obtained from fresh water—river water (35, 54), canal water (31), well water (2), pond water (68), and tap water (29). Occasionally, *Caulobacter* cells have been found fortuitously in electron microscope preparations (6, 32, 74, 30; *see also* 66); this can be attributed to the frequent presence of these organisms in distilled water which is used in the preparation of the specimens. Leifson (43) has isolated several strains from distilled water.

However, these bacteria are not restricted to the environments mentioned above; they also occur in seawater (this paper), soil (53; this paper), and the intestinal tract of millipedes (this paper).

PROCEDURES FOR ISOLATION OF CAULOBACTERS

The majority of strains isolated during the present studies were obtained from fresh water. We owe to Houwink (29) the basis for the technique which has been found generally suitable for the enrichment of caulobacters in water samples. Isolation from other sources requires different procedures. In each procedure, caulobacters are isolated under conditions suitable for growth of aerobic heterotrophic bacteria. Brief attempts to enrich caulobacters in anaerobic cultures have not been successful.

Caulobacters From Freshwater

Samples from the surfaces of ponds, streams, and a freshwater lake and from tap water were allowed to stand at room temperature (20 to 25 C) in bottles or flasks loosely covered with paper or aluminum foil. When peptone was added to a concentration of 0.01%, a surface film of bacteria, fungi, and protozoa developed rapidly, appearing quite heavy within 2 days. The surface film was examined daily in phase-contrast; the proportion of bacteria which were stalked usually reached a maximum on about the fourth day of incubation of the samples. In these developing films, the stalks were very long, sometimes reaching a length of more than 20 μ. Caulobacters were observed as individual cells occurring randomly among the microflora of the films, usually in the clumps of bacteria and frequently attached to other cells.

When caulobacters occurred in a relative proportion of about 1 in 10 or 20 cells, a sample of the surface film was streaked on 0.05% peptone-tap water medium containing 1.5% agar, and the plates were incubated at 30 C. The use of a dilute peptone medium for primary plating was essential to prevent complete overgrowth of caulobacter colonies by other organisms. Even on the dilute plating medium, other bacteria grow more rapidly than the caulobacters. The following procedure for isolation from the first plates was adopted. After 4 or more days of incubation, the plates were examined with the aid of a dissecting microscope; microcolonies thus detected were picked with sterile toothpicks and transferred as patches to plates containing the standard medium (tap water with 0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% agar). Macroscopic growth occurred in the patches within 2 days. Wet mounts were prepared from the patches and examined in phase-contrast for the presence of stalked cells. Usually 50% or more of the patches consisted of caulobacters. The isolates were purified by streaking from the growth in the patches on the standard medium.

The experience gained in the present work suggests that the following factors are important in isolating caulobacters from fresh water.

(i) Source: Water with a low content of organic material is most suitable. This favors the development of caulobacters in fairly high proportion, since the total bacterial content of such water is low. For this reason, tap water is usually a good source, or pond water after a heavy rainfall.

(ii) Nutrients: The addition of peptone to the water sample to give a final concentration of 0.01% hastens the enrichment of caulobacters. If water containing caulobacters is not enriched with peptone, but is allowed to stand for 2 months or more, the surface film will contain only a few organisms that are not caulobacters, frequently including the budding bacterium *Hyphomicrobium*.

(iii) Conditions of incubation: The peptone-enriched water sample should be incubated in contact with air without agitation to permit the development of a surface film of aerobic organisms in which caulobacters accumulate. If the sample is incubated with continuous agitation, the proportion of caulobacters is never sufficient to permit isolation by direct plating.

(iv) Detection: The recognition of *Caulobacter* cells in enrichment cultures is dependent upon microscopic detection of the characteristic stalks. This presents a technical problem due to the small diameter (ca. 0.2 μ) of the stalk, which is, therefore, close to the limit of resolution of the ordinary light microscope. In practice, stalks cannot be seen on individual living cells by ordinary illumination. However, they can be detected by phase-contrast microscopy, the technique used for all our observations of living cells. As an alternative to phase-contrast microscopy, stains can be used. The stalks are made visible in wet mounts by the addition of an equal volume of Hucker's crystal violet to the sample to be mounted. When the cells have been dried on a slide, the use of a mordanted stain is necessary; we have found Gray's flagella stain (63) satisfactory.

(v) Isolation: When the stalked bacteria have reached a proportion in the surface film which should allow them to develop as isolated colonies on a streaked plate, material from the surface film should be suspended in a small volume of sterile water, agitated to disperse the clumps of cells, and streaked on dilute peptone medium containing at least 1.5% agar. The small colonies should be removed from the plate as soon as they are visible in the dissecting microscope and patched on standard medium containing only 1% agar. After detection of caulobacters by microscopic examination of growth in the patches, pure

cultures can be obtained by restreaking on the same medium.

Caulobacters From Seawater

The seawater used successfully as a source of *Caulobacter* had been cleared by filtration and stored for use in a marine biological laboratory. Samples of the stored seawater were allowed to stand at 13 or 19 C. Casein hydrolysate, peptone, or yeast extract (0.01%) was added to some of the samples, and a duplicate of each enrichment was diluted with an equal volume of sterile tap water.

Caulobacters grew so slowly in the surface films that plates were streaked only after 7 days of incubation of the enriched samples. The peptone enrichment in full strength seawater incubated at 13 C contained the highest proportion of stalked bacteria at 7 days; therefore, the medium used for streaking was seawater containing 0.05% peptone and 1% agar. The plates were incubated at 25 C. Colonies were transferred to the peptone medium as patches, and between 30 and 40% of the patches from small colonies consisted of caulobacters. These were purified by streaking on peptone-seawater-agar plates.

The highest proportion of caulobacters was obtained in a sample of seawater diluted with an equal volume of tap water and incubated for 5 months at 19 C without added organic material. At this time, material from the surface film was streaked on casein hydrolysate (0.05%)-peptone (0.05%)-agar (1%)-seawater plates, and about 60% of the colonies consisted of caulobacters. These had evidently survived the many other forms of bacteria present in the surface film during the first few weeks of incubation.

Caulobacters From Soil

Caulobacters were detected in colonies arising on mineral-agar plates (medium of Zavarzin, 75) containing nitrate as the nitrogen source and exposed to air and methanol vapor. The plates had been streaked from a suspension of soil in a mineral-methanol medium which was to serve as an enrichment culture for *Hyphomicrobium*. The caulobacters were purified by streaking on the standard fresh-water medium, on which the colonies grew more rapidly and to a larger size.

Caulobacters From Millipedes

As observed and figured by Leidy in 1853 (41), the trichomycetous fungi that inhabit the gut of the millipede bear tufts of smaller microorganisms. On the suspicion that these tufts might be stalked bacteria, two California millipedes were dissected, and the contents of the hind-gut were examined microscopically. Stalked bacteria were indeed present, both free in the lumen and attached to the fungi, although their relative numbers were small. When washed pieces of fungi from one millipede were spread on plates of dilute peptone medium, the only bacteria which formed colonies were *Bacillus* species and one type of *Caulobacter*. Evidently, most of the microorganisms in the gut were strict anaerobes, so that the ability of the caulobacters to grow aerobically permitted their isolation despite their numerical inferiority. Caulobacters did not appear in anaerobic cultures inoculated with material from the gut. Pieces of fungal hyphae from the gut of the second millipede yielded on plating a larger number and variety of aerobic bacteria, and only one isolated *Caulobacter* colony was found.

MAINTENANCE OF ISOLATED CAULOBACTERS

Most of the caulobacter isolates are easily maintained on slants of the standard medium, and some can be maintained on glucose-mineral-agar slants. For certain isolates, the standard medium must be supplemented with riboflavine. The cultures are transferred every 5 weeks, incubated 2 to 3 days at 30 C, and then stored at 4 C until the next transfer. The caulobacters can also be stored for an indefinite time in a lyophilized state.

The marine isolates are maintained on slants of 0.1% peptone-seawater medium with 1% agar and incubated at 25 C. The marine forms remain viable at 4 C longer than the other isolates, and are transferred every 5 weeks only for the sake of convenience.

SOURCES OF ISOLATED CAULOBACTERS

Each individual colony identified as caulobacter arising on the initial plate streaked from a sample or enrichment culture has been maintained as an isolate to which a strain number is assigned. Thus, certain groups of strains in the author's collection may have been derived from a common ancestral individual within only a few generations prior to their isolation. Such strains have proven identical or nearly so in the characteristics determined in this study; accordingly,

only one strain of each such group is recognized as a unique isolate.

The source and date of sampling for each isolate are presented in Table 1. The isolates which were provided by other workers and were included in the present studies are also listed.

TABLE 1. *Sources of Caulobacter isolates**

Strain no.	Source	Sampling date	Isolated by
CB1, 2	Tap water	July, 1959	Author
CB4, 5	Pond water, SW	June, 1959	Author
CB6, 7	Lake water	June, 1959	Author
CB8, 9	Tap water	June, 1959	Author
CB10, 11, 13	Pond water, SC	August, 1959	Author
AC12	Pond water, SC	August, 1959	Author
CB15–18	Pond water, SE	January, 1960	Author
CB21, 23–29, 31, 35, 36	Pond water	February, 1962	Bacteriology class
CB37	Millipede hind-gut	March, 1962	Author
AC47, 48	Pond water, NC	October, 1962	Author
CB51, 57, 63	Pond water, NC	October, 1962	Author
CB65, 70, 79, 81, 82, 86	Pond water, SE-a	October, 1962	Author
CB66, 83	Pond water, SE-b	October, 1962	Author
CB88, 89	Soil	November, 1962	M. Macpherson
CB91	Stream water	February, 1963	Author
CB93	Millipede hind-gut	April, 1963	Author
CB-G	Well water, Kentucky	ca. 1953	Bowers et al.
CB-H	Tap water, Delft	ca. 1951	A. L. Houwink
CB-R	Contaminated *Chlorella* culture, Moscow	ca. 1960	G. A. Zavarzin
KA1–4	Pond water-tap water mixtures	1953	B. J. Bachmann
KA5, 6	Pond water-tap water mixtures	1954	Bacteriology class
CM11, 13	Filtered seawater	February, 1963	Author

* Sources of isolates obtained by the classes, Dr. Bachmann, and the author are in California. Abbreviations refer to various ponds.

GROSS MORPHOLOGY OF CAULOBACTERS

Gross Morphology of Typical Caulobacters (Caulobacter)

In electron micrographs of shadowed preparations of whole cells, Houwink (29, 31) observed that the outer surface of the stalk is continuous with the cell wall proper, and that this surface encloses a slender core of electron-dense material. His micrographs reveal substructures spaced irregularly along the length of the stalk, apparently embedded in the wall. In some of the stalks, these "Querbalken" are absent. The "button-like holdfast" is observable as a small mass of extracellular material without definite shape at the distal end of the stalk. The holdfast is not a constant feature, its position on some cells being occupied by radial filaments.

The core of the stalk is obvious in the unshadowed specimens of Bowers et al. (2), and in one preparation it extends from the cytoplasm of the cell to the distal end of the stalk. The holdfast is inhomogeneous, containing very electron-dense inclusions.

The present microscopic studies of whole cells have revealed that the characteristics of external structure of vibrioid caulobacters as described by previous investigators are shared by several morphological types. These types differ in cell shape, as was predicted by the observations of Henrici and Johnson (26); a secondary difference is in the frequency of "Querbalken" in the stalks. The type species, *Caulobacter vibrioides* Henrici and Johnson, possesses a comma-shaped cell. The stalked cell appears to give rise on division to a cell without a stalk; Henrici and Johnson suggested that the new cell would swim or float away and eventually find a substrate on which it could

settle and develop its stalk. This is the only morphological type which had been studied in pure culture, including the presumed *Caulobacter* isolate of Jones (35).

The second type described by Henrici and Johnson possesses a rod-shaped cell with rounded ends; two subtypes were recognized, in one of which the stalks are shorter and the holdfasts more obvious than in the other. Stalked cells of this type also give rise to nonstalked cells at division. The third type comprises stalked bacteria with fusiform cells. Division in this type apparently gives rise to two identical cells, since the outer cell develops a stalk before separation from the basal stalked cell is completed. This last type was usually observed to have formed extensive microcolonies on the slides, attributed by the authors to the absence of a nonsessile stage.

In the present studies, five principal morphological types were isolated. These include the *vibrioid* and cylindrical (*bacteroid*) types described by Henrici and Johnson. Strains representative of Henrici and Johnson's group of stalked bacteria with fusiform cells were not obtained, although such cells were seen on one occasion in the surface film of an enrichment culture. This type was not found on microscope slides submerged in enrichment cultures. Several strains of a similar type have been isolated. The cell is tapered, but its long axis is not curved. Unlike Henrici and Johnson's type, however, all of the *fusiform* isolates have a motile stage.

The bacteroid isolates comprise two subtypes differentiable on the basis of cell dimensions. Cells of one group are long and slender, approximately 1.5 to 2.5 μ in length when not dividing, and less than 0.5 μ in diameter. The cells of the other group are shorter and thicker, being approximately 1 μ in length when not dividing and 0.6 μ in diameter.

The two remaining principal types are not readily categorized. They share with the vibrioid type the characteristic of a curved long axis. In one type, the cells are plump and rounded and appear lemon-shaped. This cell shape is designated *limonoid*. Cells of the isolates received from Dr. Grula and Dr. Houwink are of this type, as are cells of five of the author's isolates. The fifth type is represented by cells whose shape is intermediate between vibrioid and fusiform. The cell is tapered and usually slightly curved; some of the cells in a growing culture appear vibrioid, whereas others are not detectably curved. This type has been designated *subvibrioid*.

It must be noted that the morphological features of an isolate do not always remain constant. Continued cultivation of isolates on artificial media occasionally results in morphological changes which are not accompanied by changes in physiological or cultural properties. This is particularly true of subvibrioid isolates, which tend to lose the curvature of the cell. This experience in the present work may have been due to the use of solid media for the maintenance of bacteria, the majority of which were isolated from aquatic environments. Also, the distinction between vibrioid and limonoid cells is dependent upon cultural conditions. Under adverse conditions, such as partial starvation (see cytological studies of Grula et al., 2, 22, 23), limonoid cells elongate and appear vibrioid, or when growth is slowed by toxic materials or high concentrations of sugars, vibrioid cells swell and appear limonoid. Hence, the descriptions are based on observations of liquid cultures prepared before the isolates had been subcultured more than a few times, and during the period of exponential growth at the maximal rate obtainable for the isolate.

Gross Morphology of Excentral Caulobacters (Asticcacaulis)

We have isolated a stalked bacterium which we originally described as an "excentral" type of *Caulobacter* (68). The cell is a thick, straight, nearly cylindrical rod with blunted or only slightly tapered poles. The stalk arises from one end of the cell, but is not necessarily a continuation of the long axis of the cell. Rather, it is frequently oriented at an acute or right angle to the cell axis. Its junction with the cell is not the center of the pole of the cell, and the flagellum of the swarmer stage also arises in this off-center position.

The most striking difference between the gross morphology of *Caulobacter* and of this organism is that the holdfast material does not occur at the distal end of the stalk. It is found at the stalked end of the cell, but at a site different from that at which the stalk develops. Consequently, cellular attachment does not involve the stalk.

We have found this organism to be distinct from other caulobacters in several respects other than gross morphology, and have concluded that it should not be recognized as a member of *Caulo-*

bacter. A separate genus, *Asticcacaulis*, is proposed to accomodate stalked bacteria of this type. The complete generic description is given later. Throughout this monograph, we shall use the trivial name, caulobacter, as a collective designation for members of both genera, *Caulobacter* and *Asticcacaulis*.

ILLUSTRATIONS OF GROSS MORPHOLOGY

The five principal morphological types of *Caulobacter* are illustrated in Fig. 1 to 5 in photomicrographs taken in phase-contrast. In the subsequent figures (6 to 10), electron micrographic illustrations of these types are shown. The appearance of the excentral caulobacter (*Asticcacaulis*) in phase-contrast and electron microscopes is illustrated in Fig. 11 and 12.

The photomicrographs were taken of cells growing in standard medium. For electron microscopy, cells were harvested by centrifugation, washed with water, and resuspended in water. Cells were allowed to settle on Formvar-coated copper grids, and the excess fluid was removed with an absorbent tissue. The electron micrographs are negative prints of uranium-shadowed specimens.

FINE STRUCTURE OF *CAULOBACTER*

An electron microscopic study of ultrathin sections of caulobacters was undertaken to examine the internal structure of bacteria which differentiate a peculiar external organelle. The studies of shadowed (2, 29, 31, 32, 36) and unshadowed (2) specimens had already suggested the complexity of internal structure of stalked cells, since the stalk: (i) is cellular, composed of cell wall and a core which is continuous with the cytoplasm of the cell; (ii) possesses substructures, viz., "Querbalken" and holdfast material; and (iii) is developed from a site on the cell which does not differ in external appearance from other areas, except that the flagellum is extruded at this site.

Accordingly, two strains were selected from different morphological types for this cytological study. The methods of fixation and staining were based largely on those of Ryter and Kellenberger (56). Cells were harvested from standard medium during exponential growth; they were prefixed, and fixed with osmic acid for 2 hr, suspended in agar, and stained with uranyl acetate. The stained specimens were dehydrated through a series of acetone solutions, embedded in Vestopal, and sectioned with a diamond knife on a Porter-Blum Ultramicrotome. The solutions were prepared in Veronal-acetate buffer (pH 6.0). The sections were poststained with lead hydroxide by the method of Millonig (51).

Representative sections of vibrioid strain CB2 are illustrated in Fig. 13. Within the cytoplasmic matrix of the cell are darkly stained granules, presumably ribosomes, which vary from 80 to 100 A in diameter. Light areas with the characteristic fibrillar appearance of nuclear material fixed by this procedure (37, 56) are enclosed within the cytoplasmic matrix. Electron-transparent areas occur in some sections; we interpret these as the sites where poly-β-hydroxybutyric acid had been deposited. This material is extracted during preparation of the cells for sectioning (7), leaving areas which are not stained.

Immediately surrounding the cytoplasm lies a unit membrane approximately 60 A thick, which we interpret as the cell membrane. Beyond this membrane there is a multilayered cortical structure similar to those which are observed in other gram-negative bacteria (see for examples: 8, 38, 56, 70). The outer two layers which are stained and a third unstained layer comprise a unit structure 70 to 85 A thick which seems to undulate over the surface of the cell; this wavy appearance may have resulted from irregular polymerization of the embedding material. The third cortical layer is approximately 40 A thick and is more regular in contour than the outer layers.

The internal structure observed in the sections of bacteroid strain CB11 (Fig. 14) is essentially the same as in CB2, except that the cortical layers of CB11 cells are somewhat thinner. The outer unit structure is 40 to 50 A thick, and the inner layer is 20 to 25 A. Nuclear material, ribosomes, and sites of poly-β-hydroxybutyric acid deposition can also be observed in sections of this strain.

In both strains, the cell membrane is seen to invaginate to form complex membrane intrusions similar to those found in many other bacteria, both gram-positive and gram-negative forms (15, 19, 20, 21, 25, 52, 56, 59, 69, 70, 71). In *Caulobacter*, these structures are often found in the area of constriction at which the separation of sibling cells is occurring. It is particularly significant that such a structure invariably appears in median sections of the stalked pole of the cell, and that the core of the stalk is composed of membranes con-

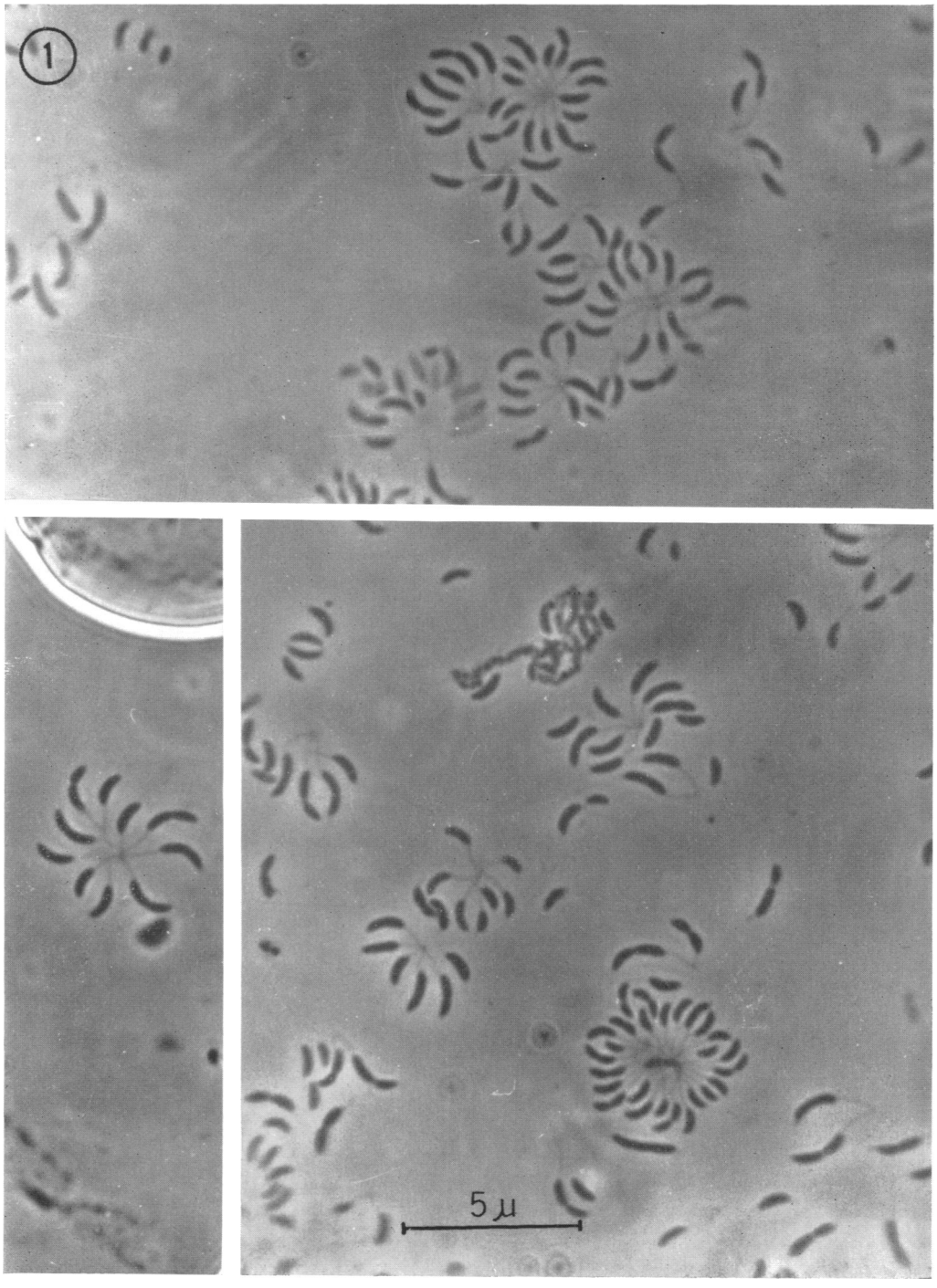

FIG. 1. *Vibrioid strain CB2. Phase contrast.*

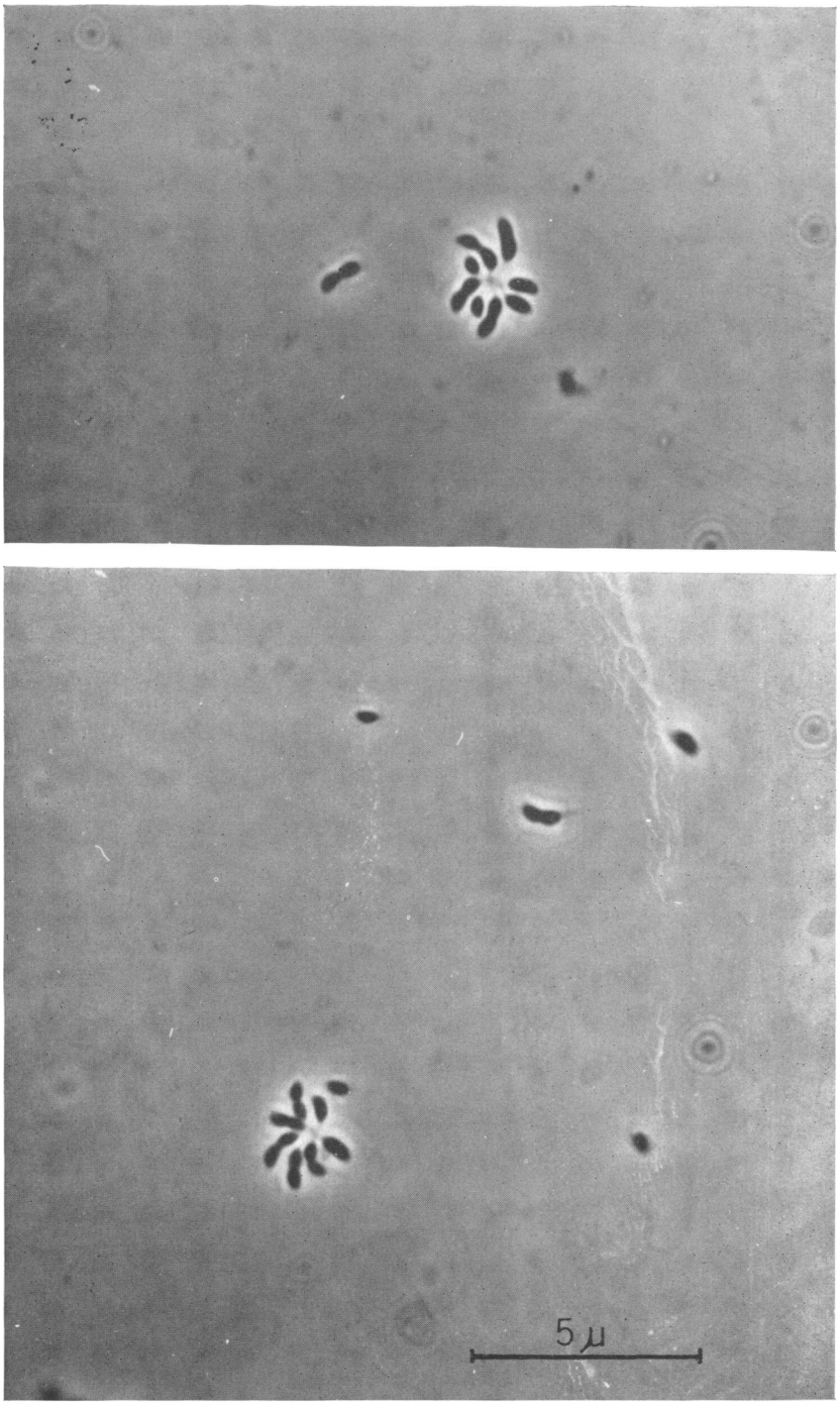

FIG. 2. *Limonoid strain CB-G. Phase contrast.*

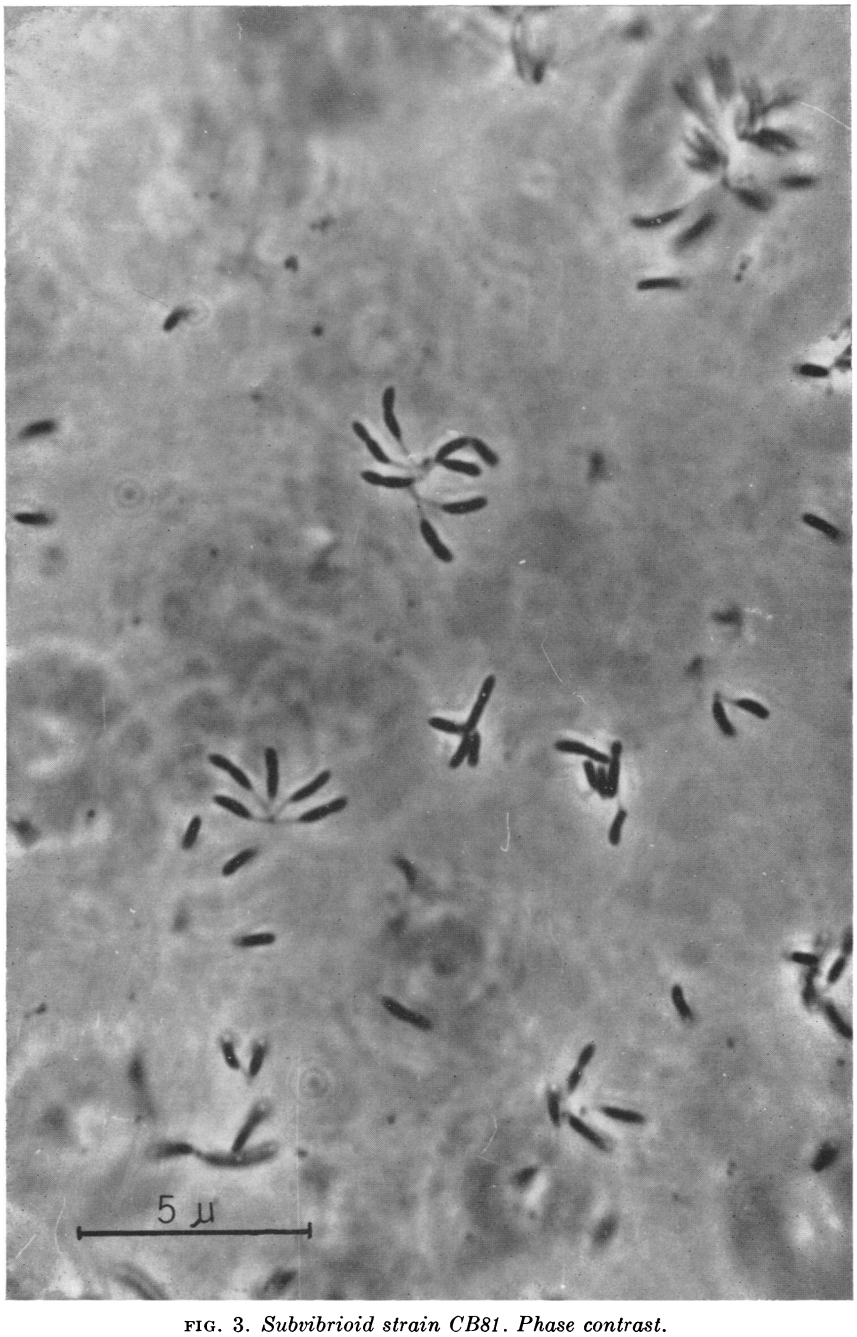

FIG. 3. *Subvibrioid strain CB81. Phase contrast.*

tinuous with the localized, polar membrane structure. Thus, the composition of the core of the stalk, as determined by examination of ultrathin sections, is predominantly membranous. This is somewhat different from the proposal of Bowers et al. (2) that the core is cytoplasmic, probably implying that the structures of the cytoplasmic matrix of the cell, such as ribosomes, would be found in the stalk; and distinctly different from the proposal of Zavarzin (76) that the core is the immobilized flagellum.

The wall of the stalk is continuous with that of

the cell. The even appearance of the outer unit structure in areas surrounding the stalk supports the interpretation of the cause of its wavy appearance around the cell, since the stalk would be more easily and evenly impregnated by the embedding material. The thick inner layer of the wall is readily observable in the juncture of cell and stalk. However, it is not obvious along the entire length of the stalk, apparently due to a progressive decrease in its thickness and its increasingly close association with the cell membrane along the stalk.

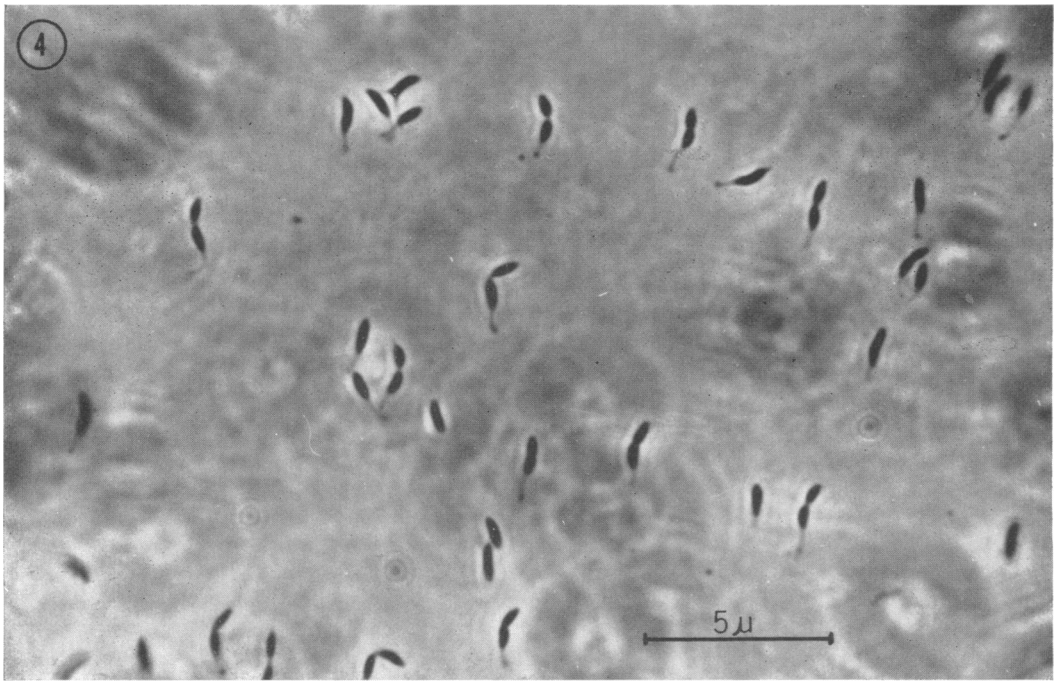

FIG. 4. *Fusiform strain CB27. Phase contrast.*

Within the wall of the stalk appear darkly stained areas of the size and location of the "Querbalken." These bands do not extend through the outer unit structure of the wall, nor do they interrupt the bounding membrane of the stalk core. Accordingly, we interpret them as annular structures which lie between the outer and inner layers of the wall.

The membrane structure associated with the stalk and from which the stalk core is derived is elaborated at the pole of the cell long before the stalk is differentiated. This is illustrated in Fig. 14, in which a section of a dividing cell reveals the older stalk membrane structure and a second such structure at the flagellated pole of the incipient swarmer cell. This is also illustrated in Fig. 13, in which the stalk of the sectioned cell has just begun to develop. The location of the holdfast material, which appears in the sections as a small mass of darkly stained spherical bodies 100 to 130 A in diameter, reveals that this is a section of a developing stalk, not a diagonal section of a fully developed stalk.

The sections of these two strains suggest that the membrane structures of *Caulobacter* are involved in the differentiation of the stalk, and possibly also in the formation of the flagellum and the holdfast. Further studies on the internal structure of caulobacters will be presented in a later paper (Poindexter and Cohen-Bazire, J. Cell Biol., *in press*).

RELATIONSHIP BETWEEN CELL DIVISION AND STALK DEVELOPMENT

The flagellated sibling of a stalked cell does not have a stalk; therefore, if the swarmer is to become a recognizable caulobacter cell, it must develop a stalk after cell division. Studies of stalk

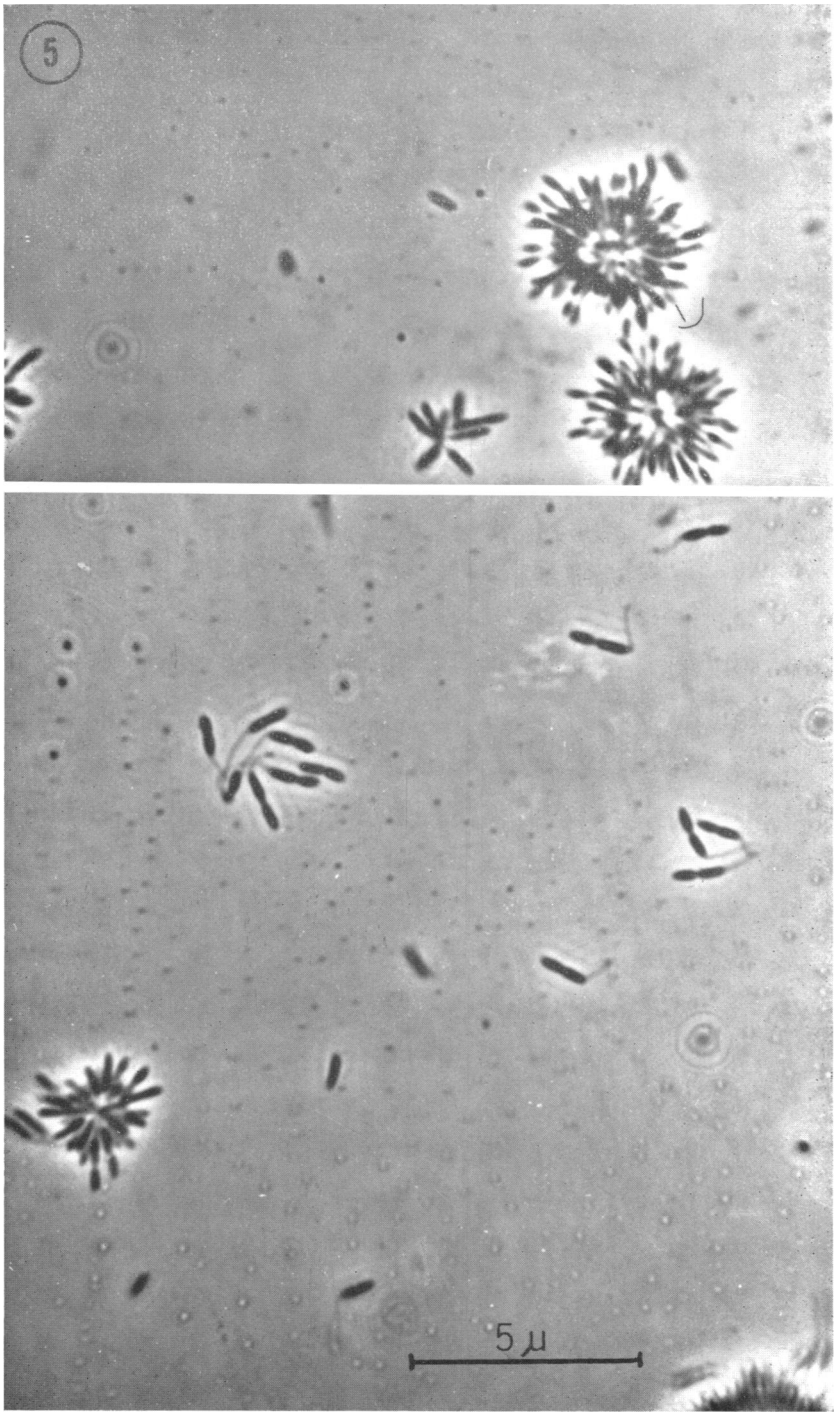

FIG. 5. *Bacteroid strain CB7. Phase contrast.*

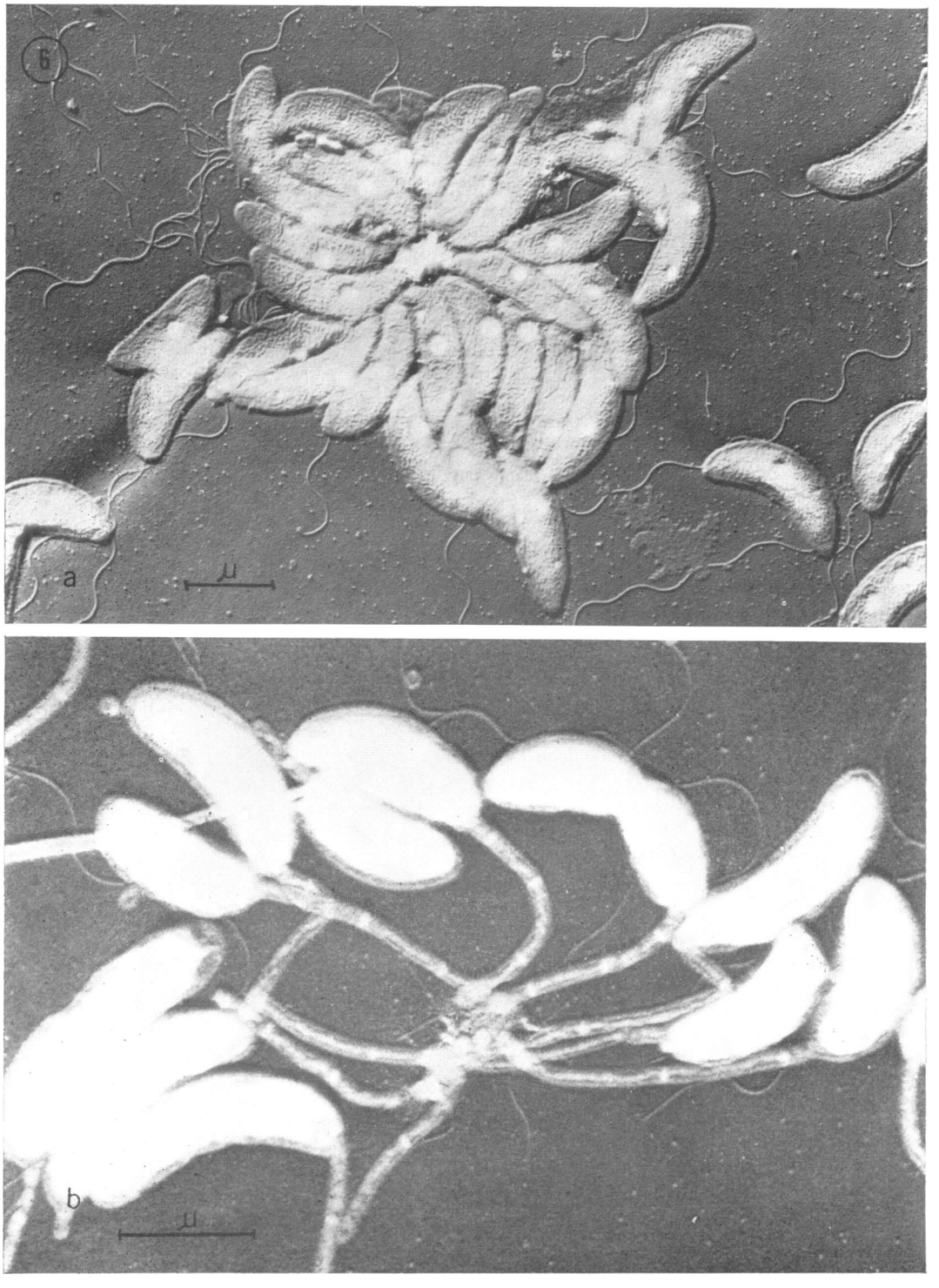

FIG. 6. *Vibrioid strain CB2. Electron micrographs. (a) Swarmer cells in a rosette, the cells adhering to the common mass of holdfast material (see Fig. 1 of reference 68). (b) Stalked cells in a rosette, the cells adhering to the common mass of holdfast material by the tips of the stalks.*

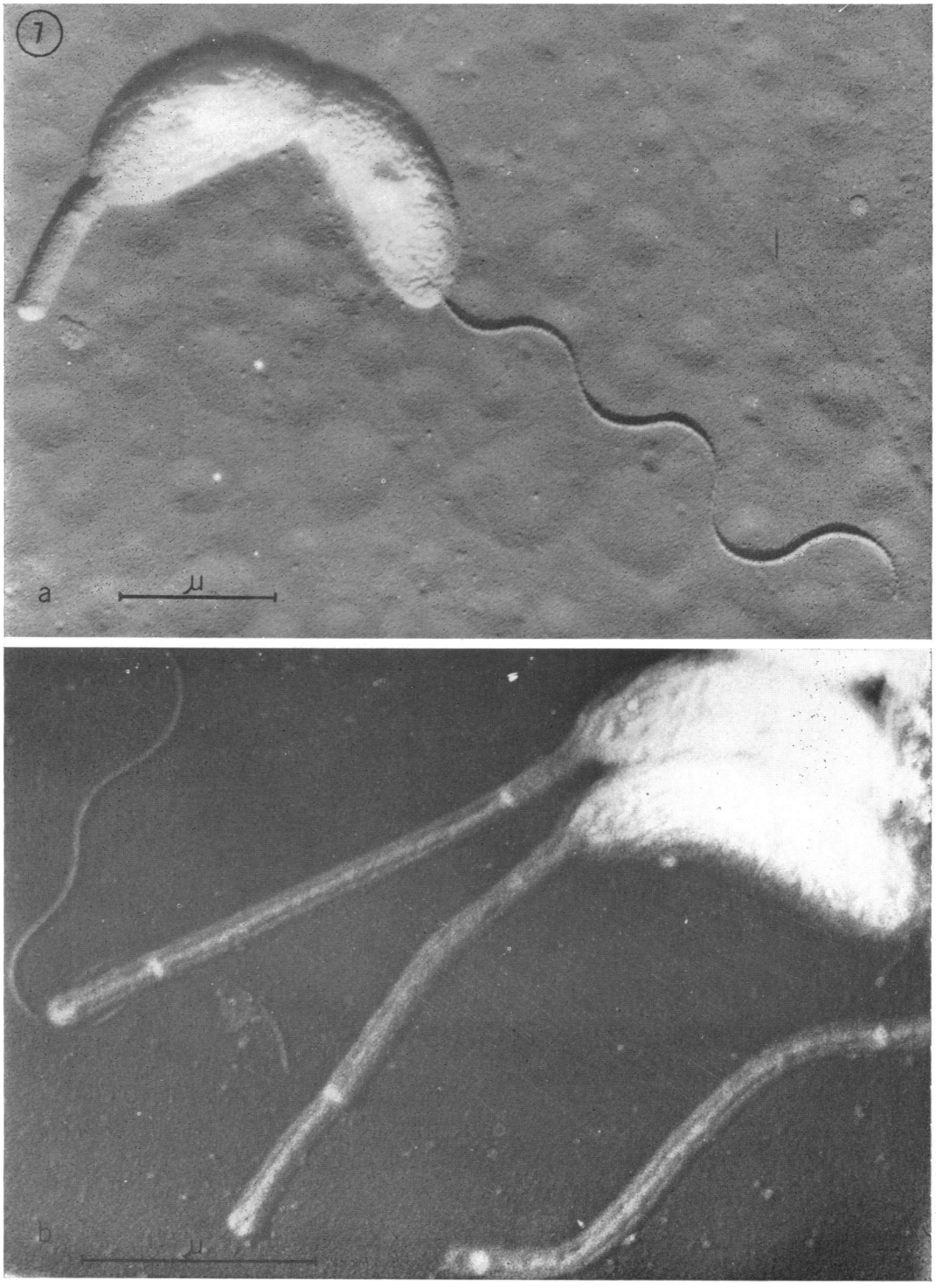

FIG. 7. *Vibrioid strain CB2. Electron micrographs. (a) A dividing cell, with flagellum at one pole and stalk at the other. (b) The long stalks of these cells have been shadowed from such an angle that the core can be distinguished readily from the flattened wall surrounding it.*

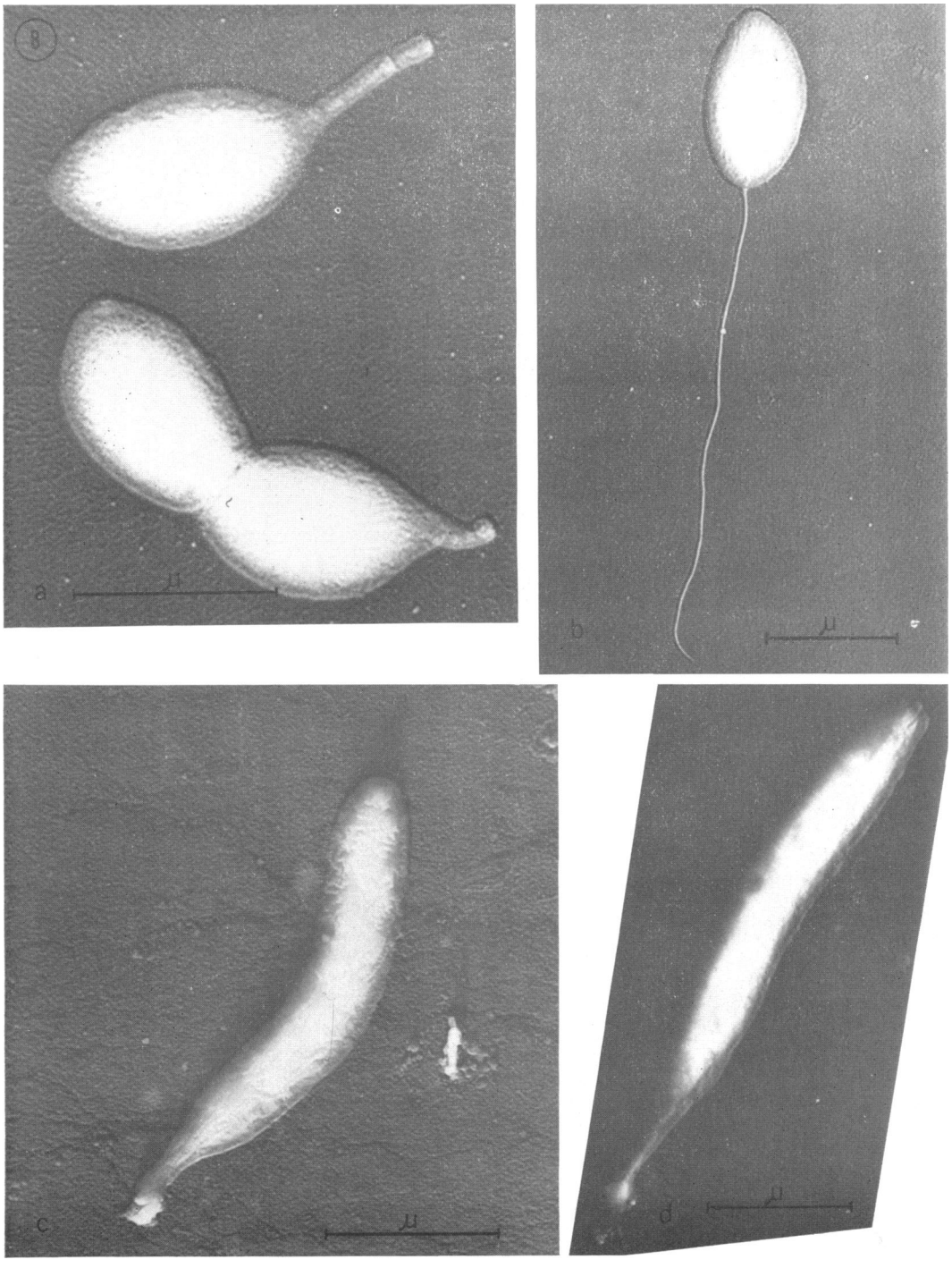

FIG. 8. *Morphological types similar to the vibrioid type. Electron micrographs. (a) Two stalked cells of limonoid strain CB-G. (b) A swarmer cell of limonoid strain CB-G. (c) A stalked cell of subvibrioid strain CB81. (d) A stalked cell of subvibrioid strain CB66.*

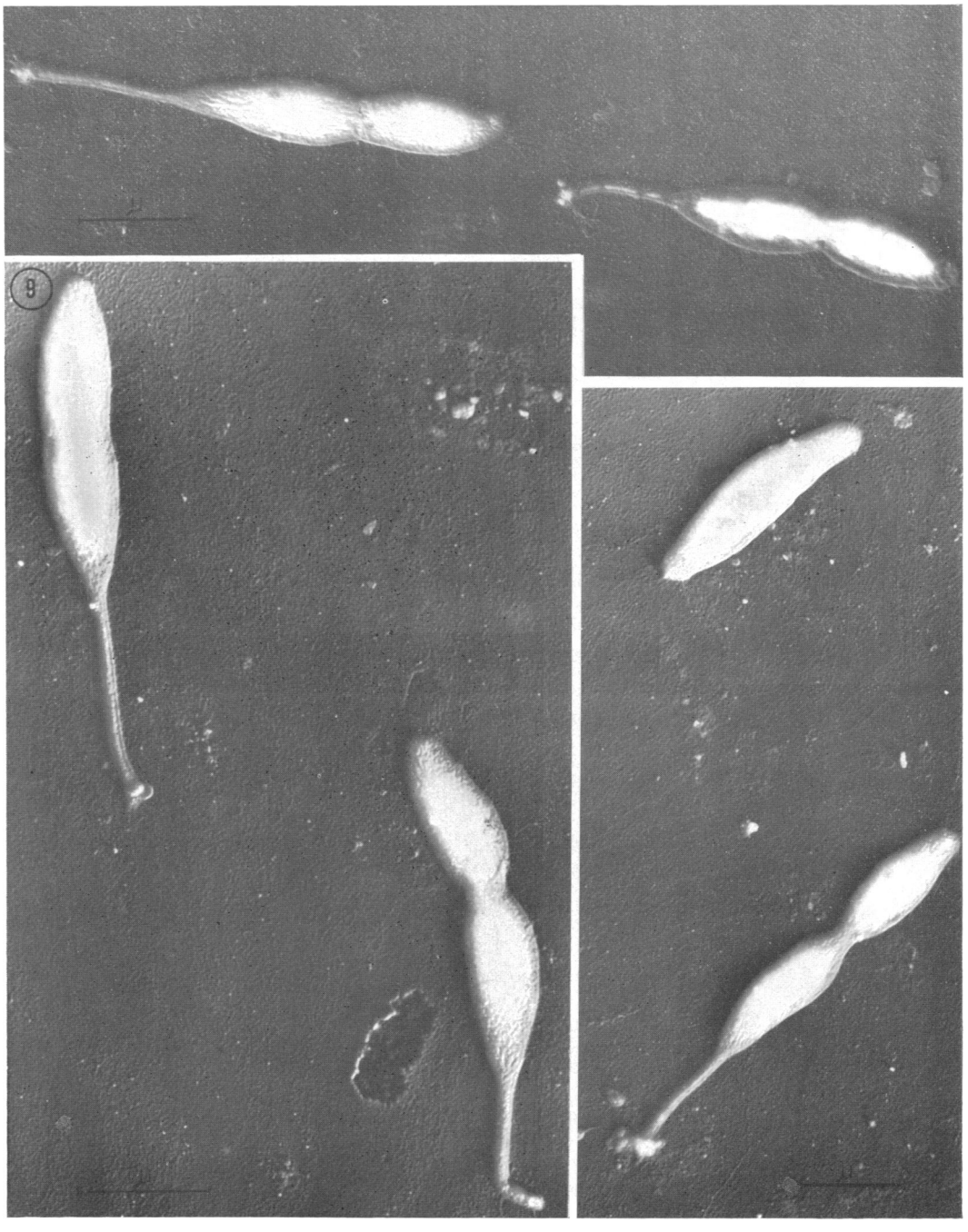

FIG. 9. *Stalked cells and a swarmer cell (the flagellum has been lost) of fusiform strain CB27. Electron micrographs.*

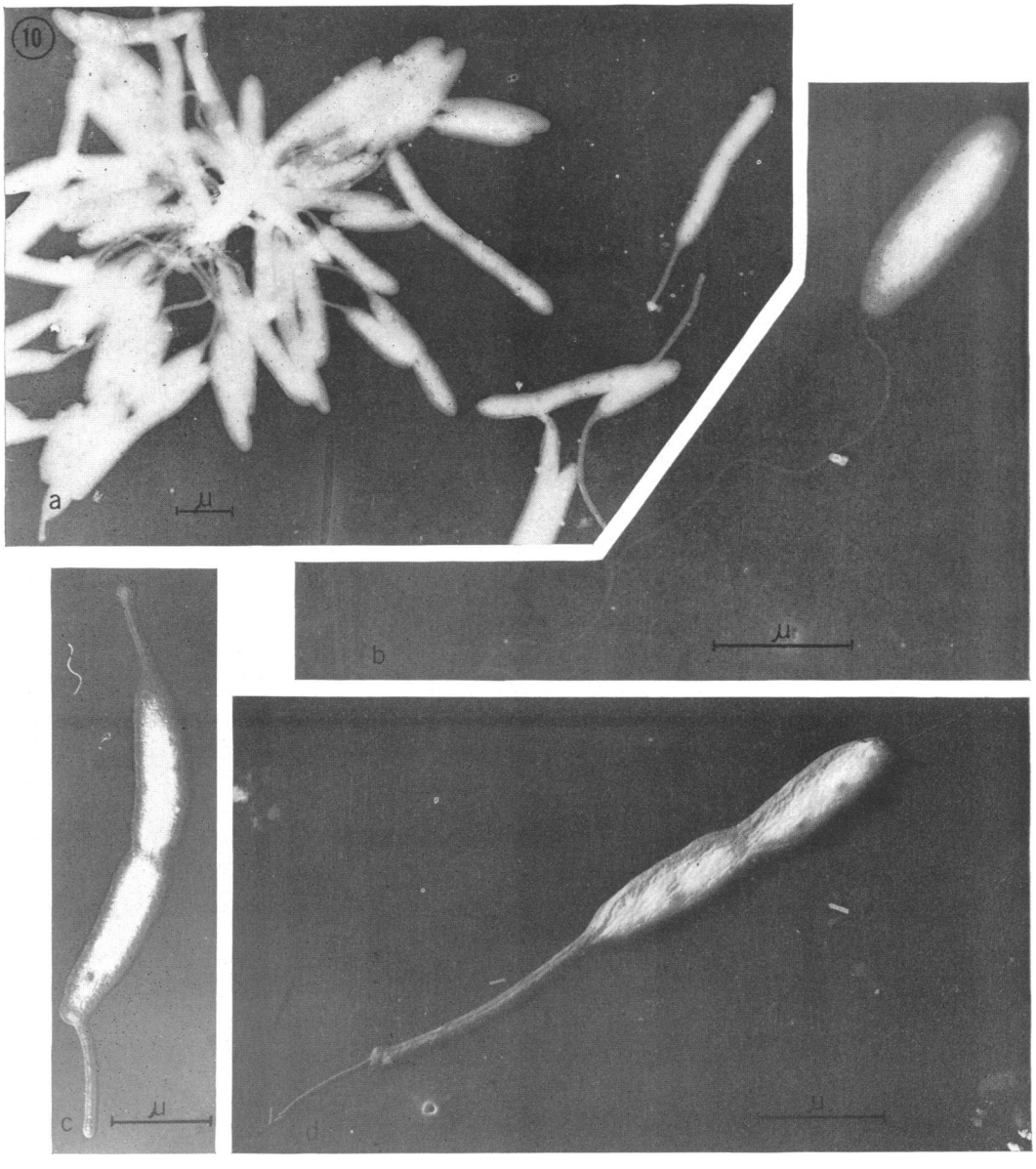

FIG. 10. *Bacteroid cells. Electron micrographs. (a) Stalked cells and a rosette of strain CB7. (b) A swarmer cell of strain CB7. (c) A dividing cell of strain CB17. A stalk has formed at the outer pole of the new cell, showing the variability of position of the stalk in this strain. (d) A dividing stalked cell of strain CB11. A remnant of the old flagellum is still present at the distal end of the stalk.*

development have not been published, although it was postulated by Henrici and Johnson (26) and by Houwink (29) that the development of the stalk is initiated by attachment of the non-stalked (swarmer) cell to a substrate. This hypothesis presupposes the presence of holdfast material on the swarmer, so that attachment can be established.

The relationships between growth, cell division and stalk development have been studied in two different ways; first, by observation of the developmental behavior of large pure populations of

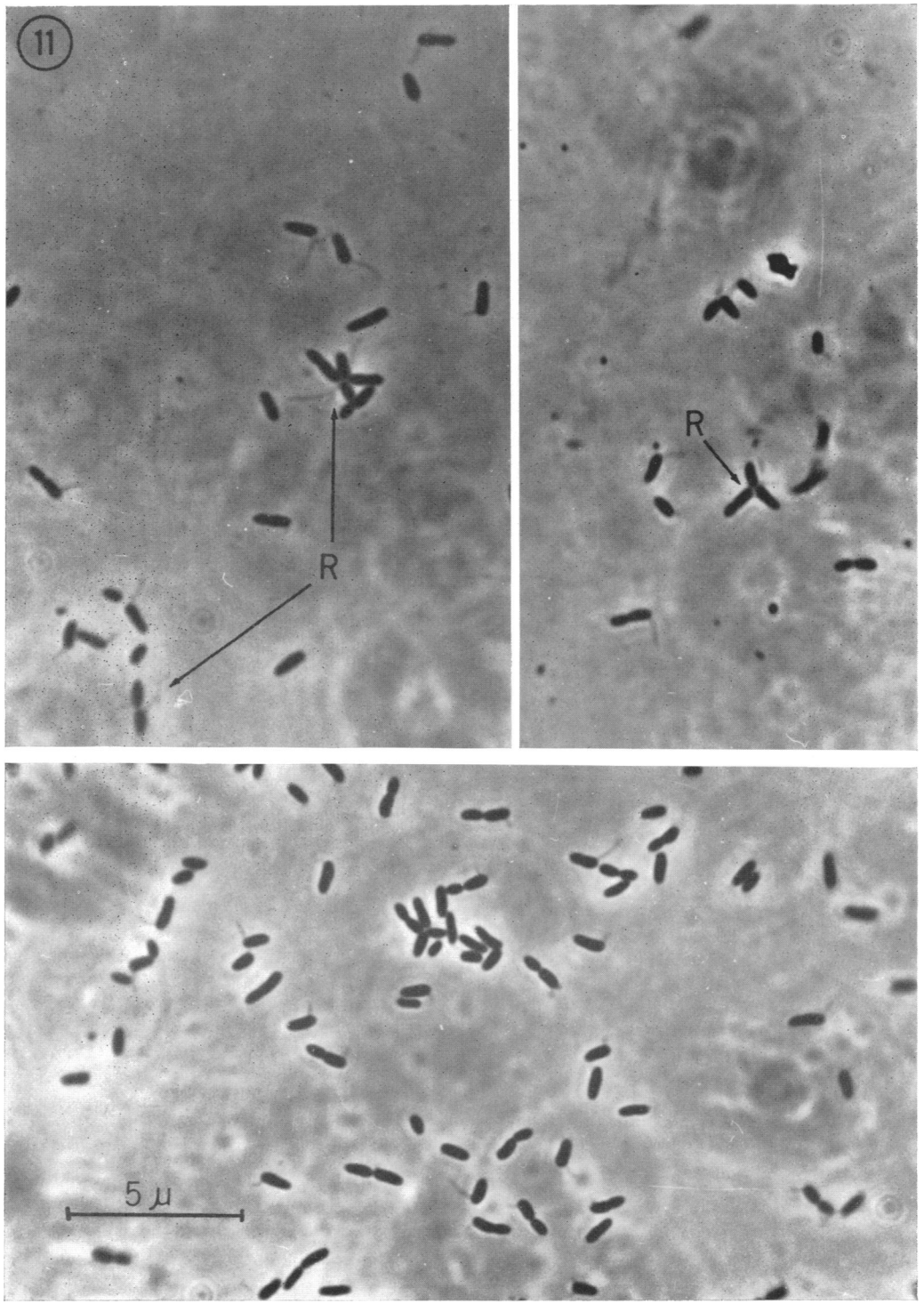

FIG. 11. *Excentral strain AC12. The stalks can be seen protruding from the rosettes (R). Phase contrast.*

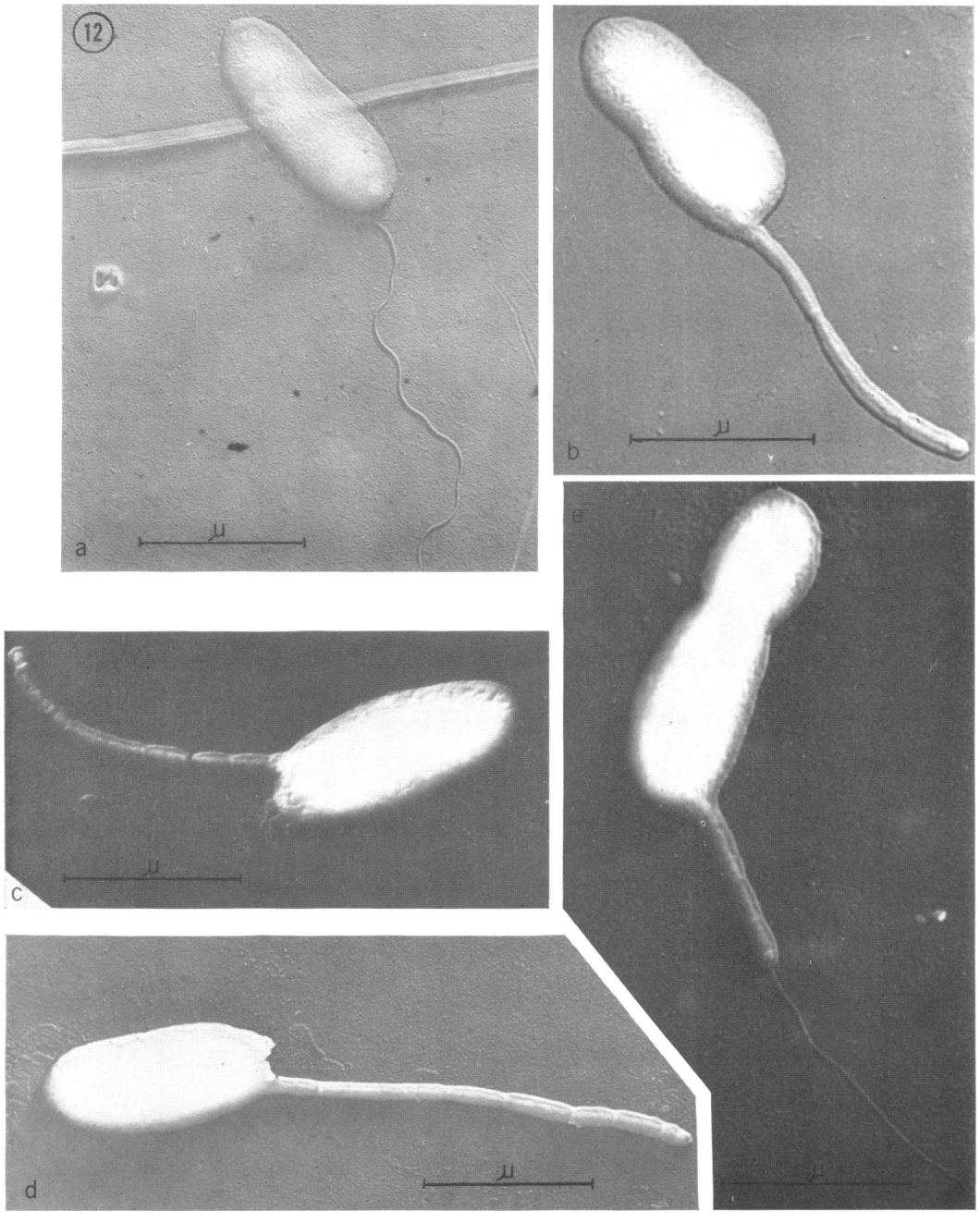

FIG. 12. *Excentral cells. Electron micrographs (see Fig. 4 of reference 68). (a) A swarmer cell of strain AC12. (b, c, d) Stalked cells of strain AC12. The holdfast material on the stalked pole of the cell is shown in c and d. (e) A stalked cell of strain AC48. A remnant of the old flagellum is still present at the distal end of the stalk.*

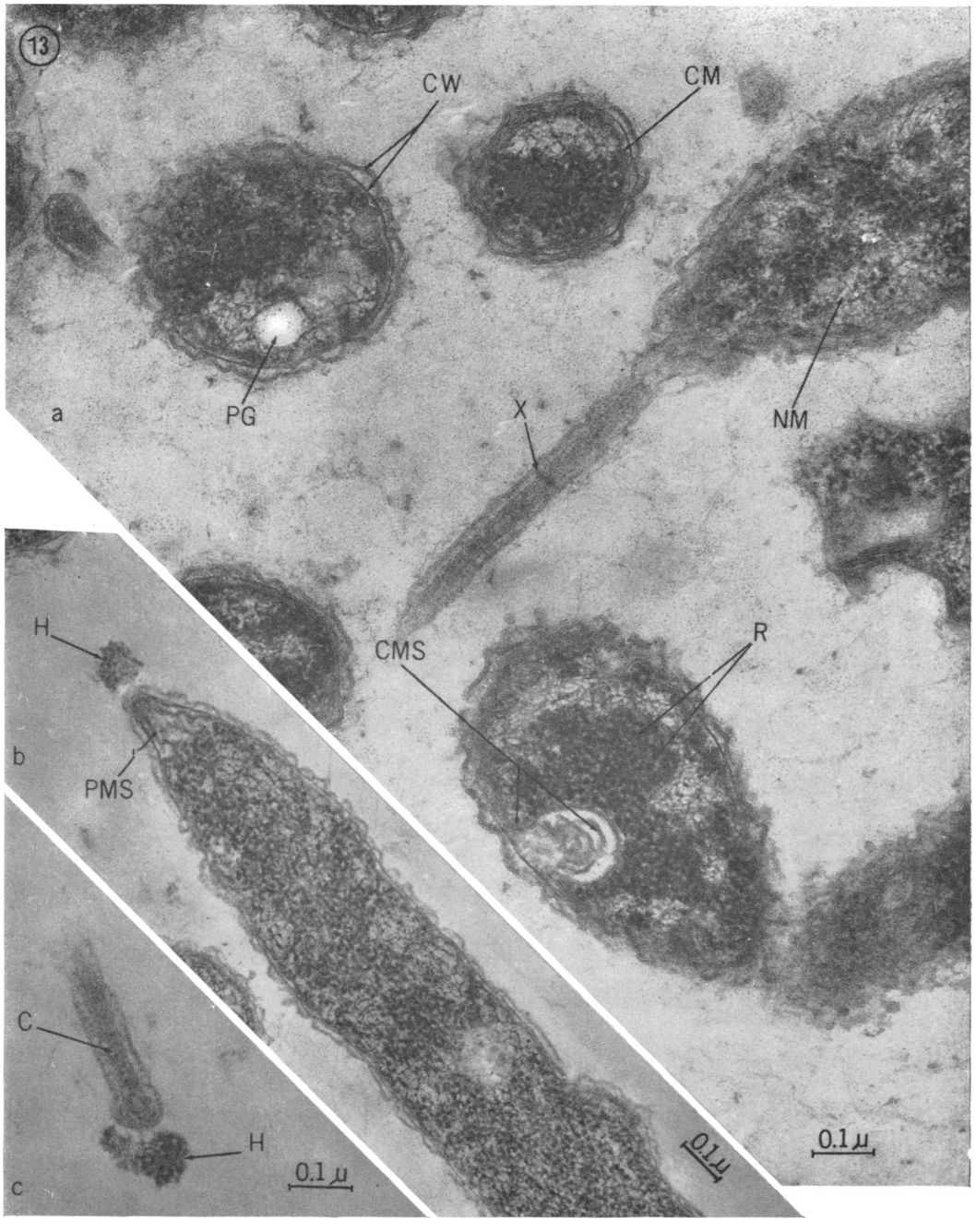

FIG. 13. *Sections of vibrioid strain CB2. Electron micrographs. CW = cell wall; CM = cell membrane; CMS = membrane structure within cell; PMS = membrane structure at base of stalk or flagellum; NM = nuclear material; PG = site of deposition of poly-β-hydroxybutyrate; R = ribosomes; C = core of stalk; X = band in stalk wall; H = holdfast material. (a) A stalked cell sectioned slightly away from the median of the juncture of cell and stalk. Another cell is sectioned in a plane which reveals the invagination of the cell membrane in the formation of the membrane structure. (b) A median section through a developing stalk. (c) A section of the distal end of a stalk showing granular appearance of the holdfast material.*

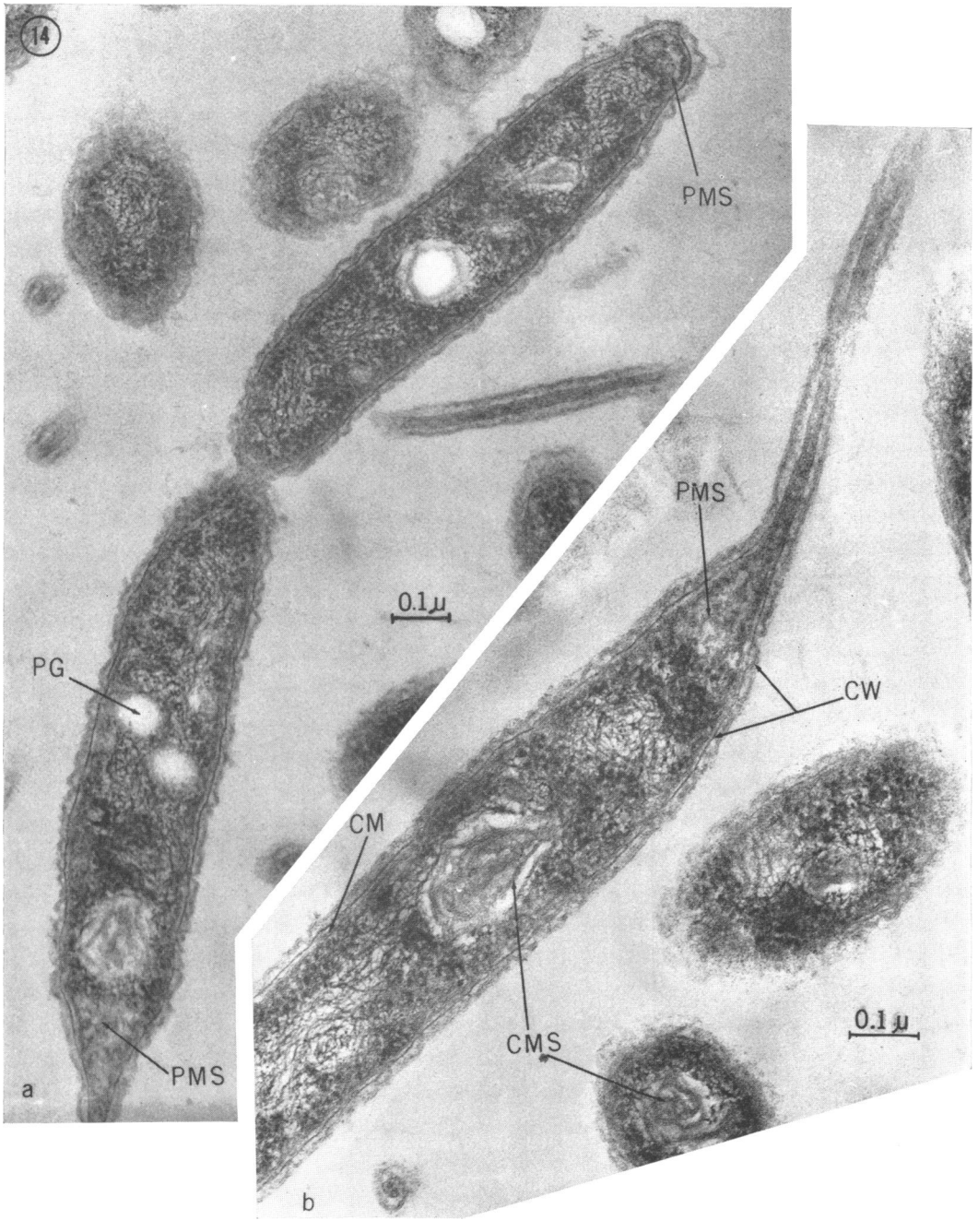

FIG. 14. *Sections of bacteroid strain CB11. Electron micrographs (see Fig. 13 legend for markings). (a) A dividing cell showing the old stalk membrane structure and the beginning of the newer one at the outer pole of the incipient swarmer. (b) A median section through a stalked cell. The membranes at the base of the stalk appear to be arranged parallel to the long axis of the cell and stalk.*

swarmers and of stalked cells, and, second, by continuous microscopic observation of clones growing in microcultures. A portion of this work has been reported (68). Both types of analysis have led to the same conclusion: the formation of a stalk is an obligatory step in the development of every swarmer cell, attached or free, and precedes the first division of the swarmer cell.

Observations on the Growth of Pure Populations of Swarmer Cells and Stalked Cells of Strain CB2

If a liquid culture or suspension of cells of caulobacter is centrifuged, the cells sediment as two distinct layers The upper layer contains stalked cells and rosettes, the lower one swarmers.

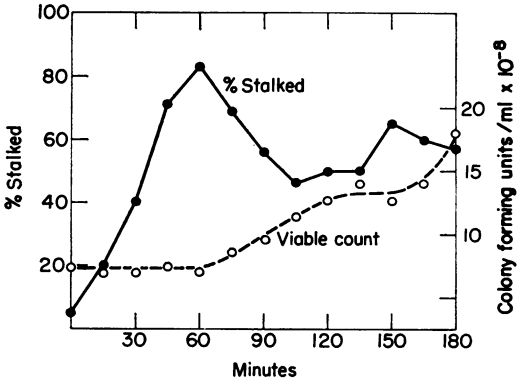

FIG. 15. *Developmental behavior in a mass culture inoculated with swarmer cells chilled during segregation. Strain CB2 (see Fig. 2 of reference 68).*

This segregation is due to an increased resistance to flow conferred on cells by the presence of stalks; swarmer cells can be sedimented in centrifugal fields in which stalked cells remain suspended. Repeated washing of the individual layers and elimination of intermediate layers yield homogeneous suspensions of the two cell types, as shown by microscopy of smears stained by Gray's method (63).

A chilled culture of strain CB2 was centrifuged and segregated at 4 C. The segregated populations were used to inoculate flasks of standard medium to a density of 6×10^8 cells per ml. The flasks were incubated on a shaker at 30 C. Samples were withdrawn at 15-min intervals for the determination of viable counts and microscopic determination of the proportion of stalked cells in the population.

In the culture inoculated with swarmer cells (see Fig. 15), the viable count did not increase during the first hour of incubation; during the following 75 min, the size of the population increased arithmetically to twice the initial level, and then remained constant for another 30 min. Thus, growth of the swarmer cells appears synchronized through at least the first division. During the 1-hr lag period, the proportion of stalked cells rose from its original value of 5% to a maximum of 83%, attained just prior to the onset of the first division; during the first division, the proportion of stalked cells dropped to a value slightly lower than 50%, owing to the liberation of a new generation of swarmer cells. A secondary,

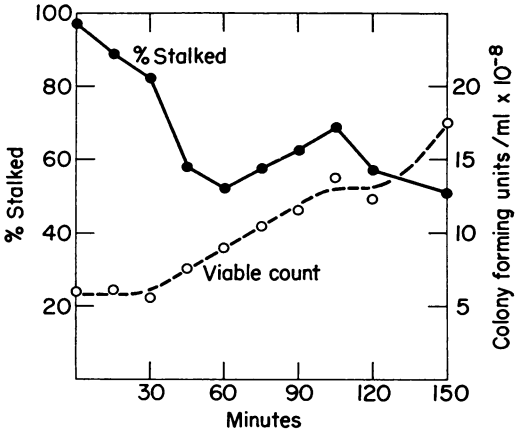

FIG. 16. *Developmental behavior in a mass culture inoculated with stalked cells chilled during segregation. Strain CB2.*

but less extensive, rise in the proportion of stalked cells occurred during the brief stationary period after completion of the first division as the new swarmer cells began to develop stalks; the maximal value attained was 65%. With the resumption of cell division, the proportion of stalked cells again dropped to a level of about 50%, and thereafter underwent only minor fluctuations as the synchrony of growth was lost.

The culture inoculated with stalked cells showed a short lag (see Fig. 16), probably attributable to physiological shock resulting from the chilling during the manipulations by which segregation was achieved. The much longer lag exhibited by the culture of swarmer cells can be interpreted by supposing that superimposed on the lag resulting from physiological shock is a lag caused

by the occurrence of stalk development as a process intermediate between recovery from chilling and the completion of cell division.

The effect of chilling was determined in an experiment in which the developmental behavior of populations segregated at room temperature was observed microscopically. Viable counts were not performed, since the frequency of rosette formation in dense suspensions of swarmers at room temperature makes the counts erratic. The observations are presented in Fig. 17. The temperature in the centrifuge cups reached 31 C during segregation; this resulted in an elimination of the lag in the culture of stalked cells, but cell division did not begin in the culture of swarmers until 45 min after transfer to growth medium. Therefore, although the lag period shown by stalked cells can be abolished when the cells are not chilled during segregation, the longer lag in division of swarmers cannot be eliminated in this way. This is most reasonably interpreted by assuming that a growing swarmer cell develops a stalk before it completes its first cell division, and that the period required for stalk development is reflected by a lag in multiplication.

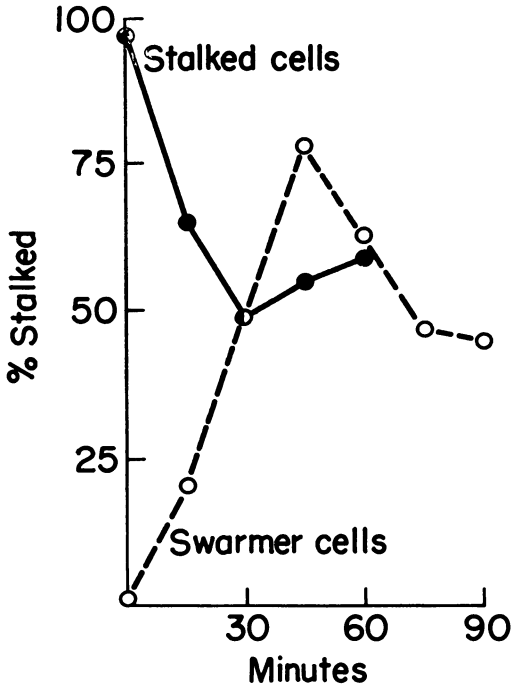

FIG. 17. *Developmental behavior in mass cultures inoculated with cells segregated at 30 C. Strain CB2.*

It should be noted that, under the conditions of these experiments, few of the swarmer cells which are chilled during segregation succeed in finding a substrate for attachment, although about 5% of the cells enter rosettes. Stalk development, therefore, takes place in a population of unattached cells.

Growth of Strain CB2 in Microcultures

Microcultures are prepared by placing about 0.04 ml of standard medium containing 1% agar in a sterile depression slide. A droplet of liquid medium containing 100 to 500 cells is placed on the agar. A sterile cover slip is laid over the depression so that a small area of the agar is in contact with the cover slip. The chamber is then sealed with VasPar, leaving the space between the agar droplet and the walls of the depression chamber to serve as a reservoir of air. Cells attached to the cover slip and the swarmer cells which they release into the thin film of fluid over the agar are observed in phase-contrast; attached cells are mapped, and their division times are recorded. The observations of strain CB2 growing in a microculture are presented in the upper part of Table 2.

In all, 90 cell divisions were observed, and 65 of these were timed from the previous division. In 33 instances, the motile sibling became attached within the field, and 10 of these were followed through at least one division. After a stalked cell had divided, giving rise to two morphologically dissimilar sister cells, the next division of the stalked sibling always preceded that of the swarmer sibling. This fact is reflected in the difference between the average division times for stalked and swarmer cells within each of the clones. Furthermore, each swarmer cell developed a stalk prior to undergoing division, supporting the conclusion drawn from the studies on the behavior of mass cultures inoculated with stalked or swarmer cells, respectively. Once a swarmer cell had developed a stalk and completed its first cell division, it proceeded to give rise to new swarmers at intervals comparable to those observed in the older stalked cells.

To make certain that determinations of division times of cells in the swarmer stage had not been restricted to physiologically sluggish swarmers unable to move out of the field of observation, the growth of a nonmotile mutant of strain CB2 (SS8) was studied under the same experimental

conditions. This mutant, obtained from an ultraviolet-irradiated population by the soft-agar technique of Leifson (42), differs from the parent strain by its lack of an externally visible flagellum. Since the nonstalked cells of this strain are lost to observation only if they float away after separation from their stalked siblings, 27 of 28 such cells attached to the cover slip and were observed through one to three divisions subsequent to the division preceding which stalk development occurred. The results of the experiment are presented in the lower part of Table 2, and are in agreement with the results of the experiment with the parent strain.

The respective mean division times of the two strains studied differ by several minutes. This may be a reflection of a change in growth rate in the mutant strain, or may have been due to the difference in room temperature on the two different days of observations, varying from 24 to 24.3 C for CB2 and being constant at 26 C for SS8. The light incident on the microcultures was reflected from the source in such a way that the cultures were not warmed above room temperature.

The data of Table 2 show clearly that the division time of nonstalked cells, whether motile or not, is 25 to 30 min longer than that of stalked cells, and that the same difference exists for each individual cell between the time required for its first division and that required for each of its divisions after it has developed a stalk. It was not possible under the experimental conditions to observe whether or not any further growth of the stalk occurred after its development during the first division cycle of the cell.

The observations on the growth of cells in microcultures are presented diagrammatically in Fig. 18 as two successive generations of a clone. Just after division, a stalked cell is of minimal length and immediately begins to elongate. When it has increased approximately twice in length, it begins to constrict near the midpoint of the cell. As the constriction proceeds, the dividing cell begins to vibrate; motion usually becomes quite violent and is obviously due to the activity of the newly formed polar flagellum. In microcultures, the incipient swarmer cell pulls the attached, stalked cell about on the cover slip; eventually it separates and swims away from the immotile stalked cell. The swarmer usually attaches to the cover slip, develops a stalk, elongates, and divides, producing an indefinite number of swarmer cells.

TABLE 2. *Division times of cells growing in microcultures*

Strain	Of the initial cell, not observed in its swarmer stage		Of cells within clones derived from initial cells			
			Swarmer stage to first completed division		Subsequent divisions	
	Clone	Time*	Clone	Time*	Clone	Time*
		min		*min*		*min*
CB2	A	97 (5)	A	132 (1)	A	100 (2)
	B	95 (5)	B	133 (4)	B	102 (6)
	D	100 (4)	D	132 (1)	D	85 (2)
	Mean	96 (14)		133 (6)		98 (10)
SS8	A	87 (4)	A	128 (4)	A	108 (3)
	C	95 (3)	C	109 (3)	C	99 (3)
	D	87 (5)	D	117 (6)	D	92 (6)
	E	94 (4)	E	124 (4)	E	92 (3)
	F	88 (4)	F	124 (3)	F	94 (2)
	G	97 (3)	G	120 (5)	G	94 (4)
	I	92 (2)	I	121 (1)	I	86 (1)
	J	82 (2)	J	107 (1)	J	84 (1)
	Mean	90 (27)		120 (27)		95 (23)

* The number of divisions for which the mean division time is expressed is given parenthetically after each mean.

Growth of Other Isolates in Microcultures

Two further isolates differing morphologically from CB2 and from each other were studied in microcultures to determine whether the developmental sequence established for the vibrioid strain and its nonmotile mutant is a general feature of caulobacters; studies of mass cultures of segregated populations of these isolates have not been performed.

The isolates selected were strains CB7 and AC48. Strain CB7 is a slender bacteroid *Caulobacter*, and strain AC48 is a typical excentral *Asticcacaulis* strain.

The following events are common to all three isolates: (i) cell division gives rise to a stalked and a swarmer sibling, (ii) the swarmer usually attaches to the cover slip shortly after separation from the stalked sibling, and (iii) the next division of the stalked sibling precedes the first division of the swarmer. The mean division times for the three isolates are presented in Table 3.

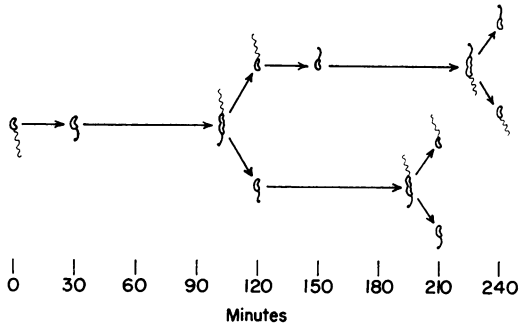

FIG. 18. *Developmental behavior within a clone. Strain CB2 (see Fig. 3 of reference 68).*

The developmental cycle of strain CB7 does not differ in any essential way from that observed in strain CB2. In both isolates, the swarmer cells develop stalks before dividing, the former process requiring a period equivalent to one-fourth to one-third the division time of stalked cells.

Division time for swarmers of strain AC48 is much longer than that of stalked cells. In all isolates of this type of stalked bacterium, the two siblings differ in size, as well as in the appendage which each possesses. The swarmer is little more than one-half the size of its stalked sibling. Hence, since the average size of cells just before division is fairly constant, the relatively long time between the separation of the swarmer from its stalked sibling and the completion of the swarmer's first division must be predominantly a period of cellular growth, as well as of differentiation. Because of the short length of stalks of AC48 on the standard medium and the effects of the agar layer on the phase coincidence of the light, it was not possible to observe that a stalk had been developed by every swarmer before its first division; this can only be surmised from the invariable loss of flagellar activity during this period, whether the cell was lying on the agar surface or had become attached to the cover slip. This is observable in excentral caulobacters, since the holdfast material does not occur at the base of the flagellum as on typical *Caulobacter* cells, and attached swarmers often continue to vibrate after attachment has been established by the holdfast material.

TABLE 3. *Division times of cells of three isolates growing in microcultures*

Isolate	Morphology	Division time (min)*		Ratio†
		Swarmer cells	Stalked cells	
CB2	Vibrioid	128 (10)	98 (55)	1.31
CB7	Bacteroid	234 (3)	188 (5)	1.25
AC48	Excentral	184 (26)	91 (59)	2.02

* The number of divisions for which the mean division time is expressed is given parenthetically after each mean.

† Division time of swarmers to division time of stalked cells.

DEVELOPMENTAL RELATIONSHIPS OF FLAGELLUM, STALK, AND HOLDFAST MATERIAL

The exact coincidence of the site of extrusion of the flagellum and of development of the stalk in both central and excentral caulobacters had suggested to us the existence of a common, localized cytoplasmic determinant for both appendages (68). The more recent findings concerning the internal structure of stalked cells (see above) suggest that the polar membrane structure may be the site of synthesis of the flagellum as well as the internal apparatus which directs the differentiation of the stalk.

The initial hypothesis was tested by electron microscopic examination of five nonmotile mutants derived from a strain (CB2) which has

flagella 2 to 5 μ long with a wavelength of 1.3 to 1.5 μ. Two of the mutants possess vestigial flagella, in the form of short stubs 0.05 to 0.25 μ long; this may reflect a limited synthesis of flagellar protein or a change in structure of the flagellum which renders it unusually fragile. The other three mutants are devoid of even such stubs.

All mutant strains develop stalks which are structurally indistinguishable from those of the parent strain, and also secrete holdfast material. The growth of one of the flagellaless mutants, SS8, was studied in microculture; as discussed above, the course of stalk development as related to cell division of SS8 was the same as that of the wild type. The internal structure of these mutants has not yet been examined.

Stocker and Campbell (65) have shown that the flagella of *Salmonella typhimurium* can be removed mechanically without impairment of cellular viability, and that rapid regrowth of flagella occurs. To determine the effects of mechanical deflagellation on the development of stalks, swarmer cells of strain CB2 were treated in a low-speed Waring Blendor for 60 sec; this treatment removed all but very short remnants of the flagella and completely eliminated motility. Viability was not affected. The cells were then inoculated into standard medium and observed at intervals for the reappearance of motility and the development of stalks. The results of three such experiments are shown in Fig. 19. Motility was estimated in wet mounts in phase-contrast, and stalk development was determined by the usual method of quantitative microscopic determination on smears prepared with Gray's stain (63).

Only in experiment 2 did any of the cells regain motility; a slight delay in stalk development was observed in this experiment. In the other two experiments, the cells did not become motile again, but developed stalks at the normal frequency. Therefore, since the reappearance of motility is infrequent, mechanical removal of the flagellum does not interfere with the development of the stalk.

In the microculture of strain CB2, attachment of swarmer cells was observed to occur soon after their separation from their stalked siblings. The swarmer cells which settled in the microscopic field attached to the cover slip within 15 min of separation. Twelve cells which swam into the field from another area of the microculture were also observed from the time of their attachment to the cover slip; their first divisions after attachment were completed within an average period of 113 min. Since the average time required for the first division of a swarmer cell is 128 min, these cells must have been free-swimming for only a short time. The nonstalked siblings of cell divisions in the microculture of strain SS8 frequently attached to the cover slip immediately after separation from the stalked siblings, and sometimes even before separation was completed. This was also observed in microcultures of strain AC48. These observations support the assumption of Henrici and Johnson (26) and of Houwink (31) that

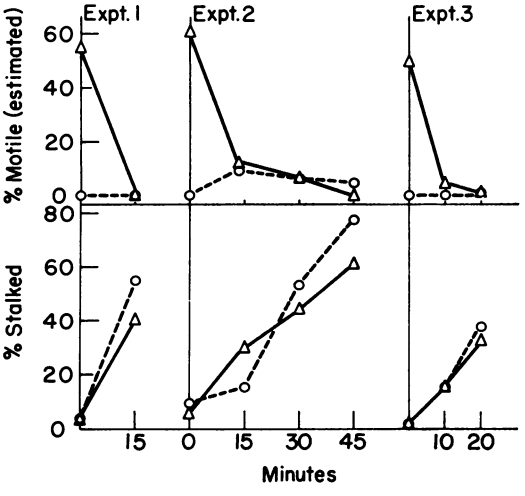

FIG. 19. *Motility and stalk development in cultures of mechanically deflagellated swarmer cells. Strain CB2.*

holdfast material is present on swarmer cells prior to the differentiation of a stalk. This is shown more directly in the electron micrograph of Fig. 6.

Holdfast material of caulobacters with central appendages is observed at the distal end of the stalk. This suggests that the localized secretion of holdfast material is intimately associated with the formation of the flagellum and subsequently of the stalk. However, the holdfast material and stalk of excentral caulobacters occur at different sites on the pole of the cell. Remnants of flagella have occasionally been seen at the distal end of excentral stalks; therefore, although holdfast material has not yet been seen on excentrally flagellated cells, it can be concluded that in iso-

lates of these caulobacters flagellum and stalk are developed at one site, while holdfast material is secreted at another.

THE PROPERTY OF ADHESIVENESS

The holdfast material secreted at the flagellated or stalked end of a caulobacter cell permits attachment of the cell to a variety of solid substrates. Attachment to glass was demonstrated by the early studies of Henrici and Johnson (26); collodion (29, 36) and cotton fibers are also excellent substrates. Of particular interest is the ability of caulobacter cells to attach to one another, leading to the formation of rosettes, and to the cells of other microorganisms. In this discussion, attention will be directed particularly to these two modes of attachment.

Rosette Formation

The occurrence of rosettes in *Caulobacter* cultures was first observed by Jones (35), who assumed without direct evidence that rosettes arise by repeated divisions of a single cell. Henrici and Johnson (26) did not observe rosettes on submerged slides, and suggested that rosette formation is "a peculiarity of artificial cultures" and is dependent on a high population density such as is obtained only in artificial cultures. This implies that they suspected that rosettes are aggregates of cells and not clones. Bachmann (*unpublished data*) did not observe rosettes in her relatively sparse enrichment cultures, but they were abundant in her pure cultures. In the present work, the same observation has been made: rosettes are absent from enrichment cultures, but are formed by every caulobacter strain growing in pure culture.

In pure cultures of most strains, rosettes are larger and more frequent on solid media than in agitated liquid cultures; in stationary liquid cultures, rosettes are particularly large and frequent in the dense surface film which is formed by all strains except the nonmotile mutants of strain CB2. These observations suggest that the motile cells are primarily responsible for rosette formation, since their aerotactic accumulation at the air-liquid interface appears to offer optimal conditions for contact and attachment.

A typical *Caulobacter* rosette may contain from two to more than 100 cells, all adherent to a common central point by the distal ends of their stalks. As seen in a light microscope, the stalks of individual cells in a rosette appear to be in direct contact with one another at the tips. However, electron microscopy shows that the stalks are physically separated from one another by the common mass of holdfast material, ca. 0.3 to 0.5 μ in diameter, which lies at the center of the rosette (see Fig. 1 and 6).

We have observed certain features of *Caulobacter* rosettes which are particularly noteworthy. If stalks are present, they are characteristically all of equal length; if they are absent, they are absent from all the cells in the rosette. Furthermore, the component cells of a mature rosette adhere to it uniquely through attachment by the tip of the stalk to the common holdfast material. These facts suggest, first, that the initiation of rosette formation normally occurs through adhesion of cells which are all at a common stage of development, and, second, that effective adhesion between caulobacters occurs only through contact between the holdfasts of the cells. This latter point is particularly well illustrated by the structure of rosettes of excentral caulobacters (see Fig. 11); in these rosettes, the cells adhere to a common mass of holdfast material by a site on the stalked pole, and the stalks, which are devoid of holdfast material, project away from the center of the rosette.

When stalked and swarmer cells of strain CB2 are segregated by centrifugation of cells harvested from a liquid culture at the end of exponential growth, the cells in rosettes are stalked, and rosettes accumulate in the upper layer that contains unattached stalked cells; the tightly packed pellet of swarmer cells is virtually free from rosettes. If the swarmer cells are resuspended in chilled medium and kept at 0 to 5 C, no new rosettes are formed. However, when the temperature of such a suspension is raised to 30 C, numerous large rosettes of swarmer cells are formed within a few minutes. Since cell division of the swarmers does not begin for at least 60 min, it is clear that multiplication is not required for rosette formation under these conditions, and that Jones' interpretation of the origin of rosettes is incorrect. The rosettes so formed are, of course, initially composed of nonstalked cells; if the swarmer suspension is then incubated at 30 C, each component cell in the new rosettes proceeds to differentiate a stalk, and after 60 min rosettes with the typical cartwheel appearance have developed.

The virtual absence of rosettes in suspensions of swarmer cells and the ready aggregation of

swarmers made it possible to study the relationship between rosette formation and population density. Swarmer cells of strain CB2 were segregated from a growing culture and resuspended in chilled water. The suspension was used to inoculate ten flasks of standard medium at different initial population densities. The cultures were incubated on the shaker at 30 C for 2 hr. At this time, a sample was removed from each flask, and smears were prepared and stained with Gray's stain; during the 2-hr incubation, stalks had been differentiated by the aggregated cells, and quantitative determination of the distribution and number of cells in rosettes was facilitated (Table 4 and Fig. 20).

TABLE 4. *Rosette formation in agitated liquid cultures in standard medium inoculated with swarmer cells of strain CB2 and incubated for 2 hr at 30 C*

Initial population density (viable cells per ml)	Per cent of population in rosettes	Mean no. of cells per rosette	Maximal no. of cells per rosette
	3.9	4.7	7
1.8×10^7	4.8	2.3	3
1.1×10^8	5.8	4.1	13
2.6×10^8	13.1	4.1	15
4.8×10^8	5.4	2.9	7
1.1×10^9	8.6	3.7	10
1.3×10^9	26.6	4.2	12
1.7×10^9	24.6	5.5	25
2.2×10^9	40.5	8.4	40
3.0×10^9	59.7	11.4	ca. 200
4.4×10^9	58.3	10.6	ca. 200

As the data show, the fraction of swarmer cells that enters rosettes remains low until the population density is at least 10^9 cells per ml. At higher densities, there is an abrupt rise in the fraction of cells that enter rosettes; an apparent plateau of about 60% is reached at a population density of 3×10^9 cells per ml. This plateau may reflect the steric difficulties involved in the formation of rosettes containing more than 200 cells; however, it is not easy to estimate the number of cells in very large rosettes, and the counts on dense populations may be unreliable. The rise is caused predominantly by an increase in the number of cells per rosette; the number of rosettes increased significantly only at initial population densities of 3×10^9 cells per ml and greater.

A priori, two different mechanisms, viz., random collision or chemotactic attraction between cells, might result in aggregation. The observed relationship between population density and rosette formation is compatible with either interpretation. However, chemotactic attraction is clearly eliminated by the occurrence of rosettes in cultures of nonmotile mutants, which form rosettes even in liquid media, though with a lower frequency than does the motile parental type. In colonies, where cells are always in contact, rosettes are as frequent in the mutant strains as in the motile parent strain.

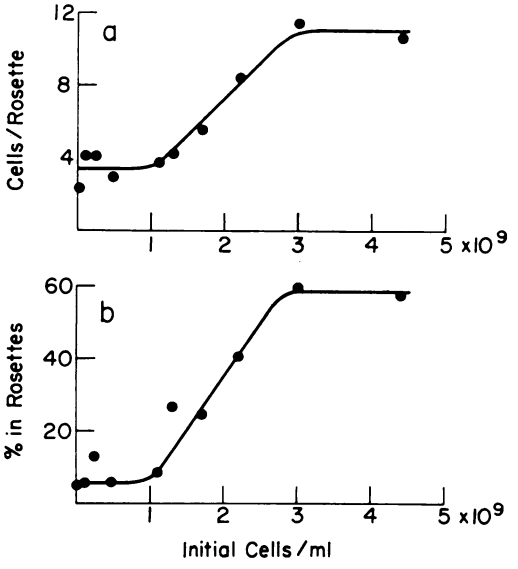

FIG. 20. *Rosette formation in agitated liquid cultures after 2 hr of incubation at 30 C. Strain CB2. (a) Mean number of cells per rosette. (b) Per cent of population in rosettes (see Fig. 6 of reference 68).*

To determine whether or not adhesion of cells in rosettes is specific, cells harvested from growing cultures of two strains belonging to different morphological groups were mixed at high population densities in standard medium. The cultures were incubated on a shaker at 30 C and examined microscopically at frequent intervals. Mixed rosettes were observed in the two-membered culture of every pair of strains tested. When the mixed rosettes first appear in a culture, they comprise nonstalked cells of each strain. After sufficient time has passed for the swarmer cells of each strain to develop stalks, mixed rosettes appear as illustrated by Stove and Stanier (68). This shows that adhesion in rosettes is not a strain-specific phenomenon.

Although these observations have established that rosettes can be formed by chance contact between swarmer cells, they do not exclude the possibility that a cell can enter a rosette after it has differentiated a stalk. Stalked cells possess the property of adhesiveness, as is readily apparent from their ability to initiate attachment to the cover slip of a wet mount. However, the mean free path of a nonmotile cell is much shorter than that of a motile cell. This factor considerably reduces the probability of effective collisions between stalked cells, and calculations suggest that rosettes should be formed predominantly by swarmer cells. This is in accord with our observations, but neither the experimental results nor the theoretical considerations exclude the possibility that cells which have already developed stalks may enter rosettes on rare occasions.

Nature of Holdfast Material

Two types of experiment were performed in an attempt to deduce the chemical nature of the holdfast material. The first type of experiment was based on studies of the periodate inhibition of stable contact formation by a variety of cells possessing receptor sites of carbohydrate nature. Such sites include the acrosomes of animal spermatozoa (14), tissue-cell receptors for influenza virus (27), and sites of conjugal contact on one mating type of *Hansenula wingei* (5) and of donor *Escherichia coli* K-12 cells (61).

Accordingly, suspensions of segregated swarmer cells of strain CB2 were incubated in a 0.001 M periodate solution and in standard medium containing 0.001 M periodate. Rosette formation was not affected. Not all of the cells survived when the concentration of periodate was increased to 0.005 M, but the surviving cells immediately proceeded to form rosettes when centrifuged from the periodate suspension and resuspended at high population density in standard medium. Furthermore, periodate treatment of the segregated stalked cells failed to disrupt already formed rosettes.

In the second type of experiment, cells of strain CB2 were incubated at 37 C for several hours in standard medium containing 5.5 μg of trypsin per ml and adjusted to pH 8.5. Rosette formation continued to occur in the presence of trypsin.

Therefore, the adhesive property of holdfast material is due neither to a periodate-sensitive carbohydrate nor to a protein which is suceptible to the action of trypsin. The chemical nature of holdfast material has not been further elucidated.

The holdfast material possesses great mechanical strength. Violent agitation of cell suspensions in the presence of glass beads in a Mickle tissue disintegrator or treatment in a high-speed Waring Blendor does not release cells from the holdfast material. The blending treatment, however, liberates cells from rosettes by breaking them from their stalks, leaving rosettes of stalks intact.

Caulobacter as "Parasite"

The ecological studies of Henrici and Johnson (26) suggested that the caulobacters are typically aquatic. Since they found caulobacters on slides immersed to a depth of 13 m, where algae are relatively rare, they inferred that these bacteria are not photosynthetic. They described the growth habit of these bacteria as "periphytic," defining this as growth on submerged surfaces.

In cultures where caulobacters and other microorganisms occur together, the stalked cells can be seen attached to other microbial cells. Such observations have led to the suggestion that caulobacters are capable of parasitizing other microorganisms, particularly other bacteria. Houwink (29) reported that simultaneous inoculation of 0.1% peptone-water with *Caulobacter* and either *Bacillus subtilis* or *B. megaterium* results in an initial predominance of the bacilli. Later, *Caulobacter* becomes predominant, and cells of the stalked bacteria can be seen attached by means of their stalks to living or lysed bacilli, although not to spores. Houwink suggested that the stalk acts as a "sucking proboscis," which enables the stalked cell to absorb nutrients from and destroy the host bacterium. However, this relationship is dependent upon the composition of the medium, since in a more concentrated medium *B. subtilis* can completely suppress the growth of *Caulobacter*.

Houwink later (31) modified his interpretation of the parasitic relationship. He concluded that lysis of the host cells in two-membered *Caulobacter-Bacillus* cultures is caused "somehow" by the attachment of the parasite. This lysis releases the contents of the host cells, which serve as nutrients for the slower-growing caulobacters. *Caulobacter* cells not attached to a host are, according to this interpretation, equally benefited by the release of these materials.

Houwink reported that, in the two-membered culture after several days, caulobacters are "exceedingly numerous, whereas the number of viable *Bacillus* has decreased ten times or even more." The growth of a pure culture of the *Bacillus* in the same medium is not described.

The question of parasitism was further investigated by Hund and Kandler (33). These workers tested a variety of bacteria as possible hosts for Houwink's *Caulobacter* isolate by preparing two-membered cultures. After 24 hr of incubation, the cultures were examined microscopically for attachment. No attachment of caulobacters to other gram-negative bacteria or to actinomycetes was observed. Eight gram-positive bacteria were tested, and all were invested by caulobacters. The most abundant attachment occurred in the two-member culture of *Caulobacter* and *B. cereus*; this organism was, therefore, selected for further experiments designed to reveal the effects of attachment.

Expressed either as the proportion of bacilli invested or the proportion of caulobacters attached, attachment reached a maximum when *Caulobacter* cells from a growing culture were mixed with *B. cereus* cells in a peptone-yeast extract medium and incubated for a period of 60 to 120 min. When a ratio of ten *Caulobacter* cells per *Bacillus* cell was used, nearly 40% of the bacilli were invested; when the proportions were reversed, approximately 30% of the *Caulobacter* cells were found attached to bacilli.

To determine whether caulobacters release nutrients from the bacilli by inducing lysis of invested cells, Hund and Kandler prepared a two-membered suspension in distilled water. In this medium, caulobacters would be capable of multiplying only if they obtained and could utilize the contents of the cells to which they attached. Relatively little attachment occurred in the two-membered suspension in water, although the usual frequency was observed in a parallel suspension in growth medium. This was at first interpreted as a consequence of the difference between the osmotic pressure of the medium and that of distilled water. However, when the effect of increasing concentrations of sodium chloride and of mannitol in the suspending fluid was investigated, attachment was found to be inversely related to the osmotic pressure. Since the proportion of bacilli invested was reduced to a greater extent than the proportion of caulobacters attached, the authors concluded that alteration of the osmotic pressure altered the cell surface of the host, making it less suitable as a substrate at higher osmotic pressures. They found greater attachment in the growth medium than would be predicted on the basis of the osmotic pressure of the medium.

To determine the effect of attachment on the viability of the bacilli, Hund and Kandler prepared two-membered microcultures. Examination of 20 such cultures gave the following results. During the period of observation, 92.3% of the free bacilli showed an average increase in size of 3.12-fold, and 2.3% of those which failed to grow were seen to lyse; on the other hand, only 30% of the invested bacilli increased in size, with an average growth of 1.55-fold, and 24% of the non-growing invested cells lysed. The proportion of bacilli invested, and the proportion of *Bacillus* cells unable to grow and eventually proceeding to lysis in a pure culture under the same conditions, were not reported. Such comparisons would be particularly significant in light of the authors' conclusions concerning the influence on attachment of the state of the host surface, conceivably variable with the physiological state of the host cell.

Zavarzina (77) reported a curious relationship involving *Caulobacter*, *Chlorella pyrenoidosa*, and a viruslike organism. Cytological alterations eventually ending in lysis of *Chlorella* cells were observed in algal cultures which contained bacterial contaminants. One of the contaminants which occurred commonly in the cultures was isolated and identified as *Caulobacter vibrioides*. It could be seen attached to the algal cells, particularly old or damaged ones, which seemed to indicate that the cytological aberrations of *Chlorella* might result from the attachment of the caulobacters. However, after three to five passages of *C. vibrioides* on a yeast extract medium, its attachment to *Chlorella* did not cause lysis of the alga. Spherical particles less than 300 mμ in diameter were found in bacteria-free lysates of the damaged *Chlorella* cultures; such particles were not found in lysates of mechanically disrupted *Chlorella* cells. The particles alone did not cause lysis of algal cells, but when *C. vibrioides* was also present, the cell damage and lysis observed in the initial *Chlorella* cultures could be reproduced. The interpretation given by Zavarzina is that "*C. vibrioides*, in the process of lysis of chlorellae, 1) per-

forms the role of carrier of the lytic agent; 2) attaches to the envelopes of the algae, damages them and renders them more penetrable for the particles of the lytic agent; 3) participates in the final destruction of the *Chlorella* cells." Zavarzina did not discuss the question of the possible advantages of this relationship for the stalked bacteria. In a more recent paper (78), she reported that kanamycin in the medium prevents lysis of the chlorellae without affecting the growth of the caulobacters.

In summary, previous workers had demonstrated that caulobacters are able to attach to cells of other microorganisms, and they had inferred from this a capacity for parasitism. In contrast to the results of the work of Stolp et al., however, the evidence for parasitism yielded by the studies of *Caulobacter* is not convincing. The following section, in which the phrase "host cell" is replaced by "invested cell," describes experimental results at variance with the interpretation that *Caulobacter* cells are facultatively parasitic.

TABLE 5. *Attachment of cells of Caulobacter strain CB2 to other bacteria*

Test species	Inoculum of test species from		Cytological and Gram reaction changes in invested cells
	Growing culture	Stationary culture	
Gram-negative			
Pseudomonas fluorescens	Rare; usually in area of constriction of dividing cells	No attachment until cells of test species begin to divide	None
Escherichia coli	Rare	No attachment	None
Azotobacter agilis	Frequent	Frequent	None; invested cells eventually clump.
Gram-positive			
Bacillus megaterium	Frequent	Occasional, then frequent as bacilli begin to divide	None; invested cells rarely clump
B. cereus var. *mycoides*	Rare; usually in area of constriction of dividing cells	Rare	None
Sarcina lutea	All packets and cells invested	All packets and cells invested	None
Propionibacterium pentosaceum	Frequent	—	None

The existence of exoparasites for bacteria which attach to host cells and induce host lysis was reported recently by Stolp and Petzold (66). In this case, the lysis of host bacteria as a result of attachment to them of the parasites and the nutritional advantage of host lysis to the parasites have been demonstrated clearly. In the first publication concerned with these parasites, it was suggested that they are a form of bacteria very similar to, if not identical with, *Caulobacter*. This confusion resulted in part from the reports of parasitism by *Caulobacter*, and in part from contamination of Stolp and Petzold's electron microscopic preparations with *Caulobacter* cells. However, the parasites of Stolp and Petzold have now been recognized as a unique group of nonstalked, motile, vibrioid bacteria (67, 67*a*).

Attachment to Other Bacteria—Qualitative Survey

Several species of bacteria were tested as substrata for attachment. Test cells were suspended in the presence of 5×10^9 CB2 cells per ml; segregated swarmer cells were used in order that rapid and frequent attachment would occur. In each two-membered culture, the density of test cells was less than 5×10^8 per ml. The medium employed was half-strength standard medium, since the stalks of strain CB2 are longer in the diluted medium and the stalked cells are more readily observed beyond the phase-contrast halo of the test cells. The cultures were examined at frequent intervals over a period of 6 hr (Table 5). Cells of *Sarcina lutea* and *Azotobacter agilis* invested with caulobacters are illustrated in Fig. 21 and 22, respectively.

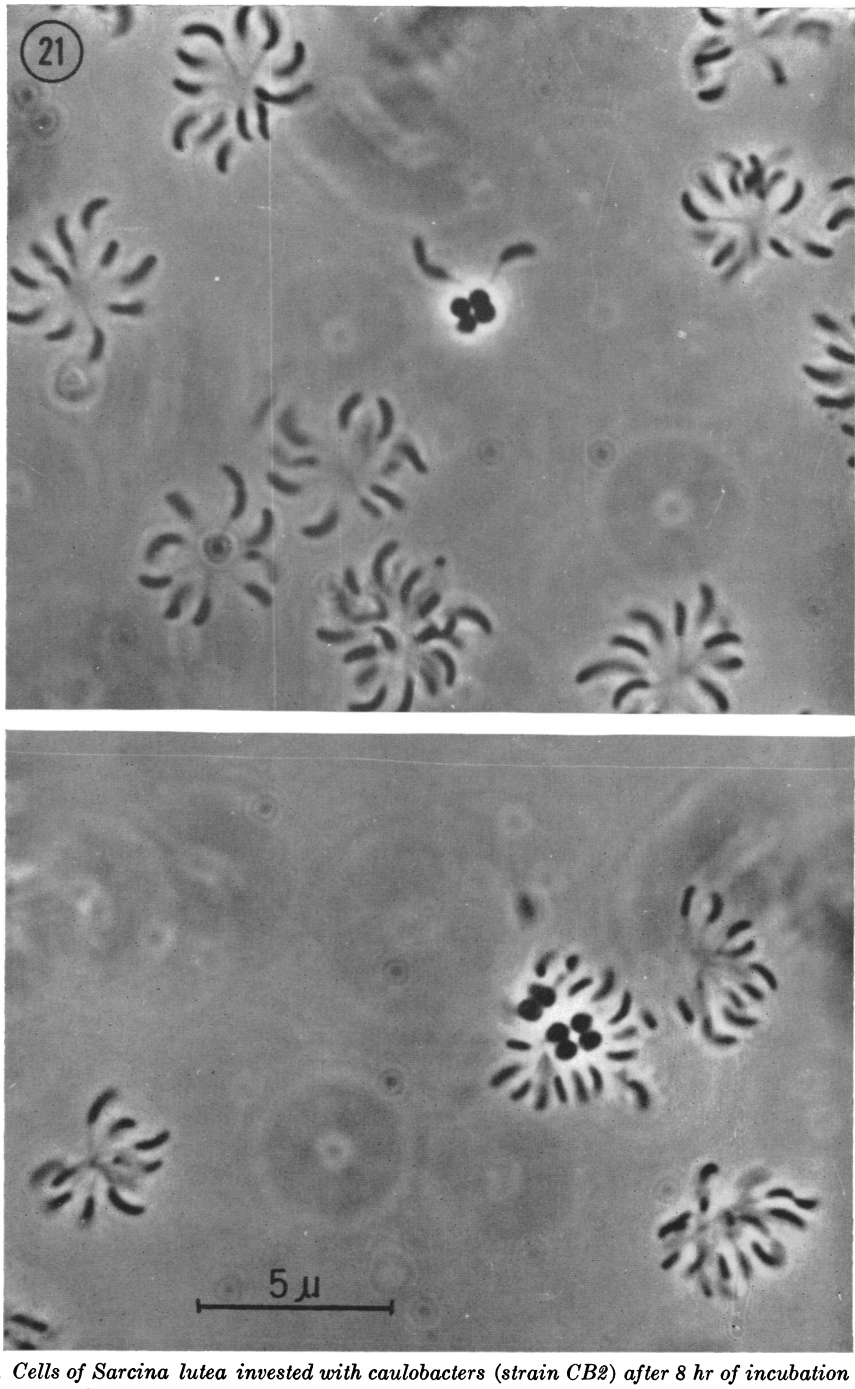

FIG. 21. *Cells of Sarcina lutea invested with caulobacters (strain CB2) after 8 hr of incubation of the two-membered culture. Phase contrast.*

In all cultures except the one containing *Propionibacterium*, the test organisms had begun to multiply before the end of the experiment. The *Caulobacter* cells had developed stalks and given rise to motile progeny in all cultures except the one with *S. lutea*, in which the caulobacters appeared to be lysing. In general, bacterial cells were found to be more heavily invested when they were

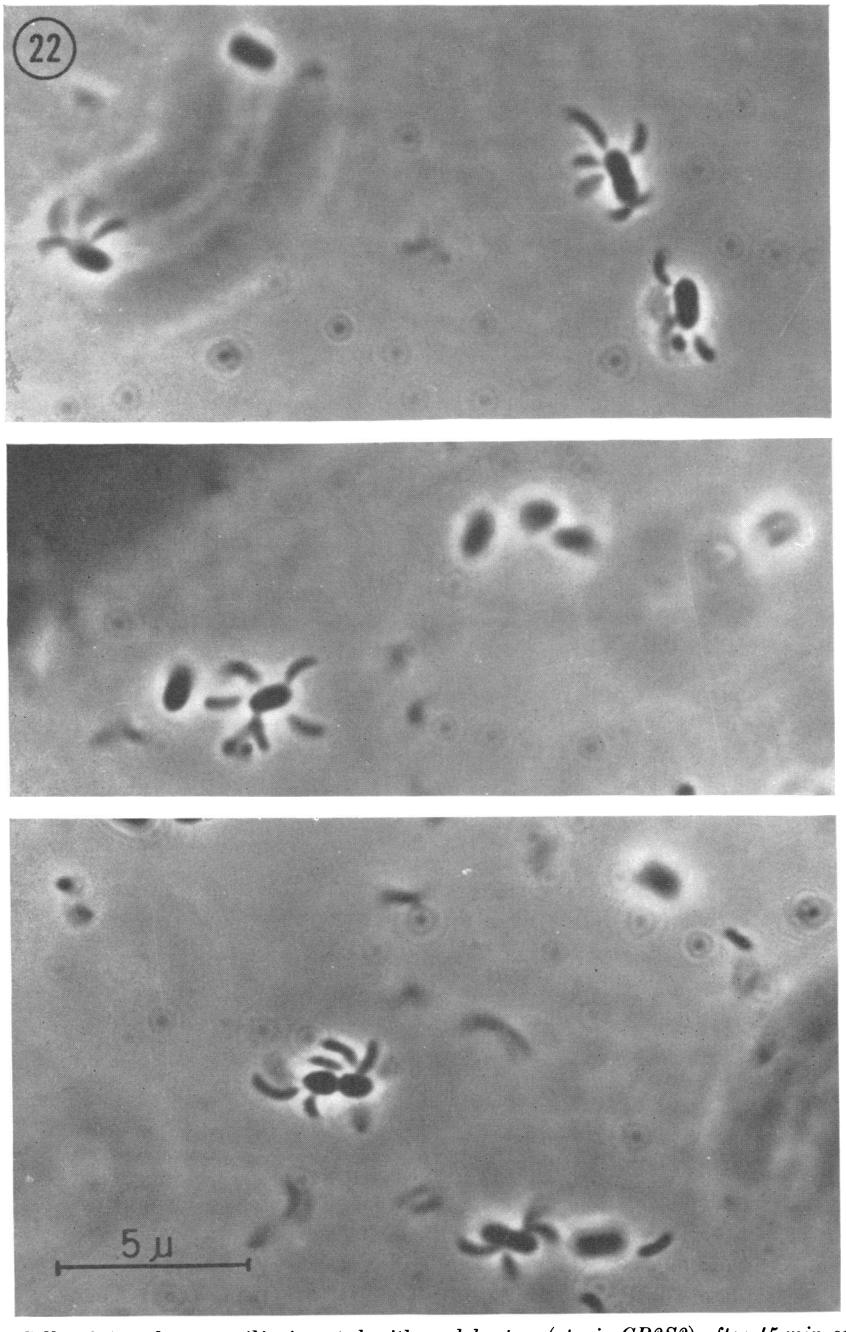

FIG. 22. *Cells of Azotobacter agilis invested with caulobacters (strain CB2S2) after 45 min of incubation of the two-membered culture in standard medium. Phase contrast.*

growing, as was observed by Hund and Kandler (33) with *B. cereus*. Although attachment is not confined to gram-positive bacteria, these were generally more suitable as substrata. These observations on the preferential investment of other organisms by caulobacters suggest that the nature of the cell walls of the substrative species is significant. However, the cell-wall compositions of

the four organisms which are substrata are sufficiently different (57, 58) to show that specificity is low.

On the basis of this survey, *S. lutea*, *B. megaterium*, and *A. agilis* were selected for further experiments. The physiological differences between *Propionibacterium* and *Caulobacter* do not permit the establishment of a two-membered culture in which both organisms could grow. *B. subtilis* was included in later experiments specifically to test the observations of Houwink (31).

Attachment to Other Bacteria—Quantitative Experiments

S. lutea as substratum. Pure cultures of *S. lutea* and of *Caulobacter* strain CB2 were prepared in standard medium; a two-membered culture containing the two organisms was prepared simultaneously. The cultures initially contained 2.3×10^6 sarcina colony-forming units per ml and 10^8 caulobacter units per ml. After 12 hr of incubation at 30 C, samples were removed from the cultures for microscopic examination and for plating on standard medium. The colonies arising on plates prepared from the two-membered culture were differentiated macroscopically as containing *S. lutea* (yellow) or CB2 (colorless).

Microscopic examination of the two-membered culture at 12 hr revealed that nearly every *S. lutea* packet and cell had at least one *Caulobacter* cell attached to it. The viable count of *S. lutea* in this culture was slightly lower than in the pure culture (2.5×10^7 per ml and 3.6×10^7 per ml, respectively). All of the *S. lutea* colonies from the two-membered culture which were examined microscopically contained caulobacters; therefore, the *S. lutea* colonies arose from invested cells and packets. The sarcinae in the colonies were invested with caulobacters. The viable count of CB2 was also lower in the two-membered (4.0×10^9 per ml) than in the pure culture (5.0×10^9 per ml); this may have been due to attachment to *S. lutea* and subsequent growth of these caulobacters in colonies which appeared macroscopically to be *S. lutea*.

Similar results were obtained in an experiment with *Caulobacter* strain CB11. After the first 10 hr of incubation, the sarcinae ceased to grow and lost their gram-positive character; in the two-membered culture, this occurrence was accompanied by an adverse effect on the viability of the caulobacters, as illustrated by the growth curves of the three cultures presented in Fig. 23. The colonies arising on plates prepared from the two-membered culture were differentiated macroscopically as containing *S. lutea* (yellow) or CB11 (orange).

In both experiments with *S. lutea*, caulobacters attached to every vegetative unit of the sarcinae without causing any cytological change in the latter that could be detected in phase-contrast or Gram-stained preparations. This, together with the fact that the same number of *Sarcina* colonies developed from corresponding samples of the pure and two-membered cultures, shows that an invested *Sarcina* cell or packet remains viable. The

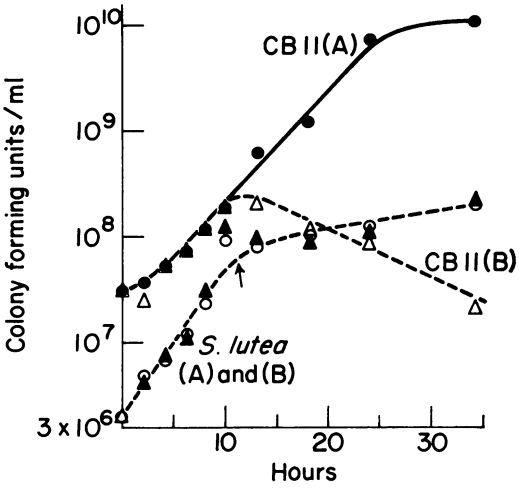

FIG. 23. *Counts of colony-forming units of Caulobacter strain CB11 and of Sarcina lutea in agitated liquid cultures in standard medium at 30 C. A = pure culture. B = two-membered culture. Arrow = transition of S. lutea to gram-negative.*

growth of *Caulobacter* was not enhanced by attachment to the sarcinae.

Bacillus species as substrata. B. megaterium and *Caulobacter* strain CB2 were grown in pure and two-membered cultures in standard medium. In the latter, the frequency of attachment increased during the period of exponential growth of the caulobacters. After 24 hr of incubation, nearly every *Bacillus* cell was invested with one to seven caulobacters. When examined in phase-contrast, some of the invested bacilli appeared to have lysed by this time, but the number of lysed cells was no greater in the two-membered than in the corresponding pure culture.

The viable counts are presented in Table 6.

The viable counts of *B. megaterium* are practically identical in the pure and two-membered cultures; therefore, the growth of *B. megaterium* is not adversely affected by the attachment of caulobacters. Many of the colonies on the plates from the two-membered culture which were scored macroscopically as *Bacillus* colonies were found, upon microscopic examination, to contain both *Bacillus* and *Caulobacter* cells; the viable counts of CB2 from the two-membered culture are, in consequence, lower than the counts from the pure culture.

In an experiment with *B. subtilis*, three flasks of standard medium were inoculated with the bacilli. To one of the flasks were added cells of

TABLE 6. *Counts of colony-forming units of Bacillus megaterium and Caulobacter strain CB2 per milliliter of culture in agitated flasks of standard medium at 30* C

Time of incubation	Pure culture of *B. megaterium*	Two-membered culture		Pure culture of CB2
		B. megaterium	CB2	
hr				
0	6.9×10^6	6.9×10^6	8.0×10^5	8.1×10^5
10	5.5×10^7	5.7×10^7	4.7×10^7	1.0×10^8
24	4.7×10^7	4.8×10^7	4.0×10^9	7.3×10^9

* Differential counts of large, matt *Bacillus* and small, glistening CB2 colonies were performed on plates of standard medium.

Caulobacter strain CB-H (the strain from Houwink), and to another were added cells of strain CB11. The flasks were incubated on a shaker at 30 C. In the culture containing *B. subtilis* and CB-H, only the bacilli grew. In this culture and in the pure culture, the bacilli began to lyse after the second day of incubation, and sporulating bacilli were very rare.

After 1 day of incubation, the turbidity of the culture containing *B. subtilis* and CB11 was nearly twice that of the pure culture of *B. subtilis*. As judged by microscopic examination of the two-membered culture, the caulobacters were predominant, and the bacilli were heavily invested with *Caulobacter* cells (Fig. 24). The invested bacilli showed no evidence of lysis. On the second day of incubation, the number of *B. subtilis* cells had increased in proportion to the caulobacters, and some bacilli had begun to sporulate. After two further days, sporulation was frequent and lysis of bacilli was rare relative to the lysis in the pure culture of *B. subtilis*. The enhancement of sporulation and prevention of lysis of *B. subtilis* by CB11 may be due to an alteration of the pH of the medium; initially 6.5, the pH is 8.4 by the time this *Caulobacter* strain reaches stationary phase.

Houwink (31) found that many of the *B. subtilis* cells in his two-membered culture lysed after several days, and that the growth of his *Caulobacter* strain seemed to be enhanced as the bacilli died. It is not clear from his discussion whether only invested bacilli underwent lysis. The results of the experiment presented above are not in accord with Houwink's conclusion that *Caulobacter* can induce lysis of *B. subtilis*.

A. agilis as substratum. Attachment of swarmer cells of CB2S2 (a spontaneous mutant of strain CB2 resistant to 0.1 mg of streptomycin per ml of standard medium) to *A. agilis* was allowed to occur in standard medium. Microscopic and viable counts of the two-membered culture, and viable counts of pure cultures of each organism, are presented in Table 7.

Spontaneous clumping of *A. agilis*, regularly observed during lag phase upon subculture in liquid media, could account for the decrease of its colony-forming units in the pure suspension. Clumping was no more frequent in the two-membered culture, and the invested cells appeared normal in phase-contrast. The decrease of *A. agilis* colony-forming units in the two-membered culture is greater than could be accounted for by clumping and by the inability of invested cells to give rise to colonies. Therefore, although the viability of *A. agilis* was affected adversely by the presence of a large number of caulobacters, the effect cannot be correlated with attachment.

The suspensions were centrifuged and resuspended in Burk's nitrogen-free medium (50). The counts of colony-forming units of caulobacters in the two cultures in Burk's medium are presented in Fig. 25. Attachment to *A. agilis* did not enhance the growth of CB2S2 over that which occurred in the pure culture, probably at the expense of reserve nitrogenous material. The viable count of *A. agilis* began to increase in the pure culture after 8 hr, and in the two-membered culture after 15 hr. The generation time (2.9 hr) and the ultimate viable count (2×10^7 per ml) were the same in the two cultures.

If a capacity for parasitism were possessed by

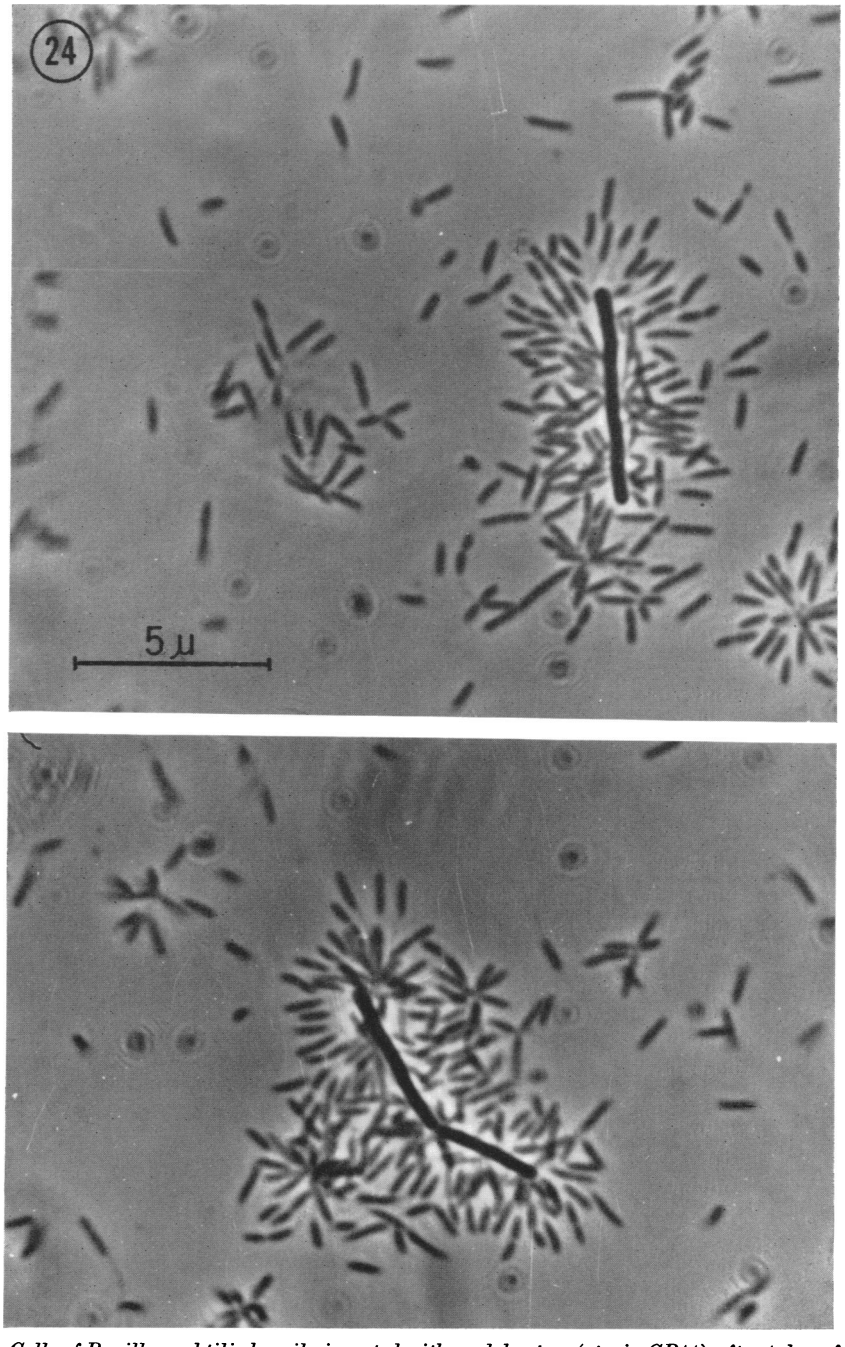

FIG. 24. *Cells of Bacillus subtilis heavily invested with caulobacters (strain CB11) after 1 day of incubation of the two-membered culture. Phase contrast.*

caulobacters, their attachment to other bacteria would result in an enhancement of *Caulobacter* growth, accompanied by lysis of the invested cells. Only if these two effects can be demonstrated would the conclusion be justified that caulobacters can parasitize other bacteria.

The results of the quantitative experiments reported here are at variance with such a con-

clusion. With each gram-positive species of bacteria tested, conditions which permitted frequent and multiple attachment of caulobacters allowed growth of the test bacteria to proceed just as in a pure culture grown under the same conditions; and, in the case of *B. subtilis* with *Caulobacter* strain CB11, the growth of the test species was enhanced by the presence of the caulobacters, an effect attributable to a shift of pH resulting from the caulobacters' growth. The only adverse effects observed were exerted on caulobacters—by *S. lutea* on strain CB11, and by *B. megaterium* on strain CB-H. *B. megaterium* also suppressed the growth of strain CB2 when glucose was present in the medium.

TABLE 7. *Counts of colony-forming units and microscopic counts of Azotobacter agilis and Caulobacter strain CB2S2 in agitated flasks of standard medium at 30 C**

Type of culture	Viable count per ml after incubation for		Per cent decrease
	0 min	20 min	
Pure culture of *A. agilis*	6.6×10^8	5.0×10^8	24
Two-membered culture			
A. agilis	6.7×10^8	2.1×10^8	69
CB2S2	2.5×10^9	1.5×10^9	41
Pure culture of CB2S2	2.6×10^9	2.3×10^9	12

* Counts of *A. agilis* were performed on plates of Burk's medium (50), of CB2S2 on standard medium containing streptomycin (0.1 mg/ml). In the two-membered culture after 20 min of incubation, 31% of the *A. agilis* cells were invested and 27% of the CB2S2 were attached.

The one instance in which an adverse effect on the test species was observed due to the presence of caulobacters was in the experiment with the gram-negative bacterium *A. agilis*. However, the effect was not dependent upon attachment of the caulobacters to *A. agilis*, and attachment to *Azotobacter* did not allow the caulobacters to grow in the nitrogen-free medium.

The results of the present studies on the alleged ability of *Caulobacter* to parasitize other bacteria have been uniformly negative, and they suggest that the attachment of *Caulobacter* cells to other bacteria is not a manifestation of parasitism. One cannot be certain that Houwink and Hund and Kandler did indeed observe such a parasitic phenomenon, since the evidence they presented is inconclusive.

Attachment to Protozoa, Algae, and Yeasts

Swarmer cells of *Caulobacter* strain CB2 were used to prepare two-membered cultures in half-strength standard medium with cells from growing cultures of each of the following microorganisms, which were provided by W. Balamuth: *Chlamydomonas reinhardi* (+), *C. reinhardi* (−), *Euglena minima*, *Ochromonas danica*, *Tetrahymena pyriformis*, *Tetramitus rostratus* (amoeba form), *Acanthamoeba castellanii*, *Crithidia oncopelti*, *Astasia longa*, and *Chilomonas paramecium*. Caulobacters attached to all but the last two of the test organisms within the 7-hr period of observation.

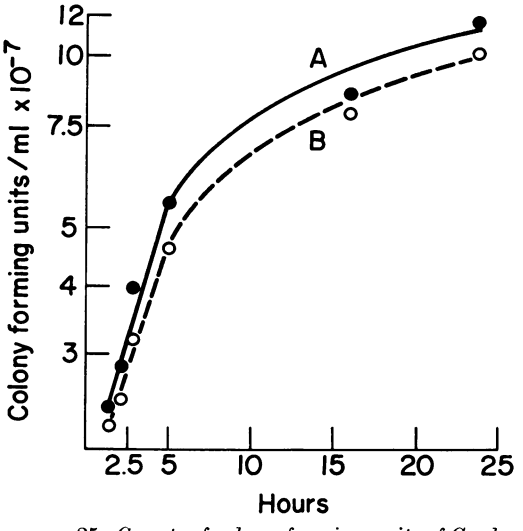

FIG. 25. *Counts of colony-forming units of Caulobacter strain CB2S2 in agitated liquid cultures in Burk's medium at 30 C. A = pure culture. B = two-membered culture with Azotobacter agilis.*

Most of the algal and protozoan cultures initially contained some cytologically abnormal cells; these were particularly susceptible to the attachment of caulobacters. In two-membered cultures of CB2 with *Ochromonas* and the *Chlamydomonas* strains, attachment to dead or damaged cells was far more frequent than to normal, motile cells; similarly, only cytologically aberrant, but not normal, cells of *Tetramitus* and *Tetrahymena* were invested. In the case of motile *Tetrahy-*

mena, the activity of the cilia prevented contact of the caulobacters with the cell surface and may have been responsible for the failure of caulobacters to attach to the motile cells. In contrast, only normal *Acanthamoeba* and *Euglena* cells served as attachment substrata; all *Crithidia* cells in the cultures appeared normal and were frequently invested with caulobacters.

Attachment of caulobacters to the test microorganisms usually occurred at any point on the surface of the test cell, but two evidences of specificity of caulobacters for restricted areas were observed. Caulobacters were seen attached to the flagella of only two organisms—*Crithidia* and the *Chlamydomonas* strains. Secondly, attachment to the cell surface was restricted to the posterior pole of *Euglena*, but to the anterior pole of *Crithidia* cells.

Attachment to yeast cells was tested with two-membered cultures of *Caulobacter* strain CB2 and *Saccharomyces cerevisiae*, *S. ellipsoideus*, a strain tentatively identified as *Nadsonia fulvescens*, and an unidentified budding yeast. Only rarely were invested cells observed, and these were almost always nonbudding.

CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS

Growth on Solid Media

On the surface of standard medium (tap water containing 0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% agar), colonies are circular, convex, and glistening. Some strains form colorless colonies, others yellow or orange. The maximal colony diameter varies somewhat with the strain, but usually reaches 3 to 5 mm after several days of incubation.

Growth in Liquid Media

In stationary broth cultures, most of the growth occurs in the form of a pellicle that develops at the surface of the medium and adheres firmly to the wall of the culture vessel. The pellicle contains motile cells, a few individual stalked cells, and large numbers of rosettes. In liquid cultures incubated on a rotary shaker, the cells are evenly dispersed throughout the culture. Therefore, growth may be measured turbidimetrically. The growth yield in shaken liquid cultures is roughly proportional to the peptone concentration from 0.1 to 1.0%. However, at peptone concentrations of 0.5% or greater the cells are unusually elongated, and their stalks are very short; the proportions of motile cells and of cells in rosettes are reduced. These observations confirm Houwink's (29) conclusion that media with a low organic content should be employed for the cultivation of caulobacters, if cells of normal form are to be obtained.

In aging cultures in standard medium, particular morphological changes occur. Cells may elongate into irregular filaments; if the cells are normally vibrioid, the filaments will be spiral. Two other forms are seen which seem to be due to difficulty in completing the separation of sister cells after division. Chains of cells may be formed, sometimes with stalks developing between cells in the chain. More frequently, the outer cell develops its stalk while still connected with the basal stalked cell; when this occurs in cells in rosettes, unattached stalks are seen protruding from the rosettes. Such cells have not been seen to complete the separation of the two siblings. In cultures in which any of these forms are frequent, rosette formation does not seem to occur and motile cells are at best rare.

Growth Rates

For growth rate determinations, liquid cultures were incubated at 30 C on a rotary shaker in flasks fitted with side arms of the dimensions of tubes used in the Klett-Summerson colorimeter. The turbidity of growing cultures could thereby be measured directly on the culture flask. A red filter (ca. 660 mμ) was used in all determinations. Dilution curves were prepared for representative strains so that Klett units could be corrected to reflect the dry weight of cell material suspended.

For the determination of growth rates by viable count, samples of liquid cultures diluted in standard medium or in tap water were mixed with 2-ml portions of melted agar medium at 45 C and poured onto plates of solid medium.

The determination of viable counts is complicated by the occurrence of rosettes in all cultures. In some instances, the first dilution of the culture to be plated was treated for 30 or 60 sec in a low-speed Waring Blendor; this resulted in an increase of viable count over that of the untreated suspension, and microscopic observation revealed that almost none of the cells were in rosettes after the blending treatment.

Release of cells from rosettes occurs by breakage of the stalks; intact cells are not liberated from the holdfast material (see above). The in-

crease in viable count obtained by the blending treatment does not account for all of the cells released in this manner, either because not all cells in rosettes are viable or because breakage of the stalk kills some of the liberated cells. Consequently, although the blending treatment increases the viable count by disaggregating the rosettes, it does not necessarily give a measure of the total viable population. No method of obtaining true total viable counts on populations other than suspensions containing only swarmer cells has been discovered.

The generation times of caulobacters in standard medium at 30 C vary from 1.4 to 5 hr, depending on the strain. The higher growth rates occur in the vibrioid and excentral strains. Although growth rates have not been determined for all strains, this generalization can be made on the basis of observations on cultures prepared for purposes other than measurements of growth rate.

Relations to Oxygen

The distribution of growing cells throughout an agitated liquid culture and the accumulation of healthy-appearing cells at the surface of stationary cultures clearly indicate the importance of an adequate supply of air for normal growth. Several strains reduce nitrate to nitrite when cultivated in standard medium containing 0.2% $NaNO_3$, plus 3% NaCl for marine isolates, in the presence of air. Some of these strains and certain others reduce nitrate anaerobically; growth occurs anaerobically but is soon halted, apparently owing to the accumulation of nitrite, which is not reduced. The presence of nitrate and nitrite was determined according to the procedures of the *Manual of Microbiological Methods* (63).

None of the strains can grow under anaerobic conditions in complex media (standard medium, or tap water containing 0.5% peptone and 0.2% yeast extract) supplemented with 0.2 or 0.3% glucose.

Catalase production was tested by smearing a small amount of growth from a 2-day slant culture on a clean microscope slide, adding a drop of 3% H_2O_2, and observing for the evolution of oxygen. This test was positive in all strains.

Effect of pH on Growth

In standard medium prepared in 0.05 M sodium phosphate buffers, growth can occur at 30 C over a range of pH values from 6.1 to 7.8, and is optimal at 6.5. On either side of the optimum, development of the pellicle on stationary liquid cultures is depressed; the cells are more elongated and relatively few are motile. The pH of unbuffered standard medium is 6.5.

Effect of Temperature on Growth

Cultures of fresh-water, soil, and millipede isolates were routinely incubated at 30 C. Growth appears normal, but slower, at 25 C. The growth of two strains was tested at 37 C in agitated liquid cultures. The vibrioid strain, CB2, grew at the rate usually observed at 30 C, and the cells appeared normal. The growth of the second strain (bacteroid, CB11) was somewhat slower than at 30 C, as determined by turbidity measurements; most of the cells were elongated, and motile cells were absent.

The marine isolates, which grew in enrichment cultures at 13 and 19 C, grew more rapidly at 25 C. Growth was somewhat slower at 28 than at 25 C.

Antibiotic Sensitivity

Droplets of cell suspensions were placed on plates of standard medium containing streptomycin (0.1 mg/ml) or penicillin G (1,000 or 5,000 units per ml); the marine isolates were tested on antibiotic media prepared with 0.1% peptone in a 3% NaCl solution. In general, streptomycin and the higher concentration of penicillin G inhibited the growth of caulobacters. Only one isolate, obtained from a millipede gut, could grow in the presence of streptomycin at this concentration. This isolate, together with a few others, could grow in the presence of the lower concentration of penicillin G.

Inorganic Constituents of Media

The base for the complex medium routinely used for the cultivation of the caulobacter strains is tap water supplemented with 0.02% $MgSO_4 \cdot 7H_2O$. The customary addition of a sodium-potassium phosphate buffer mixture was abandoned after the observation that the growth of some strains is adversely affected by this addition to complex media before autoclaving.

The $MgSO_4$ supplement is, however, essential to obtain good growth of morphologically normal cells in standard medium.

As a preliminary to the development of a suitable mineral base for synthetic media, growth in standard medium prepared in four different mineral base solutions was compared with that in the Mg-tap water medium. The chelated mineral base of Hutner (9) proved to support as good growth as Mg-tap water and was, therefore, adopted as the base for synthetic media. The carbon sources used in the synthetic media were added aseptically to the mineral base; possibly for this reason, phosphates could be used in synthetic media at a concentration of 0.02 M without adverse effect on growth.

Many of the isolates from fresh water can grow in 0.1% peptone-Mg-tap water medium containing 2% NaCl. This is true of all isolates from seawater, none of which grows in NaCl-free media and some of which can grow in the presence of 4% NaCl. The sensitivity of marine isolates to dilution of seawater was tested by inoculating liquid peptone media prepared in varying ratios of seawater and Mg-tap water. Two strains were studied further by seeding soft-agar (0.4%) media with cells growing in full-strength seawater medium and in the lowest concentration of seawater in which growth occurred. Some evidence of increased tolerance to lowered seawater concentration was observed in both strains studied (Fig. 26). The plates were counted after 6 days of incubation at 25 C.

Organic Growth Factor Requirements

Only 7 of the more than 50 caulobacter isolates of terrestrial or freshwater origin can be cultivated in a mineral medium with glucose or glutamate as the only organic substrate. The mineral base formulated by Hutner and modified by Cohen-Bazire et al. (9) was prepared at double strength and the vitamins were omitted; this base, chelated with ethylenediaminetetraacetic acid and nitrilotriacetic acid, contains Ca, Mg, Fe, and trace metals. This solution was used in the following mineral medium (per liter of demineralized water): 0.5 g of NH_4Cl, 1.74 g of Na_2HPO_4, 1.06 g of KH_2PO_4, and 10.0 ml of concentrated mineral solution. A 10% solution of the carbon source was sterilized by filtration and added aseptically to the mineral medium to a final concentration of 0.2%. When a solidified medium was desired, demineralized water with 2% agar (Difco) was autoclaved and mixed aseptically with an equal volume of double-strength mineral medium.

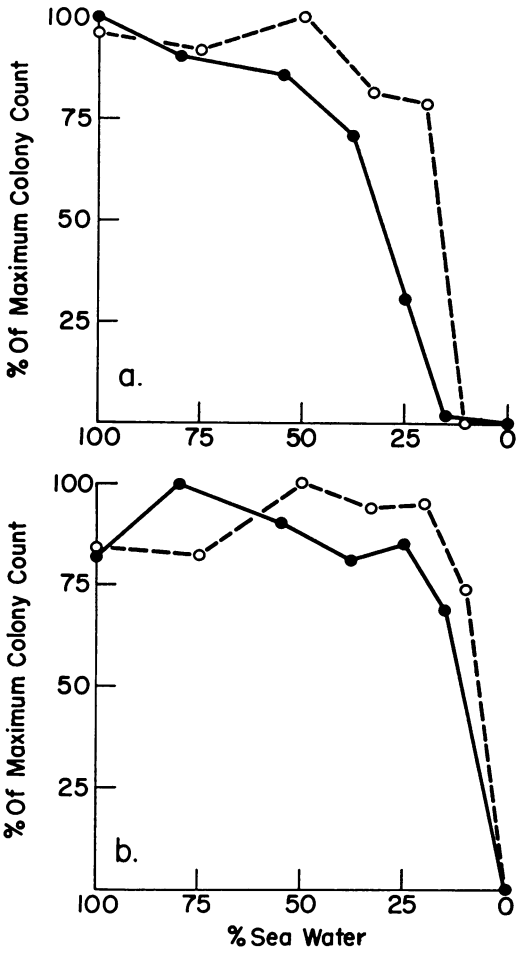

FIG. 26. *Influence of different seawater concentrations on the viable counts of marine caulobacters in 0.1% peptone media. (a) Strain CM11 plated from cultures in 100% (●) and 30% (○) seawater media. (b) Strain CM13 plated from cultures in 100% (●) and 20% (○) seawater media.*

Caulobacters were screened for response to growth factors by spotting droplets of cell suspensions on solid synthetic media supplemented with mixtures of vitamins, purine and pyrimidine bases, and amino acids. The compounds tested are listed in Table 8. If a favorable effect

was noted, growth experiments in liquid synthetic media containing glucose and one or more of the components of the mixtures were conducted to establish which ingredients were essential. A specific growth factor requirement was inferred when a constant growth rate was maintained through at least three subcultures if, and only if, the medium was supplemented with that factor.

In this way, the growth factor requirements of ten caulobacter strains were determined. Each of these strains requires a single vitamin: six strains require biotin, three require vitamin B_{12}, and one requires riboflavine. The remainder of the isolates have not been cultivated in defined media. Only 17 of these have been screened as described above; the failure of these 17 strains to respond to any of the mixtures indicates that their requirements are complex or involve materials which were not tested.

TABLE 8. *Compounds tested as growth factors for caulobacters*

Compound	Concn (μg/ml of medium)
Biotin	0.0002
Vitamin B_{12}	0.04
p-Aminobenzoic acid	0.05
Nicotinic acid, calcium pantothenate, riboflavine, and thiamine·HCl	0.1
Pyridoxine·HCl	0.2
Adenine, guanine, uracil, cytosine, and thymine	10.0
Casamino Acids, glycine; as L isomers: alanine, arginine, aspartate, cystine, glutamate, histidine, isoleucine, lysine, proline, serine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine	200.0

Utilization of Carbon Compounds

Owing to the complexity of the nutritional requirements of most of the caulobacter strains, carbon compounds to be tested as growth substrates were added to a basal medium of the following composition (per liter of tap water): NH_4Cl, 0.5 g; Na_2HPO_4, 1.74 g; KH_2PO_4, 1.06 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; peptone (Difco), 0.05 g; yeast extract, 0.05 g; and agar (Difco), 10.0 g. The low concentration of complex organic materials supports discernible growth of caulobacters in the absence of any added carbon source. For determinations with riboflavine-requiring strains, the medium was further supplemented with riboflavine (0.1 μg/ml). NaCl (3%) was present in the medium for determinations with marine isolates.

Each compound was tested at concentrations of 0.05 and 0.1%. The plates were inoculated as in the growth-factor studies. Utilization of a given compound was assumed to have occurred when growth was distinctly heavier in its presence than on the basal medium alone. Inhibition by some compounds was observed at the higher concentration only.

The utilization of starch was inferred from the ability of a strain to hydrolyze this material. Plates of standard medium containing 0.2% soluble starch were inoculated with droplets of cell suspensions. Hydrolysis was tested after 48 hr of incubation by flooding the plate with Lugol's iodine solution (63) and observing for colorless areas beneath and around the circles of growth. Some strains which appeared on the dilute complex medium to be able to utilize starch were not demonstrably hydrolytic by this test (see Table 14).

No one compound was utilized by every isolate. The compounds tested and their utilizability by the caulobacter strains are presented in Table 9. Cellulose, supplied as filter paper, was not used. Several strains were tested on poly-β-hydroxybutyric acid, but none was able to hydrolyze the polymer.

Acid production from sugars during aerobic growth was tested in standard medium containing 0.001% bromothymol blue as indicator, and 0.2% sugar. Only two strains, both of which are of the excentral type (AC12 and KA4), produced acid. Acid was produced from glucose and galactose by both strains, and from xylose by AC12. Generally, the medium became alkaline, probably owing to ammonification of nitrogenous materials present in the complex medium employed.

Intermediary Metabolism of Carbohydrates

Five strains (CB2, CB6, CB9, CB11, and AC12), each of which can utilize glucose and xylose, were selected for experiments designed to determine the presence of enzymes involved in the metabolism of carbohydrates. Cells were grown in standard medium supplemented with 0.1% glucose or xylose and harvested when the cultures had reached early stationary phase.

After being washed twice with tap water, the cells were frozen. The thawed cells were resuspended in 0.05 M sodium-potassium phosphate buffer (pH 7.0) to approximately 100 mg (dry weight) per ml and treated in a 10-kc sonic oscillator for 20 to 30 min. The sonic extract was centrifuged at 100,000 × *g* for 60 min. The supernatant liquid was aspirated from the particulate fraction, assayed for protein by the method of Lowry et al. (47), modified by a 60-min alkaline digestion of the sample, and stored at −20 C. Protein content of extracts from different strains was 2.5 to 6.9 mg/ml.

Enzyme assays were performed on the extracts thawed at 0 C. Dehydrogenase activities were determined by the procedure of DeMoss (10). Each of the extracts was tested for reduced-cofactor dehydrogenase activity; in each case, the activity was insignificant or absent, obviating correction for endogenous cofactor dehydrogenation. The procedures of MacGee and Doudoroff (48) and Kovachevich and Wood (39) were employed for the assays of 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) aldolase. The purified enzymes required in these assays were obtained from C. F. Boehringer and Soehne GmbH, Mannheim, Germany, as lactic dehydrogenase (LDH15145) and triosephosphate dehydrogenase (GAPDH15146). The results are presented in Table 10.

The presence of hexose-phosphate dehydrogenases and KDPG aldolase in all extracts, and the increase of the nicotinamide adenine dinucleotide phosphate (NADP)-linked glucose-6-phosphate dehydrogenase activity in extracts of cells grown in the presence of glucose imply that caulobacters metabolize sugars by the Entner-Doudoroff pathway (13, 48). Preliminary attempts to assay fructose-1,6-diphosphate aldolase in the extracts of CB2 and AC12 by testing for the production of triosephosphate were unsuccessful.

The hexose-phosphate dehydrogenases of CB2 and AC12 appear to be linked to either nicotinamide adenine dinucleotide (NAD) or NADP. Dilution of the extracts does not change the specific activity with either cofactor, indicating that the lower activity with NAD is not due to transhydrogenase activity. When both cofactors are present in the reaction mixture, the rate of the reaction is the same as in the presence of NADP only. It is concluded, therefore, that a single dehydrogenase is present whose affinity is greater for NADP than for NAD.

The NAD-linked xylose dehydrogenases of CB2 and AC12 appear to be induced when xylose is present in the growth medium. The other three strains do not have significant soluble xylose dehydrogenase activity, whether or not the cells are grown in the presence of xylose.

TABLE 9. *Utilization of carbon compounds by caulobacters*

Utilization	Compound
Commonly utilized	Starch, xylose, glucose, galactose, mannose, maltose, sucrose, alanine, aspartate, glutamate, proline, serine, *n*-butanol, acetate, butyrate, pyruvate, succinate, fumarate
Not utilized	Erythritol, mannitol, dulcitol, sorbitol, glycine, methionine, tryptophan, formate, oxalate, vanillin, benzoate, and anthranilate
Utilized by a minority of isolates	Arabinose, ribose, fructose, lactose, gluconate, arginine, cystine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tyrosine, valine, propionate, pimelate, lactate, malate, adipate, citrate, tartrate, methanol, ethanol, *n*-propanol, *t*-amyl alcohol, benzyl alcohol, glycerol, *p*-hydroxybenzoate, *p*-aminobenzoate, vanillate, and nicotinate

These preliminary experiments on the soluble enzymes of caulobacter cells reveal that these bacteria can metabolize sugars in a manner known to occur in *Pseudomonas* species, viz., the aerobic *P. saccharophila* (13, 48) and *P. fluorescens* (39, 40, 73), and the fermentative *P. lindneri* (*Zymomonas mobilis*) (18), and that may be present in *Acetobacter*, *Azotobacter*, and *Escherichia* (24, 40).

Reserve Materials

Caulobacter cells incubated for 36 to 48 hr in sodium-potassium phosphate buffer (0.02 M, pH 7.0) with glucose, but without a source of assimilable nitrogen, often contain large, spherical, refractile granules which are stained by Sudan Black B by the procedure of Burdon (63). Polysaccharide materials, if present, are not detectable

when these cells are stained by the method of Hotchkiss (28).

Specific tests for the formation of polysaccharide and poly-β-hydroxybutyric acid by two strains (CB2 and CB6) were carried out by a procedure similar to that of Stanier et al. (64). Cell suspensions were incubated in distilled water containing 0.2% glucose on a shaker at 30 C for 12 hr. Samples of the suspensions were centrifuged, and the cells were washed twice with distilled water. Polysaccharide was measured as acid-hydrolyzable glucose, determined enzymatically with glucose oxidase (64). After removal of ether-soluble lipids, poly-β-hydroxybutyric acid was determined gravimetrically by the alkaline hypochlorite-hot chloroform method of Williamson and Wilkinson (72). The results of the determinations are presented in Table 11.

Both strains accumulated poly-β-hydroxybutyric acid, accounting for more than 40% of the dry weight increase during the 12-hr incubation. Although polysaccharide was not detectable cytochemically in strain CB2, a small amount was detected chemically.

The storage of poly-β-hydroxybutyric acid has been demonstrated in several other species of aerobic gram-negative bacteria (12, 17, 45), as well as in *Bacillus* species (44, 72). The simultaneous accumulation of polysaccharide and poly-β-hydroxybutyric acid reserves, observed in strain CB2, has been demonstrated by Stanier et al. (64) in *Rhodospirillum rubrum*.

TABLE 10. *Specific activities in crude extracts of caulobacter cells grown in standard medium supplemented with glucose or xylose**

Enzyme	Dehydrogenases								KDPG Aldolase	
Substrate	Glucose		Xylose		G-6-P		F-6-P		KDPG	
Cofactor	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP		
Assay for									Pyruvate	Triose-P
Strain CB2										
Glucose	<1	0	1	<1	27	74			45	71
Xylose	2	0	46	2	6	17			86	73
Strain CB6										
Glucose	0	0	0	0	16	20			15	9
Xylose	0	0	2	0	9	9			13	2
Strain CB9										
Glucose	0	0	0	0	7	32			19	9
Xylose	0	0	0	0	2	15			25	22
Strain CB11										
Glucose	4	0	4	0	13	36			32	33
Xylose	0	0	0	0	19	16			60	9
Strain AC12										
Glucose	0	0	3	0	128	210	54	107	102	56
Xylose	0	0	35	0	50	91			29	22

* Micromoles × 10^3 of substrate disappearing per minute per milligram of protein. Abbreviations: G-6-P = glucose-6-phosphate; F-6-P = fructose-6-phosphate; KDPG = 2-keto-3-deoxy-6-phosphogluconate; NAD = nicotinamide adenine dinucleotide; NADP = nicotinamide adenine dinucleotide phosphate; Triose-P = glyceraldehyde-3-phosphate.

Pigments

Cells of pigmented *Caulobacter* strains were harvested from liquid cultures in standard medium, washed twice with tap water, and frozen. Thawed cells were resuspended in methanol or acetone-methanol (3:1, v/v), which resulted in immediate extraction of the pigments from the cells.

The absorption spectra of methanol extracts of

two *Caulobacter* strains are illustrated in Fig. 27. The spectra of extracts of all the yellow strains are essentially the same as that of CB10, whereas extracts of orange strains yield spectra similar to that of CB11. Although the pigments have not been identified chemically, the absorption spectra suggest that they are carotenoids.

The pigments in the alcoholic extracts can be transferred to petroleum ether; the transfer is accompanied by very little change in absorption spectra. Paper and column chromatograms reveal the presence of four to ten components in the ether solutions, the number being greater in extracts of orange than of yellow strains. Some of the components may well be isomerization or oxidation products of the naturally occurring pigments.

TABLE 11. *Accumulation of reserve materials by two Caulobacter strains during 12 hr of incubation in distilled water containing 0.2% glucose*

Determination	Strain CB2		Strain CB6	
	Initial	12 hr	Initial	12 hr
Dry weight (mg/ml)	6.36	7.70	6.70	7.79
Increase (mg/ml)	1.34		1.09	
Per cent increase	21.0		17.8	
Polysaccharide	0.04	0.22	0.04	0.02
Per cent of dry weight	<0.1	2.8	<0.1	<0.1
Increase (mg/ml)	0.18		−0.02	
Per cent of dry weight increase	13.4		—	
Poly-β-hydroxybutyrate (mg/ml)	0.63	1.67	0.23	0.69
Per cent of dry weight	9.9	21.7	3.4	8.9
Increase (mg/ml)	1.04		0.46	
Per cent of dry weight increase	77.6		42.2	

BACTERIOPHAGES

Jean Schmidt (*in preparation*) isolated several bacteriophages from chloroform-treated pond water and chlorinated sewage plated on various strains of caulobacters from the author's collection. Filtrates with lytic activity were provided by Miss Schmidt, and the specificity of the viruses for various caulobacter strains was tested by placing a droplet of virus suspension containing 10^6 to 10^8 plaque-forming units per ml on an overlayer plate of standard medium seeded with the test strain. Plates were incubated at 28 C and observed at 2 and 5 days for areas of lysis in the lawn of bacterial growth. The respective host ranges of the bacteriophages are presented in Table 14.

The specificity for caulobacters of the viruses isolated by Miss Schmidt was revealed in a survey performed by M. P. Starr and G. Cosens (*personal communication*). These bacteriophages do not lyse any of 39 species of *Pseudomonas*, six species of *Xanthomonas*, six species of *Flavobacterium*, and two species of *Erwinia*. Similarly, Starr and Cosens found that none of the caulobacter isolates is sensitive to any of 45 *Pseudomonas*, 18 *Xanthomonas*, and 2 *Erwinia* bacteriophages. Thus, caulobacters and the bacteriophages which attack them have remained as an isolated group of hosts and parasites within the natural environment which they share with these other bacteria and their respective viruses.

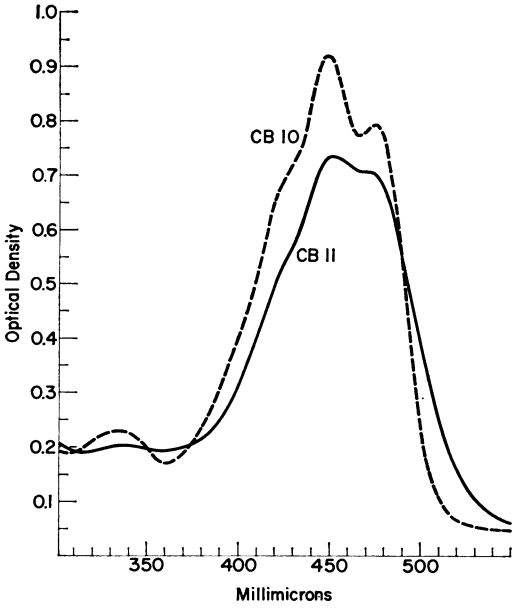

FIG. 27. *Absorption spectra of methanol extracts of yellow (CB10) and orange (CB11) Caulobacter strains.*

ECOLOGICAL INTERPRETATIONS

From our pure-culture studies, the caulobacters have emerged as a somewhat fastidious group of

aerobic chemoheterotrophs. Even under optimal growth conditions, the growth rates of most strains are relatively low; many strains require growth factors; and, for any given strain, the number of compounds that can be used as principal carbon sources is usually fairly limited. These observations immediately pose an interesting ecological question. How can one explain the widespread occurrence of the caulobacters in natural waters where they are in competition with other bacteria, notably members of the *Pseudomonas* group, which belong to the same broad nutritional category but grow more rapidly and are much less restricted nutritionally?

One feature that distinguishes the caulobacters from most other aquatic chemoheterotrophic bacteria is their capacity for attachment to solid surfaces as a result of the localized secretion of adhesive material at one pole of the cell. This holdfast enables them at attach to other microorganisms. We have found caulobacters in water samples attached to green and blue-green algae, diatoms, and to other bacteria.

Such attachment can be interpreted as a means by which the caulobacters establish themselves in close association with a continuous source of nutrients in the form of organic substances excreted by other organisms during their normal growth. Such excretion has been directly demonstrated in algae (1, 16, 46). Accordingly, it is suggested that the caulobacters should be regarded as ectocommensals of other microorganisms.

Ectocommensalism is a mode of existence that also occurs in certain higher protists, notably the trichomycetes and trichomycete-like fungi. These microorganisms secrete holdfasts by means of which they attach to snails (*Oedogoniomyces*), aquatic insects (*Amoebidium parasiticum*), algae (*Harpochytrium*), and to other trichomycetes (*Paramoebidium*) (71*a*, *b*, *c*). The holdfast of *Oedogoniomyces* is illustrated in Fig. 28a. In Fig. 28b, thalli of *A. parasiticum* grown in pure culture seem to have become attached to each other's holdfasts in a manner similar to that resulting in the formation of rosettes by caulobacters in laboratory cultures. The analogous structure and modes of attachment in caulobacters are illustrated in Fig. 1–6, 10–12, 21, 22, and 24.

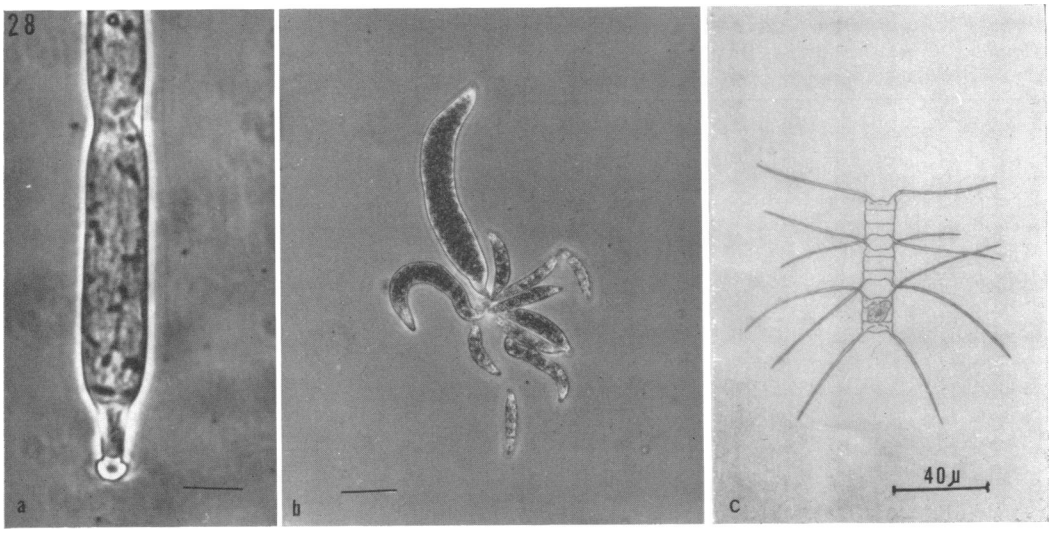

FIG. 28. *Eucaryotic protists showing analogies with caulobacters. Courtesy of H. C. Whisler. (a) The holdfast end of a thallus of Oedogoniomyces. Phase contrast (see Fig. 15c of reference 71b). (b) Thalli of the trichomycete Amoebidium parasiticum grown in pure culture, apparently in a rosette. Phase contrast (see Fig. 2B of reference 71c). (c) The appendages of the diatom Chaetoceros brevis (line drawing after Fig. 82 of reference 9a). The marker in Fig. 28a is 20 μ; in Fig. 28b and c, 40 μ.*

Henrici and Johnson (26) had demonstrated the ability of caulobacters to attach to inanimate materials by introducing artificial substrates (microscope slides) into the natural environment. They inferred from this that the caulobacters

exist in nature as sessile bacteria attached to inanimate submerged surfaces. Zobell (79), who observed the attachment of marine bacteria to glass surfaces, suggested that organic substances may be adsorbed on surfaces, this adsorption serving to concentrate nutrients in an aquatic environment. Accordingly, he interpreted the sessile habit as one which serves to provide a microenvironment richer in nutrients than the surrounding water. Although the caulobacters can be observed attached to submerged microscope slides, they are not found on submerged natural materials such as twigs, and such materials do not serve as inocula for enrichment cultures for caulobacters. The only submerged natural objects to which we have found caulobacters attached are microbial cells, and in several instances we have used these successfully as sources of caulobacter isolates. Thus, although caulobacters may in some instances attach to inanimate substrates, attachment to other microorganisms can reasonably be considered of much greater importance in their ecology.

Another factor which probably contributes significantly to the maintenance of caulobacters in natural aquatic environments is their capacity for survival in water of very low organic content. This is indicated by their prominence in samples of tap and distilled water; we have also observed that the number of caulobacters increases with the duration of storage of the samples. This increase occurs in the presence of very low numbers of other microorganisms and is evidence that the ectocommensalism of caulobacters suggested above is facultative.

The most distinctive structural feature of caulobacters is the development of cellular stalks. Stalk formation does not appear to contribute to the establishment of an ectocommensal relationship, since attachment is mediated by the holdfast, formed prior to stalk development. The functional distinctness of stalk and holdfast is particularly evident in the excentral caulobacters, where adhesion by the holdfast occurs at a site on the cell surface different from that at which the stalk is formed. At least in these strains, the stalk clearly has no function in the process of attachment. It is, therefore, necessary to consider other possible interpretations of the adaptive value of a stalk.

One demonstrable consequence of the presence of a stalk on the caulobacter cell is enhanced resistance to sedimentation. This effect underlies the procedure (see above) for the centrifugal segregation of stalked and swarmer cells. Observations on natural samples and enrichment and pure cultures show that the caulobacters habitually accumulate at the air-water interface. This is a common pattern of behavior for motile, strictly aerobic bacteria, resulting from their positive aerotaxis. However, unlike most such bacteria, the caulobacters are motile during only a small fraction of their life cycle, since motility is lost as soon as stalk development begins (see above). The stalk could play the role of a suspensory organelle in the nonmotile stage of the life cycle and thus tend to maintain the cells close to the air-water interface to which they gained access during the brief interlude of motility. The transitory appearance of a flagellated stage in each generation would then provide a means by which one of the daughter cells could relocate itself, if necessary, close to the air-water interface.

Development of specialized organelles which retard settling in an aquatic environment by offering a physical obstacle to the effect of gravity is a characteristic feature of many groups of pelagic higher protists. Particularly striking structural specializations are found in the diatoms and the dinoflagellates. Cells of diatoms such as *Thalassiosira* and *Chaetoceros* possess long, slender appendages to which a suspensory function could be ascribed. Among the dinoflagellates, the cell may be modified either by the development of long spines as in *Ceratium*, or of an expanded collar as in *Ornithocercus*. Illustrations of the dinoflagellates specialized in these ways may be found in the treatises of Doflein and Reichenow (11) and of Grassé (21*a*). The appendages of *Chaetoceros brevis* are illustrated in Fig. 28c.

Stanier (63*a*) pointed out the parallelism of structural and functional diversification in procaryotic (lower) protists—bacteria and blue-green algae—and in eucaryotic (higher) protists—protozoa, fungi, and plastid-containing algae. The caulobacters provide yet another example of cellular specializations at the procaryotic level which have counterparts in eucaryotic protists.

CLASSIFICATION OF CAULOBACTER

Historical Survey of the Systematic Treatment of the Genus Caulobacter

Henrici and Johnson proposed *Caulobacter* as the single genus of the newly proposed family Caulobacteriaceae, described as follows:

> "Stalked bacteria, the long axis of the elongated cells coinciding with the axis of the stalk. Stalks are slender, flagellum-like, often attached to the substrate by a button-like holdfast, unbranched. Multiplication of cells by transverse-binary fission. The outermost cell of a pair may form a stalk before cell division is complete. Periphytic, growing upon submerged surfaces."

This family was one of four (Caulobacteriaceae, Nevskiaceae, Gallionellaceae, Pasteuriaceae) included in a heterogeneous order, Caulobacteriales, comprising bacteria that grow attached to substrates "characteristically" by means of stalks. The members of Nevskiaceae secrete slime stalks and grow in zoogleal masses; the stalks of Gallionellaceae are encrusted with ferric hydroxide; the Pasteuriaceae form very short stalks or none at all, and multiply by longitudinal fission or by budding.

In the sixth edition of *Bergey's Manual of Determinative Bacteriology* (3), the order Caulobacteriales was reduced to a suborder, Caulobacteriineae, and placed in the order Eubacteriales. The heterogeneity of the new suborder was even greater than that of the former order, since a further family Siderocapsaceae (nonflagellated, nonstalked, encapsulated iron bacteria) was included; the family Pasteuriaceae was removed to an appendix of the suborder because of its mode of multiplication. The new suborder was defined by Breed, Murray, and Hitchens as follows:

> "Non-filamentous, attached bacteria growing characteristically upon stalks, sometimes sessile. The stalked cells are asymmetrical in that gum, ferric hydroxide or other material is secreted from one side or one end of the cell to form the stalk. Multiply by transverse fission. In some species the stalks are very short or absent. In the latter case the cells may be attached directly to the substrate in a zoogloeic mass. Cells occur singly, in pairs or short chains, never in filaments; not ensheathed. Nonsporeforming. Typically aquatic in habitat."

Bowers et al. (2) had concluded that their isolate was identical with *C. vibrioides* Henrici and Johnson, but felt that the sentence describing Caulobacteriineae stalks as secreted structures should be emended to include their observations on the cytoplasmic nature of the core of the *Caulobacter* stalk. No precise redefinition was proposed. Houwink (31) also found fault with the same sentence, restating his own conclusion that the *Caulobacter* stalk is a part of the bacterial cell. He suggested the continued recognition of the order Caulobacteriales Henrici and Johnson and the elimination from it of all families of bacteria whose stalks represent secretions. Hund and Kandler (33) agreed that a sharp division should be made between bacteria possessing secreted stalks and bacteria with stalks which constitute an integral part of the cell such as those formed by *Caulobacter*.

The position of the genus *Caulobacter* is considerably different in the seventh edition of *Bergey's Manual* (4), where *Caulobacter* is placed among the nonphotosynthetic pseudomonads. All genera within the suborder Pseudomonadineae which comprise cells that attach to substrates by means of stalks are placed in the family Caulobacteraceae Henrici and Johnson emend. Breed. The description of this family follows that of Caulobacteriineae with the addition of polar flagellation in the motile stage. Ignoring the recommendations for distinct taxonomic separation of the two known types of stalked bacteria, Breed includes four genera in the family: *Caulobacter*, *Gallionella*, *Siderophacus*, and *Nevskia*.

Present Understanding of the Distinctive Properties of Caulobacters

The single characteristic used by all workers to identify an isolate as *Caulobacter* is the presence of a slender polar stalk on at least some of the cells in the culture. Since the work of Houwink (29, 31) and Bowers et al. (2), it has been clear that the stalk is a part of the cell and is developed at exactly the site at which the flagellum passes through the cell wall. Accordingly, this type of stalk is not homologous with the secreted stalks of *Nevskia*, *Gallionella*, and *Siderophacus*.

The complex structure of the *Caulobacter* stalk has been revealed in the present studies by electron microscopy of ultrathin sections of stalked cells. The wall of the stalk is continuous with that of the cell, and the core is derived from a complex structure continuous with the cytoplasmic membrane. A swarmer cell develops a stalk before completing its first cellular division, as diagrammed in Fig. 29. The alternation of forms has been studied in detail only in one strain (CB2), but cursory observations on microcultures of two further strains (CB7 and AC48) support the conclusion that the dimorphism established for strain CB2 is common to all known types of caulobacters.

The only structures among bacteria which, upon examination by electron microscopy, appear structurally analogous to the caulobacter stalk are the filamentous outgrowths of *Hyphomicrobium* and *Rhodomicrobium*. However, these filaments have a reproductive function, since new cells are formed as buds at the tips of the filaments, whereas the caulobacter stalk does not play such a role.

In our experience, all caulobacter isolates are gram-negative rod-shaped or vibrioid cells which possess a single polar flagellum in the motile stage. Caulobacters share these morphological characteristics with pseudomonads and vibrios in general, and on the basis of these properties can be distinguished from the one other known group of bacteria with stalks which are probably cellular in nature—*Blastocaulis*. This group was described by Henrici and Johnson (26) as gram-positive, permanently immotile cocci which reproduce by budding of the cells; they have not been isolated or examined by electron microscopy.

Many of the physiological properties of caulobacters determined in the present studies are also possessed by the *Pseudomonas-Vibrio* group. The caulobacters are aerobic bacteria which, like the pseudomonas group, utilize a variety of compounds as sources of carbon for growth. Requirements for organic growth factors are common among caulobacter isolates, as among isolates of certain genera of pseudomonads (e.g., *Acetobacter*, *Aeromonas*, and pathogenic species). Aerobic metabolism of hexoses via the Entner-Doudoroff pathway had been demonstrated clearly only in *Pseudomonas* species prior to these studies on caulobacters; the occurrence of this pathway in all five of the strains tested (CB2, CB6, CB9, CB11, and AC12) is a further expression of the close similarity between these two groups of bacteria.

Classifying organisms on the basis of macromolecular similarities is a long-established practice in bacteriology. Historically, however, this has been limited almost entirely to antigenic similarities of proteins and polysaccharides; only relatively recently has sufficient information on the composition of nucleic acids become available so that these compounds can also be studied comparatively. The taxonomic implications of nucleic acid composition are not yet clear, but it is proposed by some workers (see 55, for an excellent discussion of this matter) that the results of study of composition and exchangeability of nucleic acids will permit the establishment of a sound natural system of classification of bacteria. It is of particular interest in the present discussion that the deoxyribonucleic acid (DNA) compositions of caulobacter isolates are similar to those of other genera within the suborder Pseudomonadineae.

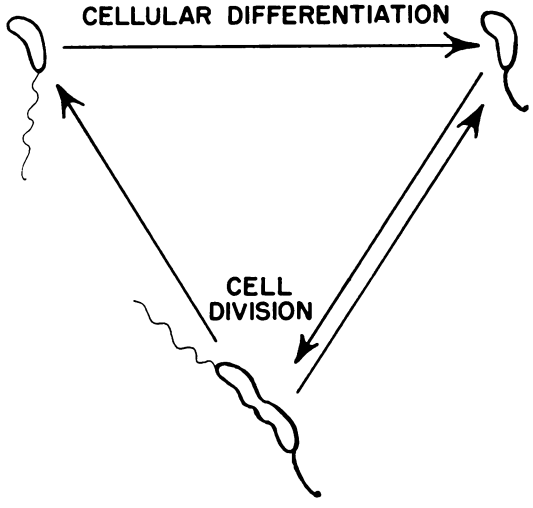

FIG. 29. *Dimorphism in caulobacters.*

The caulobacter DNA base compositions, expressed as the mole per cent of guanine plus cytosine (G + C), are presented in Table 12 (part A), along with the compositions of nucleic acids of pseudomonads (part B). The analyses of caulobacter DNA were performed by M. Mandel, by ultracentrifugation in CsCl gradient at 44,770 rev/min at 25 C, equilibrium in 20 ± 2

hr; calculations of mole percentages were based on buoyant densities of reference DNA molecules of known chemical composition. The data for other bacteria were taken from the compilation of Marmur et al. (49).

With the exception of excentral strain KA4 (*Asticcacaulis*), the G + C content of caulobacters is between 62 and 67%, a range similar to that observed in *Pseudomonas* and *Xanthomonas* species (excepting *P. cruciviae*). The lower value, 55%, found for strain KA4 is found in *Acetobacter* and *Aeromonas*. The only other genus of pseudomonads in which the DNA base composition has been determined is *Halobacterium*, which is represented by species whose G + C contents overlap the two ranges of the other genera. The values which have been determined for *Vibrio* species range from 42 to 62% G + C, revealing a considerable heterogeneity within this group.

TABLE 12. *Mole per cent guanine + cytosine of DNA molecules*

Caulobacter strain*	Morphology	$\frac{G+C}{A+T+G+C}\times 100$†
KA4	Excentral	55
KA3	Vibrioid	62
CB4	Vibrioid	62
CB21	Limonoid	64
KA1	Vibrioid	65
CB-G	Limonoid	65
CB6	Bacteroid	66
CB79	Subvibrioid	67
CB37	Fusiform	67
Species‡		
Pseudomonas cruciviae		40–42
Acetobacter gluconicum, A. ascendens		52–56
Aeromonas shigelloides, A. hydrophila, A. punctata, A. formicans, A. liquefaciens		50–60
Halobacterium IV A_2, IV A_3, II G_2		56–64
Xanthomonas pelargoni, X. hederae, X. phaseoli, X. pisi, X. percolans		62–66
Pseudomonas fluorescens, P. oleovorans, P. fragii, P. chlororaphis, P. stutzeri, P. putida, P. aeruginosa, P. tabaci, P. diminuta, P. maltiphilia, P. saccharophila		60–70

* DNA extracted from caulobacter isolates (Mandel, *personal communication*).

† A = adenine; T = thymine; G = guanine; C = cytosine.

‡ DNA extracted from isolates of genera of Pseudomonadaceae (49).

In summary, the caulobacters are more similar to the pseudomonads than to any other well-defined group of bacteria. The caulobacters and pseudomonads share principal morphological features such as polar flagellation of motile cells and gram-negativity, and certain physiological properties, among which the most significant are the intermediary metabolism of hexoses via the Entner-Doudoroff pathway and the utilization of a variety of carbon sources. Their similarity also includes the base compositions of DNA. However, caulobacters and members of Pseudomonadaceae are distinguished by their respective sensitivity to bacteriophages as revealed in the extensive survey performed by Starr and Cosens (see above), and the probable capacity of caulobacters to exist as ectocommensals of other microorganisms (see above).

Thus, the continued recognition of a family of gram-negative, polarly flagellated bacteria comprising only the stalked forms is justified on the bases of their unique capacity for cellular differentiation, their commensal relationship with other microorganisms, and the existence of bacteriophages which are strictly specific either for caulobacters or for pseudomonads.

Proposals for Revision of the Definitions of Caulobacteraceae and Caulobacter and for Recognition of Another Genus of Stalked Bacteria

The original definition of the family Caulobacteriaceae by Henrici and Johnson in 1935 better suits the observations on caulobacters made during the past 14 years than does the definition as emended by Breed (4). Therefore, it is recommended (i) that the original concept of separation of caulobacters from slime-stalked bacteria at least at the taxonomic level of the family be re-established, (ii) that the corrected spelling as Caulobacteraceae (34, Opinion 2, Appendix c) be retained, and (iii) that the definition of Caulobacteraceae Henrici and Johnson be emended as follows:

> Stalked rod-shaped or vibrioid bacteria, the unbranched stalk arising as an outgrowth of the cell; its wall is continuous with that of the cell, and its core is derived from a system of internal membranes of the cell. The stalked cell is

immotile. The cells are gram-negative. Multiplication occurs by transverse binary fission. The outer sibling of a dividing stalked cell bears either a flagellum or a stalk at its free end before cell division is complete. These bacteria occur as free cells in seawater or freshwater or soil, or attached by means of a secreted holdfast to other microorganisms or to submerged inanimate surfaces.

In light of our present knowledge, two genera would be included within Caulobacteraceae. These are *Caulobacter* Henrici and Johnson and a genus comprising caulobacters whose stalks do not possess adhesive material. A third genus might be proposed to accommodate caulobacters which divide symmetrically so that cell division gives rise to two stalked siblings; such a proposal would require isolation and cultivation of this type so that the absence of motility can be established.

In addition to the common characteristics as given in the revised definition of Caulobacteraceae, the isolates of the two recognized genera which have been included in the present studies share the following properties. They are aerobic and form catalase. Some isolates achieve a limited amount of growth anaerobically by reducing nitrate to nitrite; sugars are not fermented. The temperature range for optimal growth is around 30 C for freshwater and terrestrial isolates; a pH slightly below neutrality is most favorable. The range of utilizable carbon compounds includes sugars, amino acids, nonnitrogenous organic acids, alcohols, and aromatic compounds, but the number of compounds utilized by any individual isolate is limited. The organisms exist as ectocommensals of other microorganisms or as free-living chemoheterotrophs which frequently require organic growth factors. Resting stages (such as endospores or microcysts) are not known to occur.

The following definition of *Caulobacter* is presented in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (4):

"Stalked, curved, rod-shaped bacteria, the long axis of the elongated cells coinciding with the long axis of the stalks. Young cells motile by means of a single polar flagellum. Old cells attached to submerged objects by a stalk that is a continuation of the cell. A holdfast is developed at the distal end. Multiplication of cells is by transverse binary fission. Periphytic, growing upon submerged surfaces."

The definition of the genus can now be revised to include the life cycle and variations in cell shape. All of the freshwater and marine caulobacters obtained to date, in the present studies and by previous workers, have been isolated from water samples, not from submerged surfaces. Therefore, although the habit has previously been noted as periphytic, such a description would be misleading. The revised definition acknowledges the ability of *Caulobacter* cells to attach to solid substrates.

Bacteria of curved- and straight-rod shapes are included within this genus, although other pseudomonads are assigned to different genera on the basis of the curvature of the long axis of the cell. Our studies on the caulobacters reveal that this variation in cell shape does not justify such a division among them; indeed, the editors of *Bergey's Manual of Determinative Bacteriology* have expressed doubt that the distinction between *Pseudomonas* and *Vibrio* should be based on this characteristic (4).

Caulobacter

Unicellular stalked bacteria. The stalk arises from the pole of the cell, and adhesive material is secreted at the distal end of the stalk. Multiplication occurs by division of the stalked cell, giving rise to a nonstalked sibling which is motile by means of a single polar flagellum. This cell secretes adhesive material at the base of the flagellum, develops a stalk at this site, and enters the immotile vegetative phase. The adhesive material allows cells in either phase to attach to other microorganisms, inanimate substrates, or to each other's holdfasts to form rosettes.

The cells are rod-shaped or vibrioid. They may be colorless or contain yellow or orange pigments related to carotenoids. Several morphological types exist which are distinguished from each other by cell shape. The deoxyribonucleic acid is composed of approximately 65% guanine plus cytosine.

The definition of the second genus of caulo-

bacters is based on the characteristics which have been determined for four isolates; three of these (AC12, AC47, and AC48) were obtained from pond water during the present studies and one (KA4) was provided by Dr. Bachmann. (Two additional strains that appear to belong to the same genus have been isolated by J. L. Pate, at the University of Washington, who has kindly allowed us to examine them. One of these isolates clearly resembles the strains which we have studied; the other differs from all our isolates in both morphological and nutritional properties.) These isolates differ from the *Caulobacter* isolates in two significant properties other than those used as diagnostic distinctions between the two genera. First, none of the excentral isolates is sensitive to the bacteriophages which are lytic for several *Caulobacter* strains (see Table 14); second, the DNA base composition of the one excentral strain tested differs significantly from those of *Caulobacter* isolates (see Table 12). We propose the genus *Asticcacaulis*, defined as follows.

Asticcacaulis

Unicellular stalked bacteria. The stalk arises from a site on the cell which is not coincidental with the center of the pole of the cell. The stalk does not possess adhesive material. Multiplication occurs by division of the stalked cell, giving rise to a nonstalked sibling which is smaller than the stalked sibling and is motile by means of a single flagellum which arises in an eccentric position on the pole of the cell. This cell develops a stalk and enters the immotile vegetative phase. Adhesive material is secreted by cells in both phases at or near the pole of the cell at a site different from that at which the stalk develops. The adhesive material allows cells to attach to a variety of solid substrates, or to each other's holdfasts to form rosettes. The cells of known types are rod-shaped and colorless.

CLASSIFICATION OF ISOLATES

During the present investigations, more than 100 strains representing 56 distinct isolates have been studied with regard to their morphological and physiological properties. The characterization of these isolates has revealed differences which distinguish several types of caulobacters. It is now feasible to systematize this knowledge, and thus to propose a classification within the group.

Schemes for Classification of Caulobacter Types

The occurrence of several morphological types of caulobacters suggested that gross morphology would be a valuable determinative characteristic. Therefore, the intuitive classification of isolates is based primarily upon morphology.

The second property employed is pigmentation. The cells of many of the isolates contain yellow or red pigments in combinations resulting in various shades of yellow, golden, orange, and red coloration of the colonies. Pigmentation characteristics serve to subdivide the morphological types of central caulobacters; all isolates of excentral caulobacters are colorless.

In the classification presented in Scheme 1, two further properties are used, namely, organic growth factor requirements and, between the two types of marine isolates, sensitivity to NaCl above the normal concentration in seawater (usually slightly less than 3%). The names to be proposed for 11 of the types are presented in the last column; types for which names are not proposed have been assigned Roman numerals.

The intuitive classification was refined by a numerical analysis of similarity among the isolates. The principles of numerical classification (60, 62) require that many characteristics be determined, preferably at least 60, and that each characteristic be given equal weight in the analysis. Two sorts of characteristic were not admitted to the analysis of caulobacters: (i) characteristics used to define the group, such as cellular stalks and motility, or upon which the procedure for obtaining pure cultures is dependent, such as aerobiosis and its usual corollary, catalase production; and (ii) quantitative characteristics, such as generation time and cell size, since such characteristics must be scored within arbitrary ranges only one of which can be positive for any strain, thus eliminating the equivalence of the ranges.

The 78 characteristics which were used in the numerical analysis are listed in Table 13. Each characteristic was scored as positive or negative. The details of the determination of characteristics

will be found in the pertinent sections of this paper.

The similarity of each strain to each other strain was calculated with the aid of an IBM 9090 computer according to the following equations:

NS = number of characteristics scored positive for both strains;

ND = number of characteristics scored positive for one strain and negative for the other;

$$S = 100\left[\frac{NS}{NS + ND} + 0.005\right]$$

= similarity value.

A characteristic scored as negative for both strains or not scored for one or both of them was ignored. The similarity values (S-values) calculated for the 56 isolates are presented in a triangular matrix in Fig. 30, in which the isolates are arranged so that highly similar strains are adjacent to one another.

Groupings approximating those of the intuitive scheme appear in the numerical analysis. Three of these (*C. crescentus*, *C. subvibrioides*, and *C. fusiformis*) appear as pairs or groups within each of which the S-values are greater than 70 (70-phenon groups). Within only one of these groups (*C. subvibrioides*) is any one of the included strains related to a strain outside of the group by an S-value of 70 or more; the exceptional strain is CB81, since $S_{81.17\ (C.\ variabilis)} = 72$. A further pair appears at the 70-phenon line; these two strains (CB28 and CB31) were classified intuitively as *C. bacteroides;* on the basis of the numerical analysis, they are recognized as caulobacter type V.

Scheme 1 for classification of caulobacters

Morphology	*Pigmentation*	*Other determinative characteristics*	*Species*
Vibrioid	A. Present		
	1. Bright yellow		*C. henricii*
	2. Dark golden		*C. henricii aurantiacus*
	B. Absent		
		1. Organic growth factors not required	*C. crescentus*
		2. Riboflavine required	*C. vibrioides* H. and J.
		3. Biotin required	*C. intermedius*
Limonoid	A. Pale yellow	(riboflavine required)	*C. vibrioides limonus*
	B. Absent	(riboflavine required)	*C. vibrioides* H. and J.
Subvibrioid	A. Present		
	1. Light golden		Type I
	2. Dark orange		*C. subvibrioides*
	B. Absent		*C. subvibrioides albus*
Fusiform	A. Present		
	1. Dark yellow		*C. fusiformis*
	2. Dark golden		Type II
	B. Absent		*C. leidyi*
Bacteroid		A. Fresh-water isolates; stalk invariably central	
	1. Present		
	a. Dark golden		Type IV
	b. Bright yellow		Type V; *C. bacteroides*
	c. Orange		*C. bacteroides*
	2. Absent		Type III; *C. bacteroides*
		B. Fresh-water isolates; stalk position variable	*C. variabilis*
		C. Marine isolates	
		1. Grow in peptone medium containing 4% NaCl	*C. maris*
		2. Growth in peptone medium inhibited by 4% NaCl	*C. halobacteroides*
Excentral			*A. excentricus*

Four of the remaining groups (*C. henricii*, *C. bacteroides*, *C. vibrioides*, and *A. excentricus*) appear at the 60-phenon line, but each of these groups includes one or more strains which have S-values outside of the group greater than 60. Nevertheless, each is readily distinguishable on the basis of unique characteristics. The *C. henricii* group comprises the pigmented vibrioid caulobacters; each of three strains tested requires only vitamin B_{12} for growth in a glucose-ammonium salts medium. The slender bacteroid isolates from freshwater which utilize pentoses, acetate, and succinate are included in *C. bacteroides*. The *A. excentricus* group comprises colorless thick rods whose stalks do not possess adhesive material and are invariably excentral; each of the strains requires only biotin for growth in the defined medium.

The fourth 60-phenon grouping (*C. vibrioides*) comprises morphologically different strains. Strains CB51, CB57, and CB70 are vibrioid, whereas strains CB5, CB18, and CB-H are limonoid. All six strains are colorless. The one unique characteristic common to all strains of this group is the nutritional requirement for riboflavine. Therefore, it seems necessary to define one caulobacter type principally on the basis of physiological, rather than morphological properties. The strain received from Dr. Grula (CB-G) is assigned to this group since it is colorless, of limonoid cell shape, and requires riboflavine. Further, although it is not sufficiently similar to any other isolate to have an S-value of 60 or more, its highest S-values occur with strains of this group ($S_{G.5}$ = 53, $S_{G.70}$ = 51, and $S_{G.18}$ = 50).

Type II is represented by two strains whose similarity is low ($S_{24.26}$ = 55). One of these, strain CB26, is more similar to strain CB17 (*C. variabilis*) ($S_{26.17}$ = 61); however, since CB26 is a golden, slender bacteroid strain with an invariably central stalk, and strain CB17 is red-orange, of thick bacteroid cell shape, and its stalk may be central or excentral, it is not reasonable to recognize these two strains as representatives of the same type of caulobacter.

Strains that do not, on the basis of S-values, fall into any of the groups as defined above are

TABLE 13. *Characteristics employed in numerical analysis*

Characteristic no.	Characteristic
1–10	Utilization of arabinose, ribose, xylose, glucose, galactose, mannose, fructose, lactose, maltose, and sucrose
11	Utilization of gluconate
12, 13	Hydrolysis of starch, utilization of starch
14–29	Utilization of Casamino Acids, alanine, arginine, aspartate, cystine, glutamate, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine
30–41	Utilization of acetate, propionate, butyrate, pimelate, pyruvate, lactate, succinate, malate, fumarate, adipate, citrate, and tartrate
42–48	Utilization of methyl, ethyl, *n*-propyl, *n*-butyl, *t*-amyl, and benzyl alcohols, and glycerol
49, 50	Sensitivity to streptomycin (0.1 mg/ml) and penicillin G (1,000 units/ml)
51–57	Sensitivity to bacteriophages 1, 2, 3, 4, 5, 12, and 15
58	Long axis of cell curved
59	Stalk central
60	Diameter of cell constant
61	Grow in glucose- or glutamate-mineral medium
62	Biotin required for growth
63	Riboflavine required for growth
64	Growth factors other than biotin and riboflavine required
65, 66	Nitrate reduced to nitrite aerobically and anaerobically
67	Lipid storage products formed from glucose
68–70	Grow in 0.1% peptone-tap water, with 2% NaCl, and with 4% NaCl
71–76	Pigmentation: bright yellow, dark yellow, bright orange, dark orange, light gold, and dark gold
77, 78	Utilization of nicotinate and *p*-hydroxybenzoate

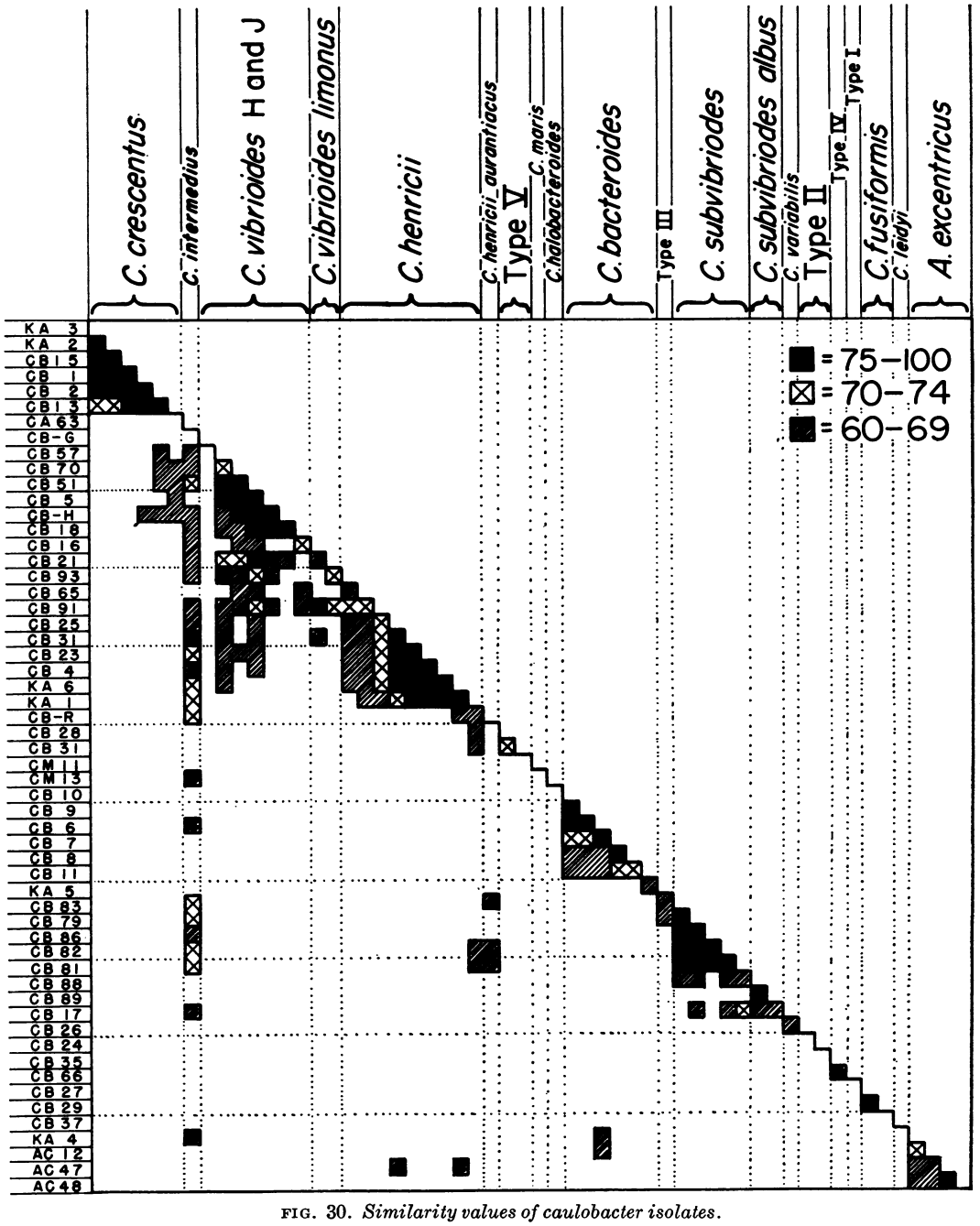

FIG. 30. *Similarity values of caulobacter isolates.*

recognizable as most similar to only one of the groups. Thus, strain CB-R is placed in *C. henricii* (as *C. henricii aurantiacus*), and strains CB88 and CB89 are placed in *C. subvibrioides* (as *C. subvibrioides albus*). Strains whose S-values show them to be approximately as similar to *C. vibrioides* as to *C. henricii* are placed in one group or the other on the basis of whether or not they require riboflavine; accordingly, strains CB91 and CB93 are placed in *C. henricii*, and strain CB16 (which is limonoid and pale yellow) is placed in *C. vibrioides* (as *C. vibrioides limonus*).

The recognition of this last subtype clarifies the placement of strain CB21, which is similar to CB16 in morphology, pigmentation, riboflavine requirement, and on the basis of S-value ($S_{16.21}$ = 62).

Several types are represented by only one isolate. Among these is an isolate (strain CB63) which is similar to strains of various types. Its S-values are 60 or greater with six strains of *vibrioides*, four strains of *henricii*, and one strain each of *variabilis*, *subvibrioides*, *bacteroides*, *halobacteroides*, and *A. excentricus;* 70 to 74 with one strain of *vibrioides*, three of *henricii*, one of *henricii aurantiacus*, and four of *subvibrioides*; and 75 with one strain of *henricii*. As a colorless vibrioid strain which requires biotin plus another factor(s) (not riboflavine), it does not share a distinctive property with any one of these groups, nor does any other strain show this intermediacy of similarity. Accordingly, strain CB63 is recognized as a caulobacter type, for which the epithet *intermedius* is proposed.

Another type represented by a single strain is *C. variabilis*. Although similar to type II and *C. subvibrioides*, strain CB17 is distinct from both of these types on the basis of its pigmentation and morphology, as discussed above in regard to strain CB26 (II).

Two strains are recognized as distinct types by both systematic approaches which have been applied. These are strains CB37 (*C. leidyi*) and CM11 (*C. maris*). Strain CM13 (*C. halobacteroides*) is also distinct, since its only S-value of 60 or greater is with CB63 (*C. intermedius*). The classification of the three remaining strains is not clear. Strain KA5 (type III) was classified intuitively with *C. bacteroides*, but it does not fall into this 60-phenon group. Further, it is similar to two strains (CB79 and CB83) of *C. subvibrioides*, although it is distinguishable from them on the basis of its lack of pigmentation and its slender bacteroid cell shape. Strains CB35 (type IV) and CB66 (type I) are of high similarity ($S_{35.66}$ = 67) and are distinguishable only on the basis of cell shape (CB35, bacteroid; CB66, subvibrioid). Neither of these strains is similar to any other type of caulobacter.

Scheme 2 presents the classification of caulobacter isolates which has been formulated by correlating the results of the intuitive and numerical approaches; this scheme differs from Scheme 1 principally in its inclusion of a larger number of physiological characteristics. The numerical analysis, heavily weighted with physiological characteristics, has justified the selection of gross morphology and pigmentation as the principal determinative characteristics, since almost all of the types distinguished on these bases by the intuitive approach are of high overall similarity. When the intuitive placement of a strain was not upheld by the S-value determinations, it was nevertheless possible to place it in a high-phenon group on the basis of selected characteristics, either morphological or nutritional, without decreasing significantly the minimal S-value of the group.

Description of Types and Proposal of Epithets

In proposing specific epithets for the several caulobacter types which can now be recognized, the problem which must first be faced is which of these types to designate as *C. vibrioides* Henrici and Johnson, the only species so far named. Henrici and Johnson's diagnosis (26) of this species was based solely on microscopic observations of morphological characteristics made during studies of organisms attached to slides which had been submerged in a natural environment. On the basis of this diagnosis, all vibrioid caulobacters would be included in this one species. However, the present collection of *Caulobacter* pure cultures contains four types (*crescentus*, *vibrioides*, *henricii*, and *intermedius*) which possess a vibrioid cell form, but which are distinguishable from one another by other, mainly physiological, properties.

The diagnosis of *C. vibrioides* was emended by Bowers et al. (2) to accommodate their observations on a pure culture of *Caulobacter* which they isolated from well water. This strain should be considered the neotype strain, and for this reason it was included in the present studies (as CB-G). However, its cell shape under conditions optimal for growth is correctly described as limonoid, rather than vibrioid. Nevertheless, it is most similar to the one *Caulobacter* type which is identified primarily by a physiological property, the requirement for riboflavine, a property included in the description of *C. vibrioides* as emended by Bowers et al. Thus, the *vibrioides* type, represented both by vibrioid and by limonoid isolates, is designated *C. vibrioides* Henrici and Johnson. The description of the type species can now be emended further to

include properties which have been determined since the work of Bowers et al.

Epithets are proposed for 11 of the remaining 16 caulobacter types. In most instances, the epithet has been selected to reflect gross morphology, as does the name of the type species. "sp. n." have been used to indicate species names proposed in this work, and "gen. n." for the one genus proposed.

Three of the caulobacter types are classifiable on the basis of the characteristics used in the intuitive classification, but the majority of the

Scheme 2 for classification of caulobacters

Principal characteristic	*Secondary characteristics*	*Species*
Riboflavine required; cells vibrioid or limonoid	Colorless	*C. vibrioides* H. and J.
	Pale yellow	*C. vibrioides limonus*
Cells vibrioid; riboflavine not required	Pigmented	
	1. Bright yellow	*C. henricii*
	2. Dark golden	*C. henricii aurantiacus*
	Colorless	
	1. Organic growth factors not required	*C. crescentus*
	2. Biotin required	*C. intermedius*
Cells subvibrioid	Pigmented	
	1. Light golden	Type I
	2. Dark orange	*C. subvibrioides*
	Colorless	*C. subvibrioides albus*
Cells fusiform	Pigmented	
	1. Dark yellow	*C. fusiformis*
	2. Dark golden	Type II
	Colorless	*C. leidyi*
Cells bacteroid	Grow in peptone-tap water media	
	1. Stalk invariably central	
	a. Pentoses are utilized	
	(1) Acetate and succinate are utilized	*C. bacteroides*
	(2) Acetate and succinate are not utilized	Type III
	b. Pentoses are not utilized	
	(1) Maltose and sucrose are utilized	Type V
	(2) Maltose and sucrose are not utilized	Type IV
	2. Stalk position variable	*C. variabilis*
	Do not grow in peptone-tap water media; grow in peptone-tap water media containing 2% NaCl	
	1. Amino acids are utilized	*C. halobacteroides*
	2. Amino acids are not utilized	*C. maris*
Stalks not adhesive; stalks invariably excentral		*A. excentricus*

The epithets and type strains are proposed in the following sections, in which each of the types is described and the representative strains are listed. Strains which are included within a particular type, but are not considered representative of the type, are referred to as "related" strains. An effort has been made to follow the recommendations of the *International Code of Nomenclature of Bacteria and Viruses* (34). The letters

properties determined for the isolates are not possessed by the strains in these three groups. The characterization of these types would necessarily be based on the absence of properties; therefore, to avoid assigning epithets to types whose physiological properties cannot yet be described in a positive manner, epithets are not proposed for types I, II, and IV.

Unless stated otherwise in the description,

the isolates are from freshwater. The physiological properties of the caulobacter types are presented in Table 14.

Genus: *Caulobacter* Henrici and Johnson, 1935

Vibrioid and Limonoid Types:

Caulobacter vibrioides Henrici and Johnson, 1935.

The neotype strain of this species, designated by Bowers et al. (2), was characterized as a vibrioid *Caulobacter* which required "riboflavin and an organic source of energy." Growth occurred in the pH range of 6 to 9; the optimal temperature was 30 C. The colonies formed were "round, smooth, slightly raised, glistening, finely granular in the center, and grayish-white with the center and reverse side becoming brownish yellow." Growth on slants was "filiform, grayish-white, glistening, and viscid." Growth did not occur deep in stab cultures, nitrate was not reduced to nitrite, and tryptophan was not converted to indole. Exclusive of the last test, which has not been used in the present study, these properties are possessed by almost all colorless vibrioid isolates, and indeed by most colorless isolates. The one exceptional property is the requirement for riboflavine, which is unique to type *vibrioides*. The reduction of nitrate to nitrite is a property which does not seem to be determinatively useful, since it occurs randomly among the types. The two strains (CB21 and CB16) of subtype *vibrioides limonus* are pale yellow; the latter strain is the only isolate whose growth factor requirements are satisfied by riboflavine alone, all the other isolates of *vibrioides* requiring an additional, undetermined factor(s).

The properties listed for *C. vibrioides* are shared by strains CB51, CB57, CB70, CB5, CB18, and CB-H; those for *C. vibrioides limonus* are shared by CB21 and CB16. The properties of CB-G are listed in a separate column. The minimal S-value within the group, exclusive of values for CB-G, is $S_{16.H} = 58$.

The neotype strain must be strain CB-G. However, strain CB51 is designated as a typical strain. Strain CB16 is the type strain of *C. vibrioides limonus* n. subsp.

Caulobacter crescentus sp. n.

This type is represented by six strains (CB1, CB2, CB13, CB15, KA2 and KA3), all of which are colorless. These are the only vibrioid strains which do not require organic growth factors. They are unique among all the caulobacter isolates by virtue of their utilization of C_1 to C_5 aliphatic alcohols. The type strain selected is CB2. $S_{\text{minimum}} = S_{13,\text{KA2 or KA3}} = 71$.

Caulobacter henricii sp. n.

The epithet for this type is a form of the name of A. T. Henrici, the principal investigator in the studies (26) which led to the recognition of the caulobacter group. Seven strains (CB4, CB23, CB25, CB36, CB65, KA1, and KA6) are representative of this type. The colonies are bright yellow. Strain CB4 is designated as the type strain. $S_{\text{minimum}} = S_{23.65} = 63$.

The growth factor requirement of three strains (CB4, KA1, and KA6) has been determined; each of these isolates requires only vitamin B_{12}. The growth rate in defined medium (glucose-mineral medium supplemented with vitamin B_{12}) has been determined for strain CB4 only.

Two strains intermediate between this type and *C. vibrioides* are recognized as more similar to *C. henricii*. They do not require riboflavine; the colonies are pale (CB91) and bright (CB93) yellow. The latter strain was isolated from a millipede gut.

A related strain (CB-R) was received from Dr. Zavarzin. Physiologically similar to *C. henricii* strains, CB-R is distinguished by its red-golden pigmentation and the unusually small size of the cells (ca. 0.8 by 0.4 μ maximum). The strain is considered representative of a subspecies, for which the epithet *aurantiacus* is proposed. The name of the subspecies is *C. henricii aurantiacus* subsp. n.

Caulobacter intermedius sp. n.

C. intermedius is represented by a single strain, CB63. This strain is readily distinguished from *C. henricii* by its lack of pigmentation, and from *C. crescentus* by its inability to grow in the glucose-mineral medium. It differs from *C. vibrioides* by its requirement for biotin, rather than riboflavine, and by its insensitivity to the bacteriophages. The epithet was chosen to reflect its high degree of similarity to several of the caulobacter types as revealed by the numerical analysis.

Subvibrioid Types:

Caulobacter subvibrioides sp. n.

The strains representing this type (CB79, CB81, CB82, CB83, and CB86) form dark-orange colonies which are translucent compared with those of other caulobacter types. The type strain selected is CB81. $S_{minimum} = S_{81.83} = 75$.

Strains CB88 and CB89, isolated from soil, are related to *C. subvibrioides*. They differ principally in their sensitivity to bacteriophage 3 and their lack of pigmentation. They are recognized as the subspecies *C. subvibrioides albus* n. subsp.; strain CB88 is the type strain of the subspecies.

Type I.

This type is represented by strain CB66. It is distinguishable from other subvibrioid strains by its light golden pigmentation and inability to utilize sugars. No specific epithet is proposed.

Fusiform Types:

Caulobacter fusiformis sp. n.

Dark yellow pigmentation and the ability to utilize butanol distinguish the strains (CB27 and CB29) of this type from the other fusiform caulobacters. Neither of these strains can utilize sugars. Strain CB27 is designated the type strain. $S_{27.29} = 82$.

Caulobacter leidyi sp. n.

The epithet for this type is a form of the name of J. Leidy, the nineteenth century biologist who observed the occurrence of microorganisms attached to the fungi in the millipede gut (41). The single strain (CB37) representing this type was isolated from a millipede gut. The colonies are colorless. Of the several caulobacter types which utilize sugars, only *C. leidyi* does not utilize sucrose. *C. leidyi* is the only caulobacter type besides *C. crescentus* which can be cultivated in a glucose-mineral medium.

Type II.

The two strains (CB24 and CB26) representing this type share the properties of dark golden pigmentation and sensitivity to bacteriophages 1 and 3 which distinguish them from each of the other fusiform types. Strain CB24 does not utilize sugars. No epithet is proposed.

Bacteroid Types:

Caulobacter bacteroides sp. n.

This type is represented by six strains (CB6, CB7, CB8, CB9, CB10, and CB11) which are heterogeneous in the properties of pigmentation and bacteriophage sensitivity. Strain CB6 is colorless; strains CB9 and CB10 bright yellow; and strains CB7, CB8, and CB11 orange. Only CB8 and CB11 are sensitive to bacteriophages 1 and 3, which were isolated on plates seeded with strain CB11. The only property unique to this group of strains is the ability to utilize gluconate. Starch hydrolysis has been tested only for strain CB11 (positive). The cell shape is slender bacteroid. The type strain is CB7. $S_{minimum} = S_{8.9 \text{ or } 10} = 62$.

Type III.

This colorless, slender bacteroid type is represented by strain KA5. Although KA5 is similar, on the basis of S-value, to strain CB11 of *C. bacteroides*, it is distinguished from the colorless strain of that group by several properties: its growth is stimulated by biotin and inhibited by 2% NaCl; it does not produce nitrite; it is lysed by bacteriophages 1 and 3; and it does not utilize ethanol. The position of this strain is not clear; thus, although it is recognized in this classification as representing a type of caulobacter, no epithet is proposed.

Caulobacter halobacteroides sp. n.

This type is represented by strain CM13. The properties of this strain are similar to those of *C. bacteroides*, but its source (seawater) and requirement for NaCl justify the recognition of this strain as a distinct type. The colonies are colorless, and the cell form is slender bacteroid. Another strain of this type differs from CM13 only in its ability to reduce nitrate to nitrite aerobically.

When plated in soft-agar media prepared in varying ratios of seawater to tap water, the colony counts of this type are greatest in 75% seawater. About 70% of the population can give rise to visible colonies in 15% seawater medium.

Caulobacter maris sp. n.

This type, like *C. halobacteroides*, is represented by a strain (CM11) isolated from seawater. It was initially distinguished from *C. halobacteroides*

TABLE 14. *Physiological properties of Caulobacter types**

Determination	Strain CB-G	*C. vibrioides* H. and J.	*C. vibrioides limonus*	*C. henricii*	*C. henricii auranticus*	*C. crescentus*	*C. intermedius*	Type I	*C. subvibrioides*	*C. subvibrioides albus*	*C. fusiformis*	Type II	*C. leidyi*	*C. bacteroides*	Type III	Type V	Type IV	*C. variabilis*	*C. halobacteroides*	*C. maris*	*A. excentricus*
Growth rate																					
Standard medium	4	3–4	2–3	2.5		1.5–2	1.4					4.5	1.2	4–5	3.5			3.5			1.5–2
Defined medium			(3.5)	4		3–4							3								3–4
Growth factors	(P_2)	(B_2)	(B_2)	B_{12}		–	(Bio)	+	+	+	+	+	–	+	(Bio)	+		+			Bio
Growth in Peptone Media containing																					
Tap water	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+
2% NaCl	–	–		–	+	v	+	+	+	+	v	v	+	v	–	–	+	+	+	+	v
4% NaCl	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitrate reduction																					
Aerobic	–	v	–	–	–	–	–	–	–	–	–	–	–	v	–	–	–	–	–	+	v
Anaerobic	–	v	v	–	–	v	–	–	–	–	–	–	–	v	–	–	–	–	–	+	v
Pacteriophage																					
1 and 3	–	–		–	–	v	–	–	–	–	–	s	–	v	s	–	–	s	–	–	–
2		v		–	–	s	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	s		–	–	v	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5, 12, and 15	s	s		–	–	s	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Starch hydrolysis	+	+	+	v	+	v	+	–	+	–	–	–	–	(+)	+	+	–	+	+	–	+
Utilization of																					
Starch	–	+	+	v	+	+	+	–	v	+	–	+	–	+	–	+	–	+	+	+	+
Arabinose	–	+	+	v	–	v	+	–	v	–	–	–	–	+	+	–	–	–	+	–	v
Ribose	+	v	+	v	–	–	–	–	–	v	–	–	–	+	+	–	–	–	+	–	–
Xylose	–	v	+	+	–	+	+	–	+	–	–	v	+	+	+	–	–	–	+	+	+
Glucose	+	+	+	+	+	+	+	–	+	+	–	v	+	v	+	+	–	+	+	+	+
Galactose	+	v	+	+	+	+	+	–	+	+	–	v	+	+	+	+	–	+	+	–	+
Mannose	+	+	+	v	–	+	+	–	–	v	–	–	+	+	+	–	–	–	+	–	+
Fructose	+	v	v	v	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	+
Lactose	+	+	v	v	–	+	+	–	–	–	–	–	–	v	–	–	–	–	+	–	+
Maltose	+	+	+	+	+	+	+	–	+	+	–	v	+	v	+	+	–	+	+	+	+
Sucrose	+	+	+	+	+	+	+	–	+	+	–	v	–	+	+	+	–	+	+	+	+

Casamino Acids	−	+	+	+	+	+	+	+	+	+	+	+	+	v	+	+	+	+	+	−	+
Alanine	−	v	+	+	+	+	+	+	+	+	−	v	+	v	+	v	+	+	−	−	+
Arginine	−	v	v	+	−	v	−	−	−	−	−	−	−	−	−	v	−	−	+	−	v
Aspartate	−	+	+	+	+	+	+	+	v	+	−	−	+	v	+	+	−	−	−	−	+
Glutamate	−	+	+	+	+	+	+	+	+	+	+	+	+	v	+	+	+	+	−	−	+
Histidine	−	+	v	v	−	v	−	+	−	−	−	−	+	−	−	−	+	−	−	−	v
Isoleucine	−	−	−	v	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	v
Leucine	−	−	−	−	−	v	−	−	v	v	−	−	+	v	−	−	−	+	−	−	−
Lysine	−	+	v	v	−	v	−	−	−	−	−	−	+	−	−	−	−	−	−	−	v
Proline	−	+	+	+	+	+	+	−	v	v	−	v	+	v	−	+	−	+	+	−	+
Serine	−	+	v	v	+	v	+	−	v	−	−	−	+	−	+	−	−	−	+	−	v
Tyrosine	−	v	v	v	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	v
Valine	−	v	−	v	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−
Acetate	−	v	−	v	+	+	+	−	v	+	−	v	+	+	−	+	−	+	+	−	+
Butyrate	−	v	v	+	+	+	+	−	v	v	+	−	−	v	−	−	−	+	+	−	−
Pimelate	−			+		+	+	−	−	−	−	−	+	−	−	v	−	−	−	−	−
Pyruvate	−	v	v	v	+	v	+	−	v	+	v	−	−	+	+	+	−	+	+	−	+
Lactate	−	v	−	v	+	v	−	−	−	−	−	−	−	v	−	+	−	−	−	−	v
Succinate	−	+	+	v	+	−	+	−	+	+	−	−	+	+	−	+	−	+	−	−	+
Malate	−	v	v	v	+	v	+	−	v	v	−	−	+	v	−	−	−	−	−	−	+
Fumarate	−	v	+	v	+	v	+	−	+	+	−	−	+	+	−	−	−	+	−	−	+
Methanol	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	v
Ethanol	−	−	−	v	+	+	−	−	v	v	−	−	−	v	−	−	−	−	−	−	+
Propanol	−	−	−	−	−	+	−	−	−	v	−	−	−	−	−	−	−	−	−	−	v
Butanol	+	+	+	+	+	+	+	−	−	v	+	−	−	−	−	−	−	+	−	−	v
Pentanol	−	−	v	−	−	+	−	−	−	v	−	−	−	−	−	−	−	−	−	−	v
Streptomycin (0.1 mg/ml)	s	s	s	s	s	s	s	s	s	s	s	s	r	s	s	s	s	s	s	s	s
Penicillin G (1,000 units/ml)		v	r	v	s	r	s	s	s	s	s	s	r	s	s	s	s	s	s	s	s

* Symbols: + = compound is utilized by, or characteristic is positive for, all strains representing the type; − = compound is not utilized by, or characteristic is negative for, all strains representing the type; v = compound is utilized, or characteristic is possessed by, some strains but not by others; s = sensitive; r = resistant; Bio = biotin; B_2 = riboflavine. Growth rates are expressed in hours per doubling of turbidity at 30 C. Growth factors indicated parenthetically are known to stimulate growth, but do not support growth through several serial subcultures in defined media.

by its sensitivity to a dilution of seawater to 25%, at which concentration the viable count is 30% of the count in full-strength seawater medium. Colonies are not formed in 10% seawater medium. These two types are further distinguished by the inability of *C. maris* to hydrolyze starch or to utilize organic acids, by its reduction of nitrate to nitrite under anaerobic conditions, and by its ability to grow in peptone medium containing 4% NaCl. The appearance of the colonies and the shape of the cells are the same as those of *C. halobacteroides*.

Type IV.

This type, represented by strain CB35, is unique among the slender bacteroid caulobacters by virtue of the dark-golden pigmentation of its colonies and its inability to utilize sugars. No epithet is proposed.

Caulobacter variabilis sp. n.

Two properties of this type, which is represented by strain CB17, distinguish it from all other caulobacters. The colonies are dark red-orange, and the position of the stalk is variable, being either central or excentral on different cells even within a clone. Physiologically, it shares several properties with strains of *C. bacteroides*, including sensitivity to bacteriophages 1 and 3; the numerical analysis revealed that it is also similar to *C. subvibrioides* and one strain (CB26) of type II. The cells are of thick bacteroid shape.

Type V.

The two strains, CB28 and CB31, which represent this type are equally similar, on the basis of S-values, to *C. bacteroides* and to *C. variabilis*. The cell shape is closer to that of *C. variabilis* than to the long, slender cell shape characteristic of *C. bacteroides*. However, the position of the stalk is invariably central. The colonies of this type are bright yellow. $S_{28.31} = 73$. No epithet is proposed.

Genus: *Asticcacaulis* gen. n.

Asticcacaulis excentricus sp. n.

The colonies of the four strains (AC12, AC47, AC48, and KA4) representing this type are colorless, and each of the strains requires only biotin as a growth factor. The position of the stalk is invariably excentral. The type strain is AC48. $S_{minimum} = S_{12.47} = 61$.

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