BACTERIOLOGICAL REVIEWS, Dec. 1973, p. 479-521 Vol. 37, No. 4
Copyright © 1973 American Society for Microbiology
Printed in U.S.A.

Growth, Structure, and Classification of *Selenomonas*

V. VICTOR KINGSLEY AND JUDITH F. M. HOENIGER

Scarborough College and School of Hygiene, University of Toronto, Toronto, Canada

INTRODUCTION 479
HISTORICAL ASPECTS 480
TECHNIQUES FOR CULTURING SELENOMONADS 481
Anaerobic Technique 481
Organisms and Media Employed 481
Composition and Preparation of MPB Medium 482
Isolation Methods for Selenomonads 482
Determination of Growth 482
GROWTH OF SELENOMONADS 482
GENERAL MORPHOLOGY AND CYTOLOGY OF SELENOMONADS 484
Size and Shape of Cells and Presence of Flagella 484
Selenomonad Nuclei 487
FINE STRUCTURE OF BOVINE AND HUMAN SELENOMONADS 489
General Considerations of Overall Structure 489
Electron Microscopy of Flagella 489
A Note on the Flagella of S. palpitans 490
Characterization of Selenomonad Cell Wall 491
Isolated cell walls of Selenomonas ruminantium 495
Chemical characterization of murein 497
Natural lysis of ruminant selenomonads 497
A Note on the Cell Wall of S. palpitans 499
Plasma Membrane and Its Variations 499
Membranous intrusions 499
CLASSIFICATION 505
Numerical Taxonomy 508
Serology 509
DNA Base Ratios 511
INTERPRETATIONS 513
CONCLUSIONS 517
LITERATURE CITED 518

INTRODUCTION

The morphologically distinct microorganism *Selenomonas* von Prowazek, 1913 (45) (family *Spirillaceae*) exhibits a "crescent moon" shape with a characteristic tuft of flagella situated in the middle of the concave side of the cell. Early investigators of the morphology of *Selenomonas* seem to have perpetuated a number of inconsistencies. Serious, although understandably unsuccessful, attempts were made at placing the organisms into an appropriate taxonomic niche. These attempts were based on wrong information and have necessarily led to considerable confusion.

Apart from aspects of classification, other fundamentally biological questions arise, such as the behavior of the concavely placed flagellar tuft during cell division. This problem was tackled theoretically and practically with other bacteria at first by Bisset (4), but no other group of organisms provides a better working basis for such a study than the selenomonads, simply because of the site of the flagellar tuft.

Another problematic area concerns the nucleus of selenomonads. Robinow (90) showed that the chromatin bodies of *Selenomonas palpitans* are in close proximity to the flagellar tuft. In many cases it appeared that the flagella may even originate within the nuclear area. Jeynes (43), and later Jeynes and Bisset (44), categorically stated that a true nucleus appeared to be present and divided in synchrony with the cell.

Thus, the unusual type of flagellation, the mention in *Bergey's Manual* (6) that the flagella are tapered, and the possibility of the flagella originating in a nuclear region all con-

tribute to the fundamental classification of the selenomonads.

This review emphasizes aspects of classification, growth, and structure of *Selenomonas ruminantium* and *Selenomonas sputigena*, with some marginal studies of *Selenomonas palpitans*, for purposes of comparison.

HISTORICAL ASPECTS

The organism known to bacteriologists as *Selenomonas* (family *Spirillaceae*) is now believed to be one of the first bacterial organisms to have ever been historically documented, having been observed by van Leeuwenhoek in 1683 (23). Two centuries after van Leeuwenhoek observed the motile cells in his own mouth the organism was named *Spirillum sputigenum* by Miller (67) and subsequently described by him as "rods, curved like commas, which show very active spiral movements" (68). Loewenthal (59) believed that motility was due to the presence of one flagellum placed on the concave side of the cell. As this single flagellum sometimes appeared frayed, it was suggested that the organelle might actually consist of many smaller fibers (36). When these anaerobes were first cultured in horse serum agar, observations were made which suggested that *S. sputigenum* might actually have a flagellar tuft on its concave side, although it was not clear at that time whether the flagella originated as a compact tuft on the concave surface only or were sometimes displaced, giving the impression of a peritrichous arrangement (71).

Simultaneously, the taxonomic niche of the organism was under study. Categorical statements were made denying any connection with the genus *Spirillum*, and even with the whole range of bacteria primarily because of the absence of transverse fission and the apparent flexibility of the cell wall (36, 59). Others concluded that *S. sputigenum* represented simply a stage in the development of fusiform bacilli (77), an inference which was vehemently contradicted by Mühlens (71).

The controversy and uncertainty of classification of this and morphologically similar microorganisms was continued by the publication of drawings which represented various stages of *S. sputigenum*-like organisms observed in the rumen of some herbivores (82). The cells were reminiscent of an organism previously described and known to protozoologists as *Ancyromonas ruminantium* (15, 49). A new generic name was proposed (82): *Selenomonas*, meaning "moon shaped unit," obviously alluding to the overall morphology of the organisms in the genus, but not designating any species within the genus. Morphologically similar organisms were observed in the stomach of goats, and although similarities to the protozoon *A. ruminantium* were again noted, the organism was placed into still another genus, *Selenomastix ruminantium* (111). Similarly, wild guinea pigs were observed to harbor in their ceca *Selenomonas*-type microorganisms, and although these were partially described, no specific epithets were given (21, 27), until Simons first observed (97) in the cecal contents of guinea pigs and later named (98) such species *Selenomonas palpitans*. Unfortunately, Simons did not describe the species, leaving this study to his student Boskamp who published an account of his observations (5), and gave credit for naming the organism to his teacher. Boskamp did not culture *S. palpitans*, but observed the organism in cecal washings. He was the first to suggest that *S. palpitans* was morphologically similar to *S. sputigenum* (the oral species), saying that the term *S. sputigenum* was taxonomically invalid. However, he cautioned against the renaming of *Spirillum sputigenum*, *Selenomonas sputigena*, because of lack of any concrete evidence, stressing the necessity for comparison of *S. palpitans* to the organism found by Kerandel (49), because "possibly a new species" could be involved.

The taxonomic order which apparently was taking place was disturbed by Wenyon (108) who, in his textbook *Protozoology*, described and illustrated *Selenomonas ruminantium* from the cecum of guinea pigs (sic), designating it as a protozoon. However, it was not until *S. sputigenum* was studied in pure culture by Dobell (23) that the organism was transferred to the bacterial genus *Selenomonas*. An investigation of sheep selenomonads, followed by a morphological description of the organism, was performed by Moir and Masson (69). The first isolation, culture, and detailed study of *Selenomonas ruminantium*, where it was recognized, was by Bryant (8) who showed that Huhtanen and Gall's HD strains (37), unrecognized by them, also belonged to this species. MacDonald (60) described in detail two strains of *S. sputigenum* and reintroduced the old controversy concerning the classification of this and related species. He recommended that both forms be included in *Bergey's Manual* as recognized species of the genus *Spirillum*. However, in a review article, Lessel and Breed (56) countered, suggesting that the genus *Selenomonas* be recognized and that *Spirillum sputigenum* be listed as one of the three species of this genus, but that the latter's name be changed to *Selenomonas sputigena*. MacDonald and Madlener (61) were not satisfied with the proposed change in classifica-

tion and carried out detailed studies of *S. sputigenum*, using dark-field microscopy, ordinary light microscopy of organisms stained for their flagella, and electron microscopy of shadowed preparations. These workers proposed that, because of the seemingly random distribution of the flagella over the circumference of the cell, *S. sputigenum* should not be grouped with *Selenomonas*.

In the meantime, electron micrographs of shadowed preparations of *S. palpitans* were published by van Iterson (40), showing a striated pattern to the cell wall and the insertion of the flagellar tuft on the organism's concave side.

Selenomonads were isolated by micromanipulation by Purdom (83), and their role in the biochemistry of proteolysis in the rumen was investigated by Abou-Akkada and Blackburn (1) and by Fulghum and Moore (28). A number of workers studied selenomonad growth (16, 47, 58), cell walls (32), and their relationship to flagella (44). An investigation of the biosynthesis of phospholipids and selenomonad involvement was published by Kamio et al. (46). Another worker isolated and described a new giant selenomonad species (81). A study by Hirsch (Bacteriol. Proc., p. 30, 1971) asserted evolutionary (taxonomic) relationship with *Gallionella*. Most recently, selenomonads in the cecum of rats were described by Ogimoto (76).

TECHNIQUES FOR CULTURING SELENOMONADS

Anaerobic Technique

The most important prerequisite for culturing selenomonads is the exclusion of oxygen both during the preparation of media and during the transfer of the organisms. Essentially the method of Hungate (39) was followed by use of an oxygen-removing apparatus, and by incorporating suggestions of K. A. Pittman, then at U.S. Department of Agriculture, Beltsville, Md. Similar oxygen-removing columns have been described by Hungate (39) and recently by Latham and Sharpe (53).

Organisms and Media Employed

The rumen organisms examined were *Selenomonas ruminantium* strains HD 1, HD 4, PC 18, GA 31, GA 192, no. 6, no. 17, and CHR. (For sources and references pertaining to these strains consult Table 3, p. 509.) Strains no. 6 and no. 17 were received in a lyophilized condition and were reconstituted in rumen fluid-glucose-cellobiose agar (RGCA) medium (9) and grown anaerobically in the presence of carbon dioxide at 37 C. The strain designated as CHR was isolated in Toronto from fresh rumen juice. The other strains were at first obtained from K. A. Pittman and employed for preliminary studies. Later the stock cultures were replaced by the same strains, obtained from M. P. Bryant, University of Illinois, Urbana. Both sets of strains were received as stabs in RGCA rumen medium stubs (9).

Three strains of the oral organism *Selenomonas sputigena* (*Spirillum sputigenum*), namely F2, B, and ITK (see Table 3), were freshly isolated by use of the method of MacDonald and Madlener (62).

S. palpitans could not be grown in pure culture (but see recent report by Ogimoto [76]), and was always obtained from the ceca of recently killed guinea pigs. *S. sputigena* and *Selenomonas ruminantium*, however, were grown with ease once the techniques of anaerobic transfer and anaerobic conditions had been mastered.

S. sputigena grew well in fluid thioglycolate (Difco). For *Selenomonas ruminantium*, however, the following types of media were investigated: (i) thioglycolate medium (Difco), (ii) Hungate's anaerobic medium (38), (iii) RGCA medium of Bryant and Burkey (9), (iv) medium no. 4 of Hobson and Mann (33), (v) supplemented RGCA medium of Bryant and Robinson (10), (vi) isotonic medium of Munch-Petersen (72), and (vii) the volatile fatty acids medium of M. P. Bryant (personal communication, 1965), called here MPB.

The MPB medium was adopted for several reasons: (i) it was clear and almost colorless when reduced; (ii) with most strains this medium gave the highest yield of growth as judged by measurements of optical density; (iii) it was a reproducible, well-standardized medium; (iv) it was comparatively easily prepared; and (v) *S. sputigena* grew well in this medium, so that this organism could be compared with *Selenomonas ruminantium*.

Stock cultures of ruminant selenomonads were maintained anaerobically in 1.5% MPB agar medium; for oral selenomonads, fluid thioglycolate (Difco) was used.

S. sputigena was transferred once every 7 to 10 days, and kept at room temperature, whereas stock cultures of *Selenomonas ruminantium* could be kept for 5 to 7 weeks in the form of stab cultures in the refrigerator. Ruminant selenomonads on RGCA medium were deep frozen, and growth was normal even after 10 months of storage. Lyophilized cultures of both ruminant and oral selenomonads in MPB broth grew normally when reconstituted, but had a longer lag phase.

Composition and Preparation of MPB Medium

During the preparation of the medium the exclusion of oxygen was partially achieved by (i) sterilization at 121 C for 15 min in a stoppered round-bottomed flask, the medium having been boiled under a stream of carbon dioxide from which all traces of oxygen had been removed beforehand by passage over hot copper, and (ii) by the inclusion of cysteine mono-hydrochloride in the medium. The composition of the MPB medium, to make 300 ml, is as follows: glucose, 300 mg; Trypticase (BBL), 1.5 g; KH_2PO_4, 300 mg; sodium acetate (F.W. 136.1), 1.2 g; resazurin (0.1%), 0.3 ml; yeast extract (Difco), 600 mg; distilled water, 279 ml.

To the above mixture is added 3 ml of volatile fatty acids (VFA) solution made up as follows. One milliliter each of butyric acid, iso-valeric acid, and *n*-valeric acid is transferred to a flask and made up with water to 100 ml. (It was shown later that only the straight-chained acids, especially *n*-valerate, are needed for good growth [47, 103].) The solution must be mixed well, adjusted to pH 6.7 to 6.8, and stored tightly stoppered in a refrigerator. The glass assembly is tightly clamped and autoclaved at 121 C for 15 min.

At the same time, additional (freshly made) solutions are prepared in separate screw-capped test tubes and consist of the following: (i) 8% aqueous solution (wt/vol) of Na_2CO_3 (15 ml); (ii) 2.5% (wt/vol) aqueous solution of cysteine-hydrochloride (3 ml). The head space is flushed with oxygen-free CO_2 and the solution is autoclaved. After sterilization, both solutions are cooled to approximately 50 C and then added to the sterile medium under anaerobic conditions. The complete medium is stoppered and stored at room temperature.

Isolation Methods for Selenomonads

Rumen selenomonads were isolated from fresh rumen juice, obtained from a slaughter house (Canada Packers, Ltd., Toronto), by the roll tube method of Hungate (38) but using RGCA medium (9).

The isolation of oral selenomonads on sodium lauryl sulfate agar (SLS) followed the procedure of MacDonald and Madlener (62). Material obtained from scrapings between gum and tooth was placed on SLS agar plates and incubated for 5 to 8 days in an anaerobic jar under hydrogen.

Note that all our attempts to isolate *S. palpitans* from the cecum of guinea pigs and grow it in pure culture failed. In addition to the several media used in the culture of rumen and oral selenomonads, MPB medium supplemented with sterile cecum juice of a guinea pig was tried, the latter being at final concentrations of 3, 5, 7, and 10%. Selenomonads from ground squirrel were described by Barnes and Burton (3) and those from the cecum of rats were described by Ogimoto (76).

Determination of Growth

Generally, for optical density (OD) measurements of the growth of selenomonads, the inoculum consisted of 0.1 ml of an overnight culture in 9.9 ml of freshly prepared and well-reduced MPB medium.

All selenomonads were grown in rubber-stoppered test tubes (18 by 150 mm) containing 10 ml of MPB broth. Growth was routinely measured by OD readings made on a Coleman Junior spectrophotometer model 6A (Perkin Elmer Corp.) set at a wavelength of 600 nm against a blank of reduced MPB broth. Incubation was at 37 C.

To obtain a more complete picture of the growth cycle of rumen selenomonads, the OD was correlated with direct cell count and bacterial nitrogen.

A sufficient number of MPB broth tubes was inoculated so that OD, direct count, and bacterial nitrogen determinations could be performed simultaneously on identical tubes incubated together.

Growth measured as OD was related to dry weight of cells for various phases of the growth cycle. The cells were centrifuged at 17,300 × *g* for 20 min in a Sorvall refrigerated centrifuge, model RC-2B (Ivan Sorvall, Inc., Norwalk, Conn.). After washing in saline, the weight of the pellet was determined by drying to constant weight at 105 C.

Bacterial nitrogen was determined spectrophotometrically after digestion and Nesslerization (14). Ammonium sulfate (anhydrous) was used as a standard.

Cells were killed by exposure to 5% formaldehyde to stop motility, and direct microscope count of the total number of selenomonads per milliliter of culture was determined with a Bright Line Improved Neubauer Hemacytometer (Clay Adams, N.J.), using phase-contrast illumination.

GROWTH OF SELENOMONADS

Typical 6-day growth curves for the ruminal strains HD 1 and no. 17 are illustrated in Fig. 1. Basically similar growth curves were obtained with other rumen strains. The growth curve for an oral strain (ITK) of *S. sputigena* is also shown in Fig. 1. There are at least two growth

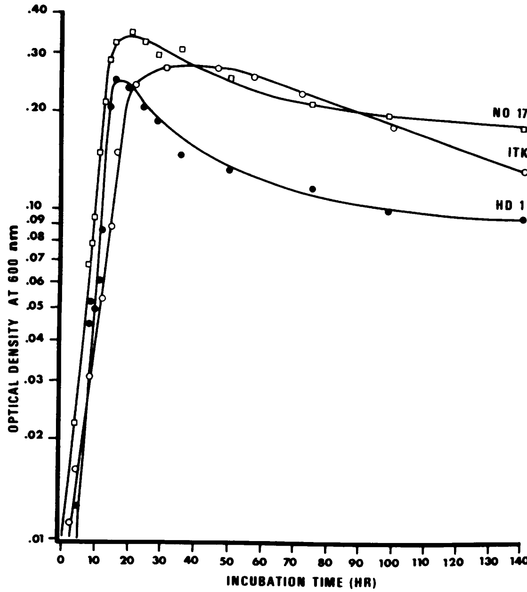


FIG. 1. *Growth curves (OD) of selenomonad strains HD 1, no. 17, and ITK. (For methods of growth see text.) Note that the ruminant strains HD 1 and no. 17 differ in their decline phases from that shown by the oral strain ITK in which no cell lysis was observed.*

characteristics in which these two groups of organisms differ from one another: (i) the generation times are slightly different (the generation time of *Selenomonas ruminantium* strain HD 1 was 75 min, that of *Selenomonas ruminantium* strain no. 17 was 2 h, and that of *S. sputigena* strain ITK was approximately 2.5 h), and (ii) both the stationary and the decline phases are dissimilar.

Selenomonas ruminantium strains showed a peak of growth which was immediately followed by a phase of decline, whereas a true stationary period occurred during the growth cycle of the oral strains. The rather drastic decline in OD of the rumen organisms was thought to be due to autolysis. Dry weight determinations were correlated with OD readings during growth. Figure 2 shows that the OD curve corresponds closely to the dry weight curve only during the exponential phase. Although the dry weight curve is similarly exponential in the phase of decline, this does not seem to be the case with the OD curve, possibly due to some optical property of the spheroplasts.

Correlation of OD with nitrogen content and total count of cells is shown in Fig. 3.

A comparison of doubling rates of the above parameters in MPB medium provides additional information (Fig. 4). The values are

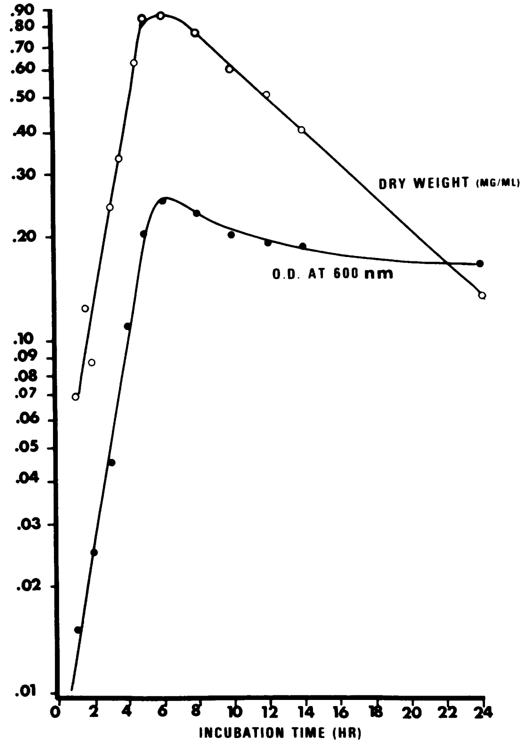


FIG. 2. *Relationship of OD_{600} to dry weight (mg/ml of culture) of growing cultures of Selenomonas ruminantium HD 1. (For method of growth and comparison of the two parameters see text.) Note that the OD parallels the dry weight only during the exponential phase, whereas the two curves do not correspond to each other in the phase of decline.*

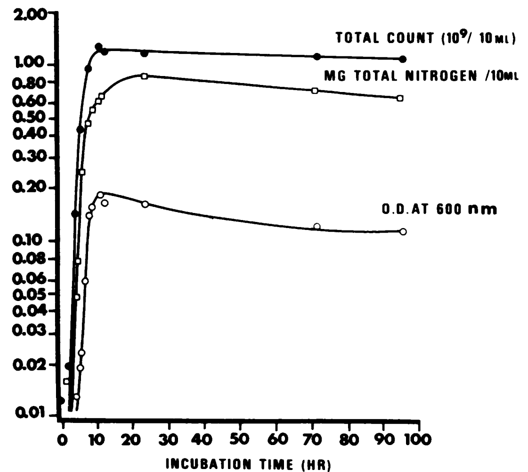


FIG. 3. *Comparison of the parameters of growth expressed as OD, nitrogen content, and total count in Selenomonas ruminantium HD 4. All three curves show good degree of correlation and as such any one parameter could serve singly as a good criterion of growth.*

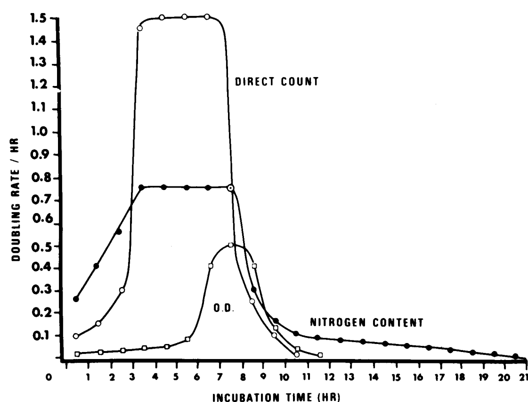


FIG. 4. *Comparison of "doubling rates" per hour in the three growth parameters expressed as OD, nitrogen content, and total count from the data in Fig. 3. by Fig. 3.*

plotted as "doublings per hour" from data in Fig. 3. The direct count doubling rate exhibits a lag, but becomes steady for a period of 4 h in the exponential phase (horizontal portion). The nitrogen content curve, however, shows an immediate rise. Although the rate of doubling of both the nitrogen content and the direct count begins at the same time, the former attains an ultimate steady rate of 0.75/h as compared with 1.5/h for the latter. It is assumed from these data that double the amount of nitrogen is required for each dividing cell and that each fission process into two cells is reflected in an apparent decrease of nitrogen doubling rate.

GENERAL MORPHOLOGY AND CYTOLOGY OF SELENOMONADS

Boskamp (5) published drawings of *Selenomonas palpitans* in which he showed the overall shape of the organism and the site of insertion of the flagellar tuft. He also performed gross morphological as well as some cytological studies on stained specimens taken from the cecum of guinea pigs, the main habitat of this organism. At first *S. palpitans* was considered to be the type species, but later *S. sputigena* was established as the type species (45).

Size and Shape of Cells, and Presence of Flagella

The microscopy studies presented here are concerned mainly with two selenomonad species, *Selenomonas ruminantium* and *S. sputigena*, although *S. palpitans* has also been examined to a limited degree in living and stained preparations (see also Robinow [90]). Although phase contrast studies of living organisms revealed that both *Selenomonas ruminantium* and *Spirillum sputigena* are spiral in shape, the latter more so than the former, the cell shape of all three species is crescentic in dried preparations (see also Ogimoto [76]). This helical characteristic has not been observed in the much plumper *S. palpitans* (see also Robinow [90] and Jeynes and Bisset [44]). The spiral structure of the first two organisms is emphasized in "long forms" (47) which were also occasionally observed in our pure culture.

Another morphological variant observed in cultures that had passed into the stationary and decline phases was a round form, and the number of these increased with time of incubation. The diameter of these globular forms, which were sometimes weakly motile, scarcely exceeded the width (see below) of the normal helical organisms. Such cells could readily be observed in older cultures, first because of their spheroid shape, and secondly because of their less dense appearance in phase contrast preparation. These globular structures were later found to be spheroplasts. Such spontaneously occurring spheroplasts in selenomonads have also been observed by Hobson et al., (34).

Selenomonas ruminantium cells are usually single, but may occur in short chains (Fig. 5). The cell width ranges from 0.9 to 1.1 μm between strains. The greatest variation is found in cell length. The length of the organisms within a single strain was quite uniform. However, the variation between strains was much more pronounced: strain HD 1 was 2.95 ± 0.37 μm long; strain GA 192 was 5.61 ± 0.12 μm long. The range of sizes obtained for *S. sputigena* strains was comparable to that of the ruminant strains: length of strain F 2 was 3.67 ± 0.57 μm; strain ITK was 5.21 ± 0.16 μm long.

The gross morphological characteristics of various selenomonad species and strains of the same species do not remain constant. Thus the length of the organism depends on various conditions and should not constitute a taxonomic parameter. For example, nutritionally deficient selenomonads may form long, sinuous cells (47). This was confirmed by numerous phase contrast observations of both species. Long, motile cells were observed which were not only helical in shape, but exhibited at each turn of the helix a beating flagellar tuft. No separation into shorter individual cells could be discerned (Fig. 6).

From this observation it can be assumed that, under certain conditions of growth, the synthesis of cross walls and the mechanism regulating the separation of daughter cells in *Selenomonas ruminantium* and *S. sputigena* are more sensi-

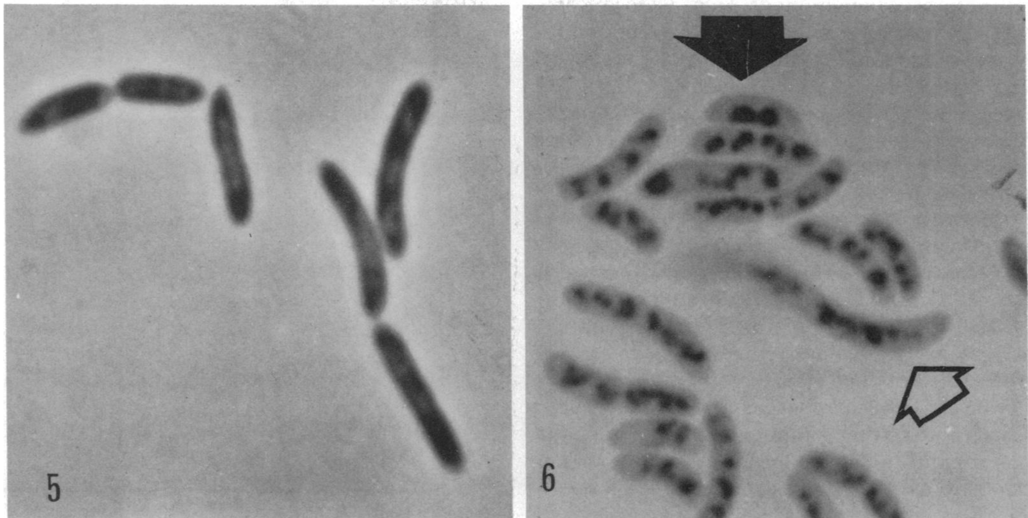


FIG. 5. *Selenomonas ruminantium HD 1. The cells were suspended in 22% gelatin to show nuclear areas in growing cells. Phase contrast.* ×*3,500.*

FIG. 6. *Giemsa stained cells of Selenomonas ruminantium HD 1. The empty arrow points to a long, helicoid S. ruminantium cell. The rest of the cells are either single or in the process of division. The full arrow indicates a cell similar in appearance to S. palpitans as photographed by Robinow (90).* ×*3,500.*

tive to nutritional deficiencies than is flagellar synthesis and flagellar function. In electron micrographs such cells may appear peritrichously (63), an observation which may have led some investigators to faulty conclusions regarding the flagellation of these organisms.

The above observations suggest that, for purposes of characterization, it would be more informative to use the smaller rather than the larger dimensions, since the latter may reflect a poor growth environment. Similarly, a descriptive term such as "size of non-dividing cells" (6) has no real meaning since in most bacterial cells nuclear division precedes cellular division and, what is of more importance, cellular division and subsequent separation may never take place, although the organism may have elongated fivefold.

During a preliminary study of media which could be employed for growing ruminant selenomonads, it was found that commercially supplied thioglycolate medium (which is normally used for the propagation of oral selenomonads) supported the growth of ruminant selenomonads rather poorly. The two criteria employed in the evaluation were the general appearance of the cells and their motility. In thioglycolate medium the growing cells became progressively less motile, so that generally after 48 h of incubation no motile cells were present. Other media tested at the same time not only supported the growth of the organisms but also maintained or preserved the motility of the incubated cells. To resolve this problem of the loss in motility observed in thioglycolate medium, a series of tests was set up excluding from the complete thioglycolate medium one constituent at a time. Three ruminant selenomonad strains (HD 1, HD 4, and no. 6) were employed in this study. Actively motile cells were evident in that set of tubes from which only glucose had been excluded. The usual concentration of glucose in thioglycolate medium is 0.5%. Other media tested were RGCA with 0.2% glucose, and a modified RGCA containing 0.05% glucose. Both of these media not only supported growth, but most cells were motile. It was concluded, therefore, that 0.5% glucose is inhibitory to both growth and motility of ruminant selenomonads in thioglycolate medium. Whether such nonmotility was due to an absence of flagella or to a nonfunctioning of these organelles already present was a problem which could easily be solved by examining negatively stained preparations in the electron microscope. All of these cells were found to be flagellated; however, the number of flagella per cell varied. Thus, a suspension taken from the thioglycolate test medium containing no glucose showed cells with more numerous flagella per cell than did suspensions from the other media. In contrast, control cells grown in a complete thioglycolate medium exhibited the least number of flagella per cell. It was concluded that a high concentration of glucose inhibits the synthesis of flagella in *Selenomonas ruminantium*.

Ruminant selenomonads that were successively subcultured five times in normal thioglycolate medium exhibited poor growth and were nonmotile; yet when transferred to the same medium without added glucose, luxurious growth occurred and the cells regained their motility. This finding further supports the view that the loss of flagellar function in these organisms in the presence of high glucose concentration, although not an irreversible process, is a glucose-sensitive phenomenon. Changes in pH were minimal during this process.

A second observation concerning flagellation and growth was made when total counts of rumen selenomonads were correlated with motility as observed by phase contrast microscopy. It was noted, then, that some selenomonads were nonmotile, but an unduly large proportion of such nonmotile cells was in the process of cellular division.

Counting was performed and the following criteria were used. Mother and daughter cells were scored as one individual if they were still attached to each other. Obviously dead cells were not included in the count. Immediately before the organisms were withdrawn from the test tubes, an OD reading was recorded and later correlated to the percentage of motile cells present. The data are presented in the form of a graph (Fig. 7) which shows the change during growth (as OD) and the corresponding change in the percentage of motile cells. Results similar to those with strain HD 1 (shown in Fig. 7) were also obtained with strains no. 17 and GA 192. After preliminary experiments, the test was repeated on a total count of 200 cells (as compared to a count of 50 for the preliminary test), with similar results.

It is apparent from these data that ruminant selenomonads grown in MPB medium progressively lose their motility up to the end of the exponential phase. The proportion of motile cells in the culture thereafter increases. It may be concluded that, during active reproduction (exponential phase), when the rate of cell division is highest, selenomonad cells dispense with motility and that they regain their motility when active growth is over (stationary and decline phases).

Again the question must be asked: is the decline of the number of motile cells during the growth due to the absence of flagella on the cells, or is this decrease due to the nonfunctioning of flagella already present? If the latter were the case, the number of flagella per cell would be expected to remain fairly constant during the life of a cell. If, however, the former hypothesis were true, the number of flagella per cell would

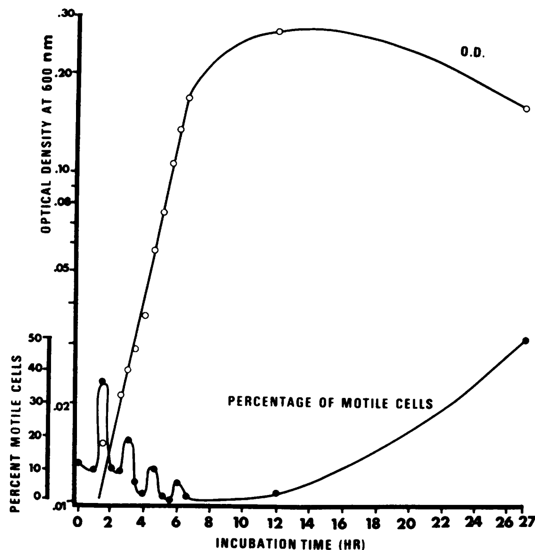


FIG. 7. *Percentage of motile cells of Selenomonas ruminantium HD 1 present during growth. The OD curve shows the usual ruminant selenomonad growth profile. Note that the majority of motile cells is present at the beginning of incubation. The percentage of such cells, however, decreases as incubation continues. A cyclic phenomenon is operative here and coincides with the generation time for the organism. The culture as a whole exhibits virtually no motility by the end of the logarithmic period of growth.*

be expected to decrease proportionately.

Experiments were carried out to correlate the OD and the number of flagella per organism as observed in electron micrographs of negatively stained cells. The procedure was to inoculate an overnight culture of a ruminant selenomonad strain into MPB broth. The cells were then incubated, and small samples were withdrawn and examined. OD readings were recorded immediately before samples were taken. The cells were negatively stained with phosphotungstic acid and examined in the electron microscope. This procedure gave a good correlation of the growth curve to the number of flagella per cell. The number of organisms examined varied from preparation to preparation but was never fewer than 50. The numerical results are given in Table 1 and are presented graphically in Fig. 8. The fairly large standard error (Table 1) reflects a nonsynchronous population. It can be seen that, immediately after transfer into fresh medium, the number of flagella per cell decreases. However, a subsequent increase occurs at the beginning of the exponential phase, followed by a sudden decline which lasts until the beginning of the stationary phase. The largest number of flagella per cell is present in the case of orga-

nisms in the early phase of exponential growth.

It should be noted that occasionally unseparated, but dividing, mother-daughter cells do not possess an equal number of flagella per cell, i.e., usually one cell has no flagella, whereas the other shows a large number of these organelles. Results obtained by Ingram and Kingsley (Abstr. Proc. Can. Fed. Biol. Soc. No. 135, 1972) with nonsynchronous cultures of strain GA 192 indicate that, in *Selenomonas ruminantium*, the numerical segregation of flagella to dividing cells is a statistically random process so that, although the tuft of flagella has a good chance of segregating into two numerically equal halves, one will find in the same culture a number of dividing cells in which one daughter cell may inherit the complete maternal tuft of flagella, whereas the other daughter cell will show no flagella at all.

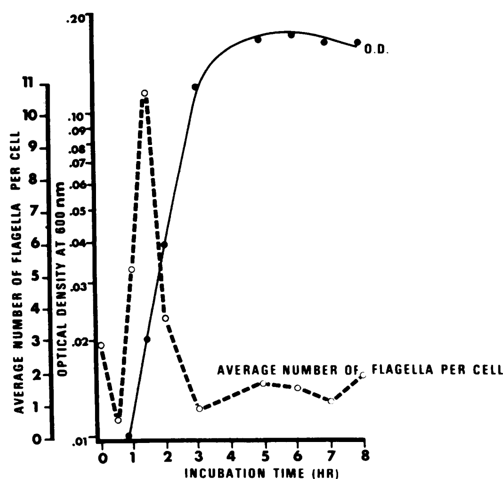


FIG. 8. *Relationship of OD of culture to average number of flagella per cell in growing Selenomonas ruminantium HD 1. At beginning of incubation there is an increase in the average number of flagella per cell, followed by a drastic decrease. Comparing results of this graph to those presented in Fig. 7, it appears that motility of S. ruminantium cannot be observed when cells have fewer than three or four flagella per cell.*

Selenomonad Nuclei

Cytological observations on both *Selenomonas ruminantium* and *S. sputigena* have been mainly concerned with the nuclear apparatus and its division during growth. Jeynes (43) categorically denied that *S. palpitans* and *Selenomonas ruminantium* were bacteria. He based his conclusions on the characteristics of nuclear division in the two organisms, which were entirely unlike any known scheme of nuclear division in bacteria. In our studies, preparations of living cells were suspended in 22% gelatin according to Mason and Powelson (66). Parallel preparations were stained with Giemsa as outlined by Robinow (87, 88).

Phase contrast microscopy clearly showed the multinucleate nature of growing cells of selenomonads. The chromatin bodies appear as clear areas (Fig. 5) in the living cell. In the shorter cells usually only two such areas are present, but in the longer organisms the nuclei appear as a string of beads.

TABLE 1. *Number of flagella per cell of Selenomonas ruminantium HD 1 correlated to OD during growth*

Incubation time (h)	Flagella per cell ± SE	OD (600 nm)
Inoculum	2.9 ± 2.5	0.005
0.5	0.5 ± 0.5	0.005
1.0	5.2 ± 4.3	0.010
1.5	10.6 ± 1.4	0.020
2.0	3.7 ± 1.7	0.041
3.0	0.9 ± 0.3	0.120
5.0	1.6 ± 0.9	0.165
6.0	1.5 ± 0.3[a]	0.170[a]
7.0	1.2 ± 0.3[a]	0.160[a]
8.0	1.9 ± 0.9[a]	0.161[a]

[a] Results obtained from a second series of experiments.

More detail was present in Giemsa preparations (Fig. 6, 9, 10). One hour after inoculations, the nuclear material of *Selenomonas ruminantium* begins a condensation process in the cell and forms a well-delineated body that stains deeply. Most of the cells, however, still do not possess a compact nuclear area, but rather a large nuclear area. It must be noted that many of the spheroplasts that have been transferred with the inoculum seem to recover and follow a similar type of condensation of the nuclear material.

By the second hour of growth no spheroplasts transferred in the inoculum can be observed. Although there still can be seen a certain proportion of chromatin-devoid or chromatin-pale cells, the majority have now undergone not only a distinct organization of the nucleus into a compact body, but also have passed through one or even two nuclear divisions (Fig. 10).

Generally, after inoculating 0.1 ml of a 24-h culture into 9.9 ml of fresh medium, the culture reaches exponential phase within 3 h. In this active state of growth individual cells possess from three to eight distinct nuclei. The majority of nuclei appeared centrally located, exhibiting "dumbbell" or "saddle" shapes (89). Occasionally, cells were seen in which the stained nu-

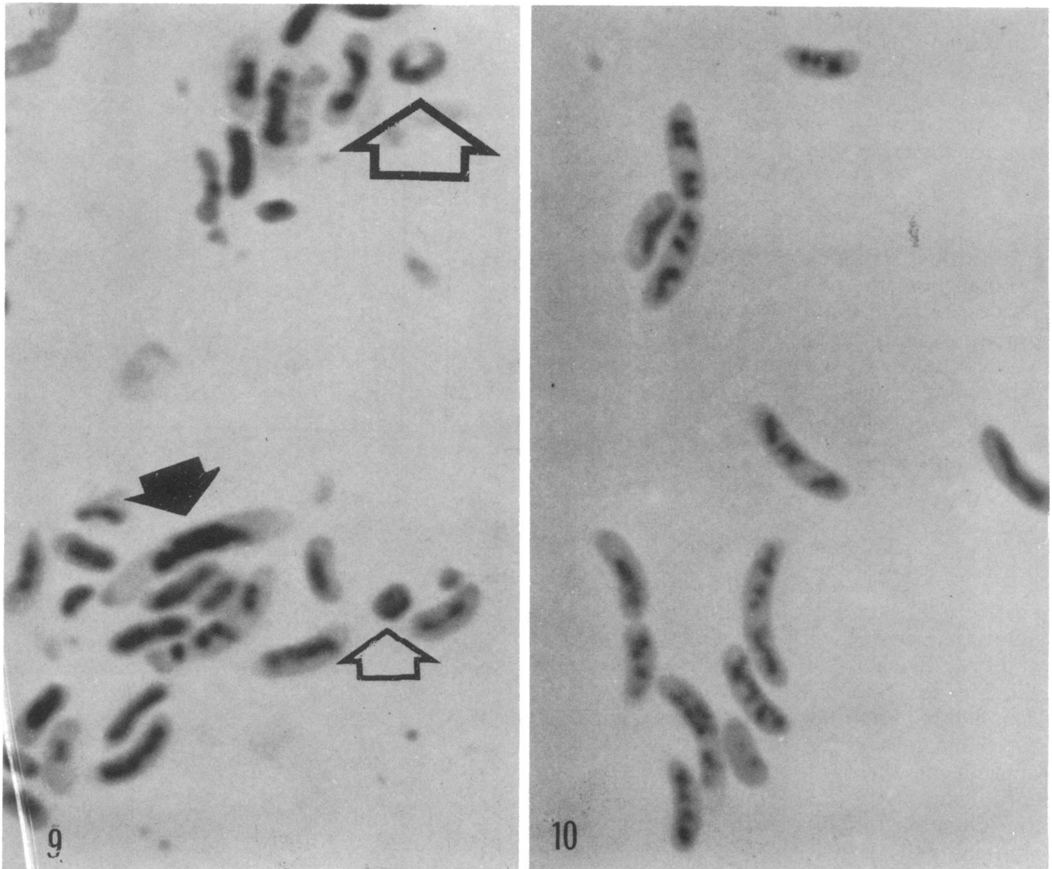


FIG. 9 and 10. *Giemsa-stained cells of Selenomonas ruminatium HD 1.*

FIG. 9. *Note the long cell (full arrow) with a condensed nuclear area. Empty arrow indicates circular nuclear material in spheroplasts. ×3,500.*

FIG. 10. *Well-delineated nuclear areas of growing Selenomonas ruminantium cells after 1 h of incubation. Many cells appear to have undergone fission and illustrate the quasi-multinucleate nature of dividing ruminant selenomonads also characteristic of other bacteria. ×3,500.*

cleus was very closely apposed to the concave side of the organism (dark arrows in Fig. 6, 9, and 10). Such cells are reminiscent of drawings published by von Prowazek (82) and by Boskamp (5) and of photomicrographs of *S. palpitans* prepared by Robinow (90).

At the peak of the stationary phase, which usually occurred 6.5 to 7.5 h after inoculation, the nuclei gradually lost their saddle and dumbbell shapes, and shrank into small, compact, globose structures.

At 18 h (phase of decline), individual chromatin areas became beaded and appeared to be connected by a thin Giemsa-positive strand. Some cells exhibited Giemsa-pale nuclei, whereas nuclei in other cells did not stain at all. A number of cells were found that obviously had begun to lyse. These could be distinguished from normal healthy cells not only by their pleomorphic appearance, but also by the granular and vacuolated appearance of their cytoplasm. Some nuclei were found to stain much more intensely. This was usually the case with cells where one, two, or all of the beaded nuclei coalesced into a long or ovoid body occupying the entire cell. This picture was strongly reinforced in 24 h after inoculation, when no distinct nuclear areas could be clearly distinguished. Similarly, under phase contrast the cells appeared "washed out," exhibiting no definite nuclear areas.

One of the more interesting series of observations was made of naturally and spontaneously occurring spheroplasts, usually present after 24 h. Three sets of samples were studied for this purpose: (i) 1.5-h cells containing spheroplasts of the inoculum; (ii) 24-h cells; and (iii) cells more than 14 days old. Observations showed no

gross differences in the nuclear structure between the various samples of spheroplasts. All spheroplasts stained well with Giemsa, and in all cases the chromatin body was peripherally placed, giving the appearance of a doughnut. In some instances, a semicircle of chromatin could be seen; in still other cells, a small segment of the chromatin was missing (Fig. 9). There was always a large number of crescentic cells that failed to give the characteristic Giemsa reaction.

The main results of the present studies point to the fact that, under the light microscope, the nuclear bodies of selenomonads look and behave like other bacterial nuclei, as well documented by Robinow (87-90).

FINE STRUCTURE OF BOVINE AND HUMAN SELENOMONADS

Two strains of *Selenomonas sputigena* (*Spirillum sputigenum*) from the oral cavity of man were first described by MacDonald (60). Later, MacDonald et al. (63) made extended observations of this particular species with special reference to flagellation, and included some electron micrographs of shadowed specimens. Similarly, van Iterson (40) published two electron micrographs of shadowed specimens of *S. palpitans*. The first electron micrographs of *Selenomonas ruminantium* were taken by Kanegasaki and Takahashi (47) by use of shadowed preparations.

The photomicrographs of *S. palpitans* by Robinow (90) suggested the intriguing possibility that the flagellar tuft might be implanted in or near the dividing nuclear region. In fact, the whole question of flagellation in selenomonads appeared to set the organisms apart, since bacteria are generally spoken of as (i) having no flagella, or (ii) being either polarly or peritrichously flagellated. It was inferred that a flagellar tuft arising from the middle of the concave surface of the organism must confer on the cell some unusual properties. Thus, the possession of a tuft of flagella became not only an important criterion in the classification of this organism, but a separate characteristic of general interest in the study of the fine structure of selenomonads.

General Considerations of Overall Structure

A negatively stained specimen of a typical selenomonad is shown in Fig. 11. This is ruminant strain HD 1 and illustrates both the morphology of the organism and the site of the insertion of the flagellar tuft, i.e., towards the center of the concave surface. This cell possesses 16 flagella, most of which form a single fascicle. This number of flagella was most frequently observed in preparations of selenomonads taken from fresh bovine rumen contents.

When the organisms form spheroplasts, the crescent-shaped cells change into globose forms (Fig. 12). Again the number of flagella is 16, and although they are all laid down on the supporting film of the grid in the same general direction, they do not seem to exhibit a closely coordinated tuft of flagella.

Both *Selenomonas ruminantium* and *S. sputigena* when sectioned were found to be typically bacterial in their fine structure. A representative thin section of each species is shown in Fig. 13 and 14. In Fig. 13 the cell was prepared by standard Ryter-Kellenberger (R-K) fixation, whereas in Fig. 14 the cell was fixed with glutaraldehyde and postfixed by the R-K procedure. The nucleoplasm (Fig. 13) appears fibrillar and is surrounded by an electron-dense cytoplasm. The ribosomes are approximately 10 nm in diameter and appear to form a network of dark, dense bodies. The cytoplasm is surrounded by a characteristic plasma membrane and the cell wall layers are located immediately outside the plasma membrane. In Fig. 13 and 14, the cell wall is composed of at least three layers which can be differentiated in the electron microscope. In the middle of the concave side of *S. sputigena*, stubs of longitudinally sectioned flagella are inserted through the cell wall (large arrow in Fig. 14).

Electron Microscopy of Flagella

Some disrupted cells showed to advantage the insertion site of flagella (Fig. 15). Flagella were measured to have an average length of 7.5 μm, with a wavelength of 3.5 μm, and a pitch of approximately 0.4 μm. The flagella form a fascicle and presumably function in unison.

Flagellar substructure can often be observed in shadow-cast preparations of sonically treated specimens of *Selenomonas ruminantium* (Fig. 16). In such preparations the flagella are 28 nm thick, yet, contrary to the statement in the present edition of *Bergey's Manual of Determinative Bacteriology* (6), they are not thicker at the base, but of uniform dimensions throughout the entire exposed length (Fig. 17). When compared to flagella not sonically treated, any increase in diameter of the flagellum must be ascribed to the method of preparation. Measurements of such preparations show that there is a repeating substructure approximately every 14.5 nm. Although a number of investigators have focused attention on the helical features apparent in some preparations of bacterial fla-

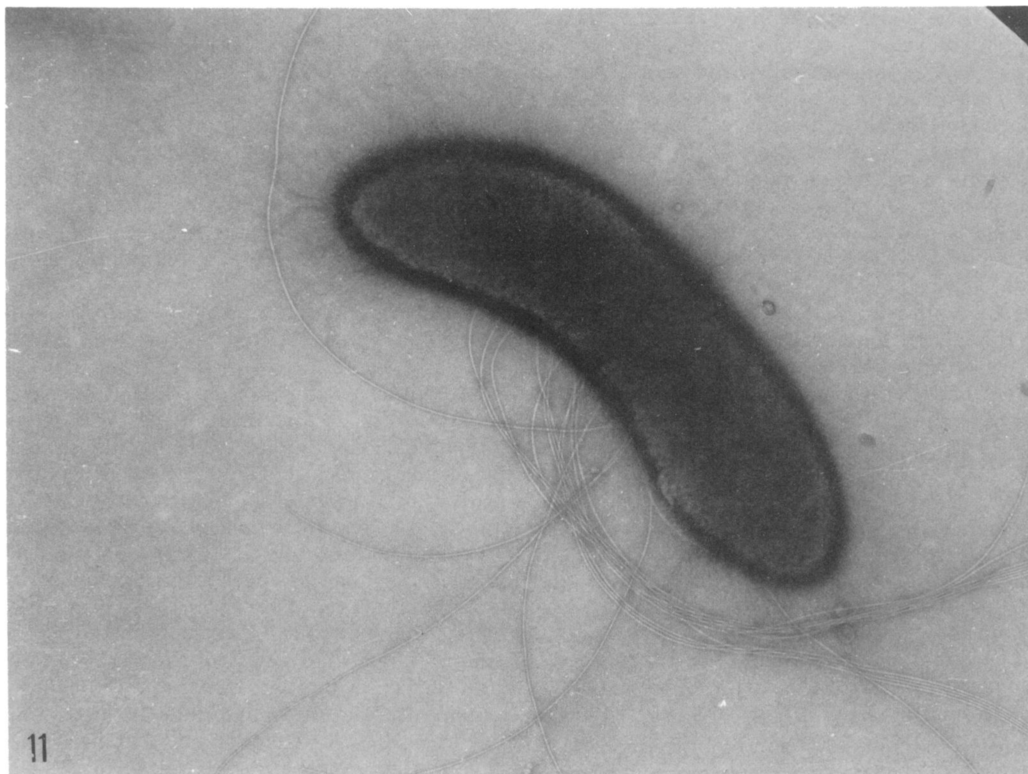


FIG. 11. *Selenomonas ruminantium HD 1 with a full complement of flagella. Note the vibrio-helicoid nature of the organism and the insertion site of the flagella. Negatively stained with phosphotungstate.* ×*18,000.*

gellar filaments (for recent reviews see Smith and Koffler [99] and Doetsch [22]), it has not yet been established that bacterial flagella are made up of fibrils. It is also known that when bacterial flagella are exposed to a variety of chemical and physical agents, thin, distinctly helical subfibrils can be observed (Abram et al., Abstr. Biophys. Soc. No. WC5, 1964). In the above sense, geometric reconstruction of selenomonad flagella reveals that there cannot be more than two "fibrils" per flagellum, if periodicity, angle of turn, and the thickness of the flagellum are taken into consideration.

In Fig. 12 the flagellar insertion site in the spheroplast is seen to be linear, so that the flagella emerge from the cell side in a neatly separated pattern, rather than as a compact tuft. This spatial distribution of the proximal ends of the individual flagella originated within the nonspheroplasted cell (Fig. 18). The flagella of this selenomonad are observed to penetrate the cell wall with the distance between the insertion sites of the 15 individual flagella showing a remarkable uniformity, i.e., a center-to-center distance of 68 nm. Here the thickness of the flagella is approximately 17 nm.

A Note on the Flagella of S. palpitans

Since *S. palpitans* was not available in pure culture, a study of motility and of the number of flagella per cell during growth could not be performed. However, results obtained with negatively stained preparations of the contents of guinea pig cecum (the habitat of *S. palpitans*) yielded a number of important observations. Thus, a typical cell of *S. palpitans* (Fig. 19) is approximately 4.5 μm long when measured in a straight line from end to end, and 1.5 μm at its widest point. The cell exhibits a strictly coordinated tuft of 22 flagella. The wavelength of the flagella is approximately 7 μm, with an amplitude of about 1 μm. The surface of the cell does not appear to be smooth, but this could be an artifact of preparation. The flagellar tuft or the individual flagella are not enclosed by a sheath. The flagella are of the same thickness throughout their free length, and are not "thicker at the base" as previously stated (6). Some flagella appear shorter than others, but the ends of these flagella could possibly have been broken off during preparation as is suggested by the presence of two or three loose

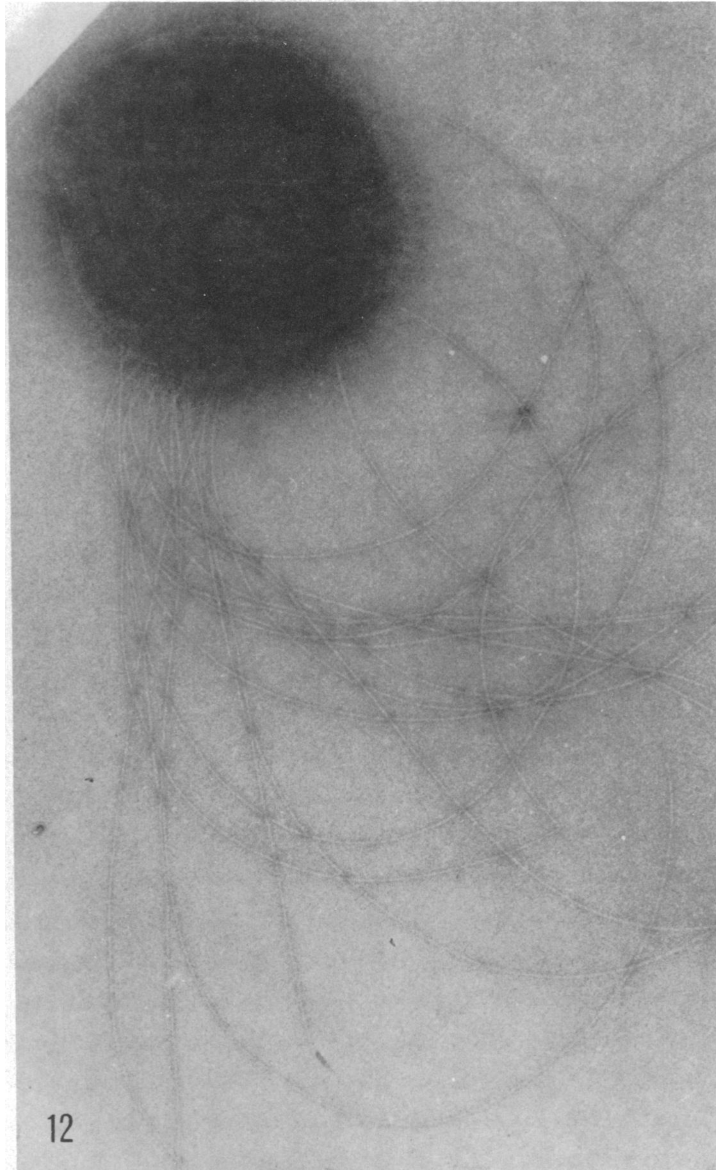


FIG. 12. *A spheroplast of Selenomonas ruminantium HD 1 with flagella still attached. Negative stain (phosphotungstate).* ×25,000.

flagella in the micrograph.

In this connection, it is important to note that the diameter of the flagella of *S. palpitans* contrasts with the much thicker and more complex flagella of eukaryotic organisms. This observation supports the view that *S. palpitans* is a bacterium and not a protozoon as suggested by Wenyon (108), Jeynes (43), and by Wilson and Miles (109).

A higher magnification of the proximal portion of the flagellar tuft of a lysozyme-treated cell of *S. palpitans* is shown in Fig. 20, and reveals that the average diameter of the negatively stained flagella is 15 nm. The flagella of *S. palpitans* are all bunched together in a single area, rather than forming a line of insertion parallel to the cell surface as is the case in *Selenomonas ruminantium*.

Characterization of Selenomonad Cell Wall

Thin sections of normal selenomonads (Fig. 13 and 14) reveal a multilayered profile to the

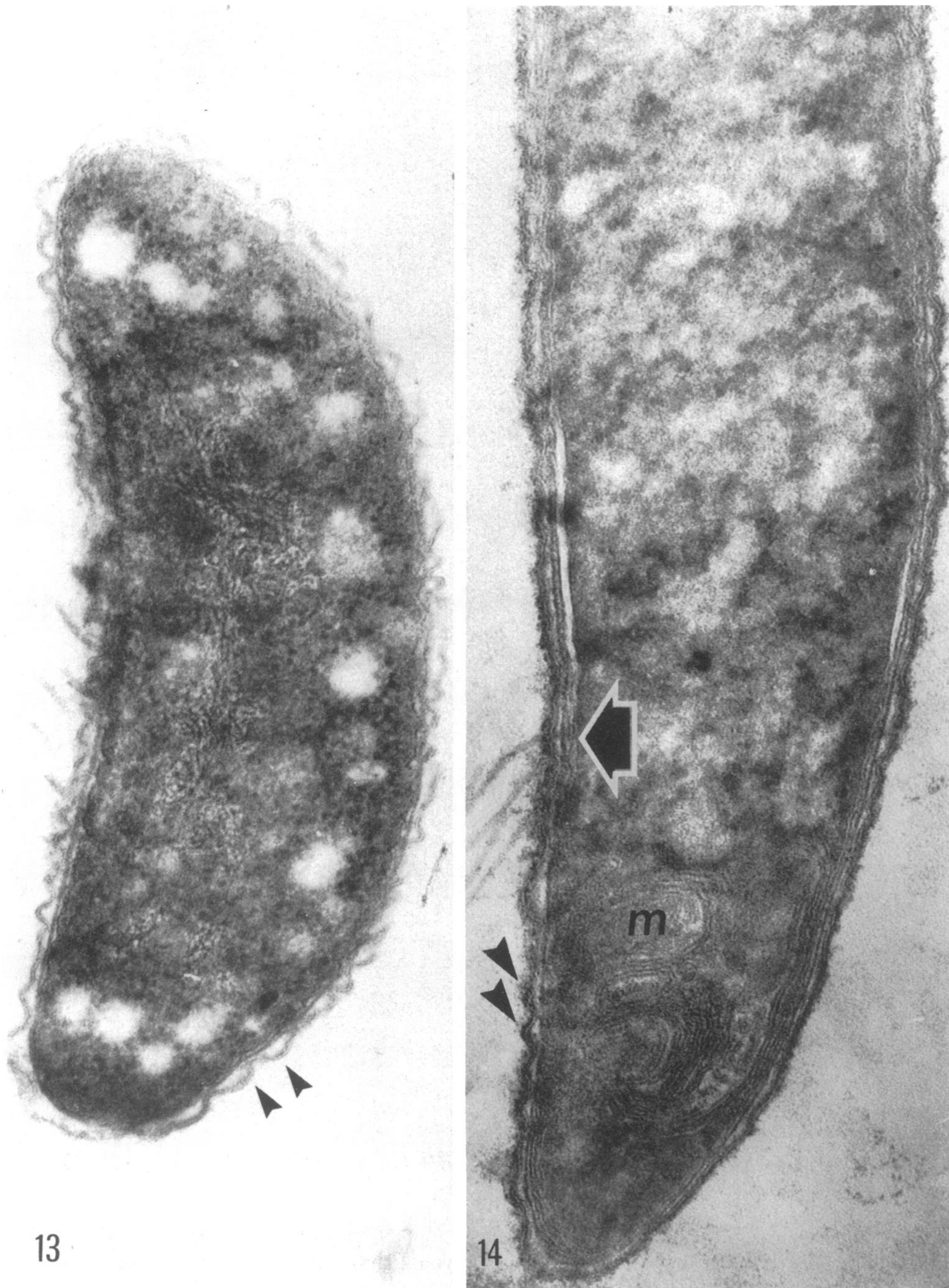
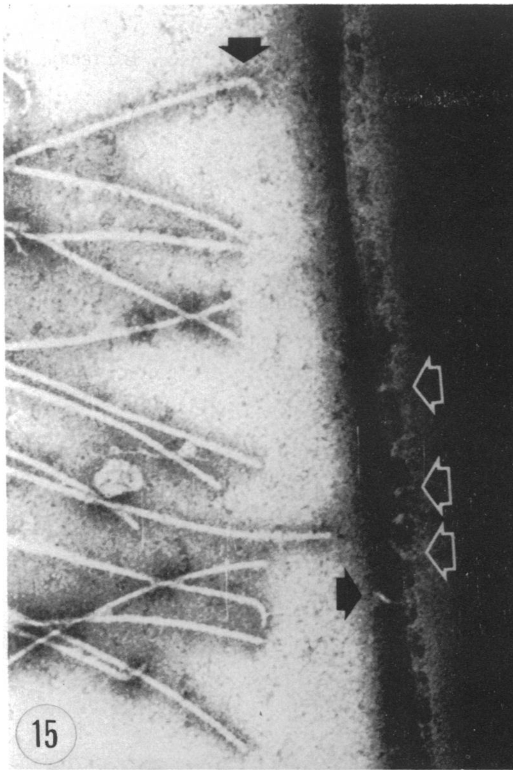
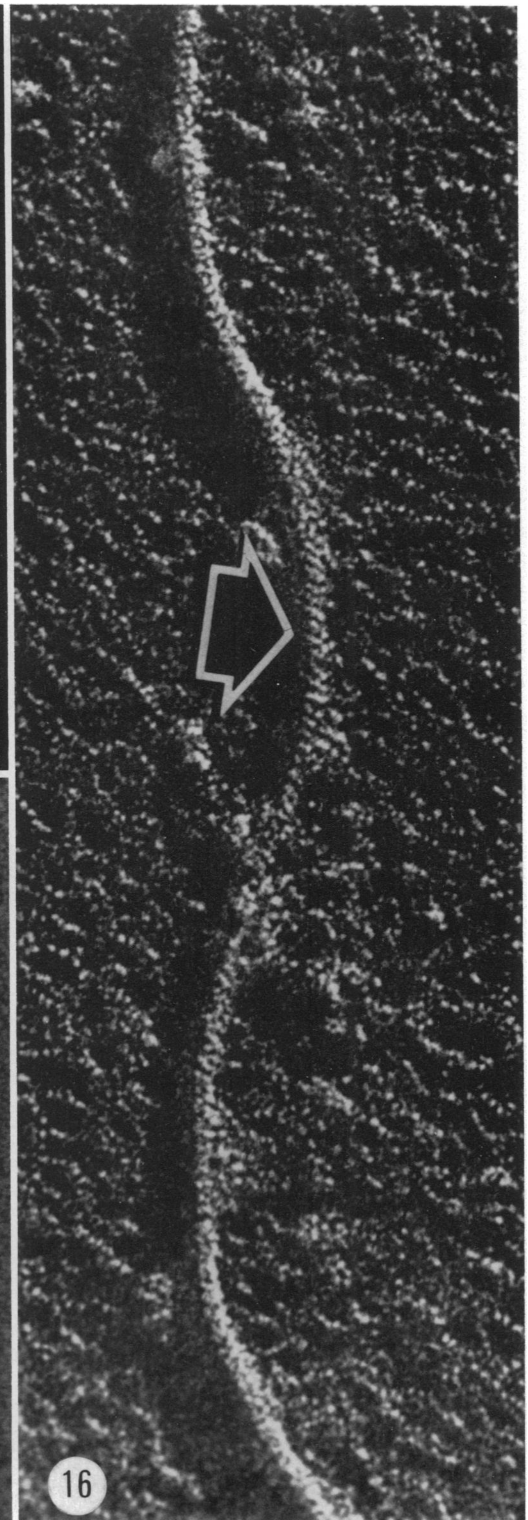


FIG. 13 and 14. *Comparison of structure in representative selenomonads.*

FIG. 13. *Thin section of a typical cell of Selenomonas ruminantium (HD 1). Note irregular, undulating outer layers of typically gram-negative cell wall (pointers). Insertion sites of flagella cannot be seen in this section. R-K fixation.* ×*40,000.*

FIG. 14. *Thin section of oral strain Selenomonas sputigena B. A number of flagella (arrow) penetrate the cell wall and the plasma membrane. A large mesosome (m) is seen in the lower half of the micrograph. Undulating cell wall profile can also be observed (pointers). Glutaraldehyde fixation. Postfixation with R-K.* ×*70,000.*





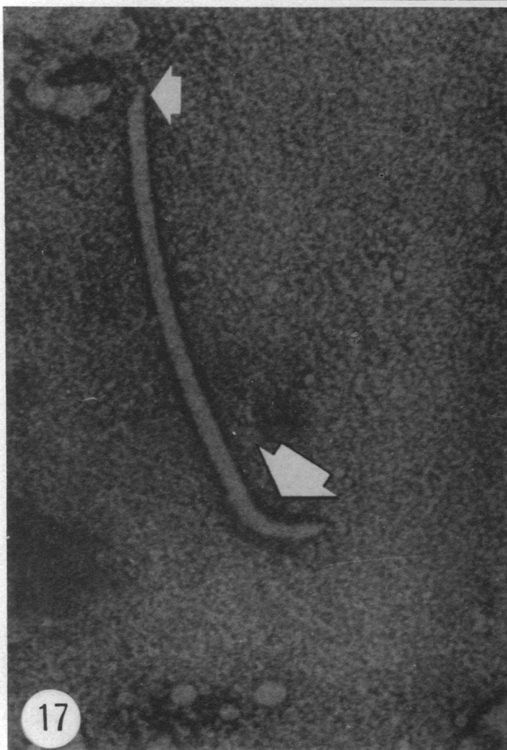


FIG. 15, 16, and 17. *Flagella of selenomonads.*

FIG. 15. *Flagellar tuft separated from a cell of Selenomonas ruminantium GA 31. Note typical bacterial flagellar hooks (full arrow). Some parts of the torn-off flagella are still inserted in the cell (empty arrows). Negative staining (phosphotungstate). ×64,000.*

FIG. 16. *A sonically disrupted flagellum from Selenomonas ruminantium HD 4. Arrow points to area showing flagellum fibrils to advantage. Shadow cast with platinum. ×117,000.*

FIG. 17. *An isolated selenomonad flagellar hook (large arrow) with part of the flagellar filament from a sonically disrupted Selenomonas ruminantium HD 4. Note that the filament ends in a sharp point (small arrow) at the site of fracture. Phosphotungstate. ×100,000.*

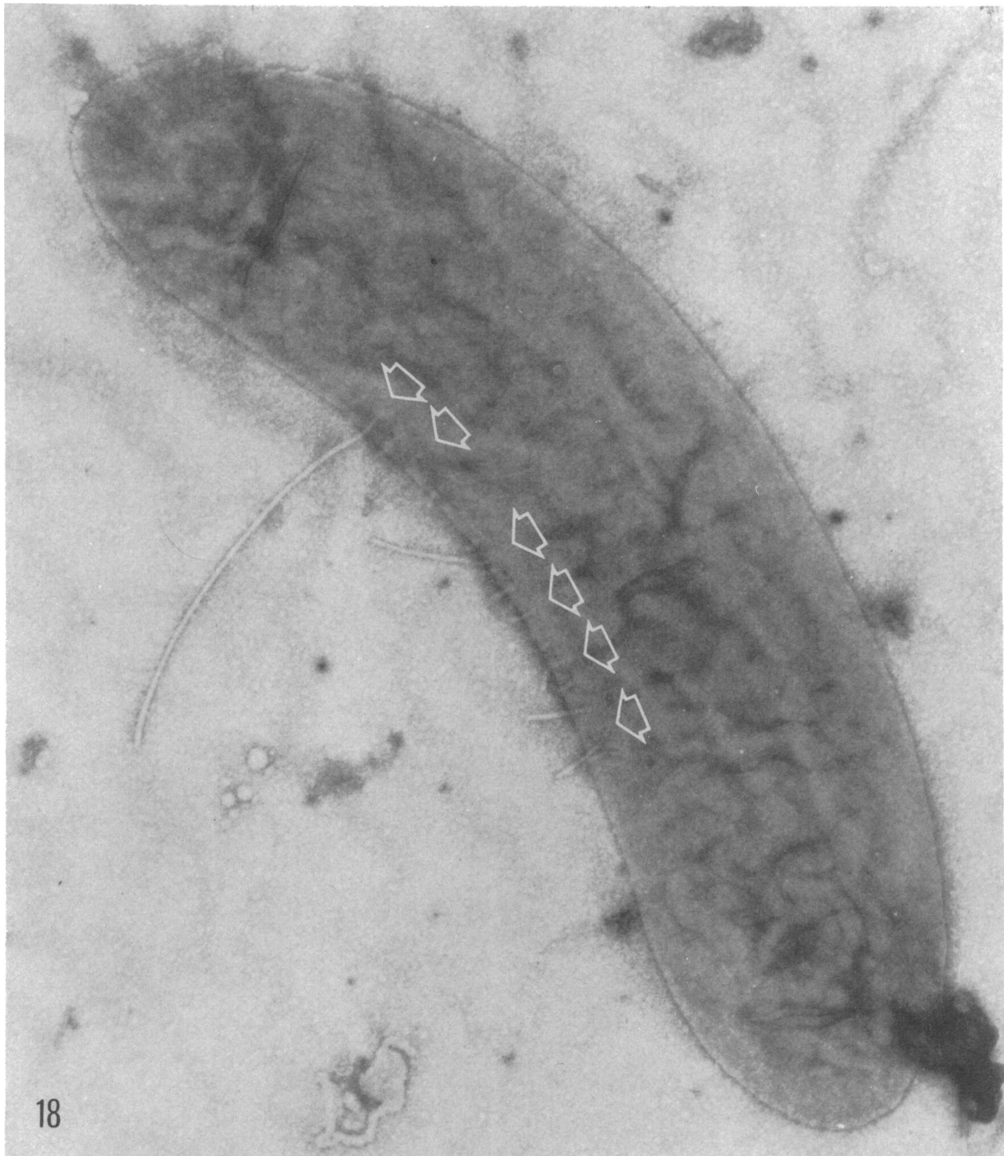


FIG. 18. *Arrangement of flagella in Selenomonas ruminantium no. 6. Arrows point to insertion sites of flagella on this organism. Most of the flagella have been torn off when the cell was exposed to the action of Ballotini beads in a Mickle disintegrator. Note the linear arrangement and equal spacing between the flagellar stubs remaining in the cell. Phosphotungstate. ×61,000.*

cell wall which is typical of gram-negative bacteria (54). Both *Selenomonas ruminantium* and *S. sputigena* exhibit a convoluted outer double layer that contacts the inner (murein) layer only occasionally. The murein layer in *S. sputigena* varies in width from approximately 9 to 12 nm. Similar results were obtained with *Selenomonas ruminantium*. The outer double layer appears to have the characteristic profile of lipopolysaccharide and lipoprotein as described by Murray et al. (75). Some loosely packed material adheres to the ripples of the outer layers in *S. sputigena*, whereas in *Selenomonas ruminantium* such material is lacking. The outer layers in both organisms measure 10 to 15 nm across. This would indicate that the cell walls of selenomonads are almost twice as thick as those given for the much studied *Escherichia coli* (75).

The total cell wall thickness of selenomonads

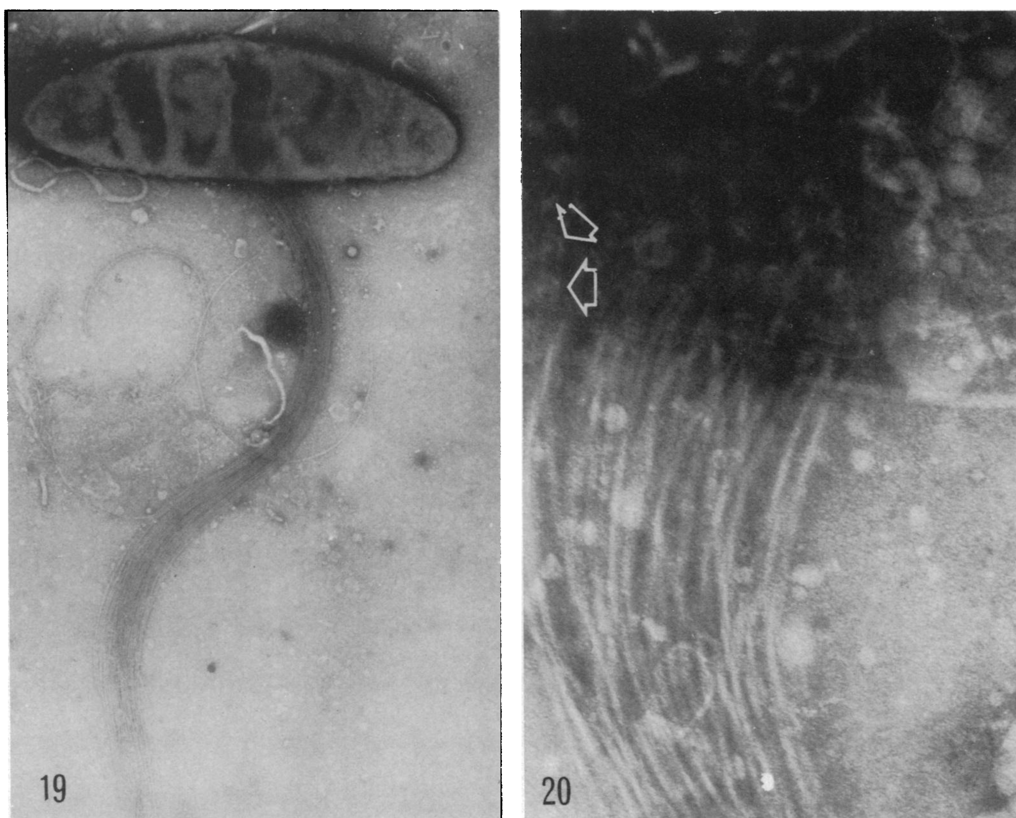


FIG. 19. *Typical cell of Selenomonas palpitans from the cecum of guinea pigs. Note the well-coordinated flagellar tuft. Phosphotungstate.* ×*15,000.*

FIG. 20. *Insertion site of the flagellar tuft in Selenomonas palpitans. Arrows indicate individual insertion areas of what appear to be prokaryotic-type flagella. The flagella seem to be inserted in the cell in a random spatial arrangement. Compare to linear arrangement of flagella shown for Selenomonas ruminantium in Fig. 18. Phosphotungstate.* ×*90,000.*

would thus be 19 to 27.5 nm.

Shadow-cast preparations of normal cells of *Selenomonas ruminantium* and *S. sputigena* did not reveal the surface pattern observed in *S. palpitans* by van Iterson (40).

Isolated cell walls of Selenomonas ruminantium. Early log-phase cells of the ruminant selenomonad strain HD 1 were disrupted in a Mickle disintegrator by shaking with 0.2 mm Ballotini beads for 10 min at a shaking amplitude of approximately 2.5 inches (approximately 6.35 cm). Several suspensions of cell walls were combined, kept at 4 C, and prepared for observation in the electron microscope as outlined by Salton (94).

Such broken cells of *Selenomonas ruminantium* HD 4 after disintegration and incomplete purification are seen in Fig. 21. One of the cells is disfigured by the physical forces employed and shows a partially broken cell wall. It appears that some cytoplasmic material is still present within the cell (full arrows). The lines of fracture in the broken cell run straight across the cell only and not parallel to the long axis of the organism (empty arrows). All such preparations regularly showed cell wall fractures in this direction only. This would indicate that, in the selenomonad cell wall, the weaker links susceptible to mechanical disruption may be those parallel to the smaller circumference of the cell.

Mechanically disrupted portions of selenomonad cell walls are shown in Fig. 22. It is not known which side or which of the multiple layers of the cell wall the figure depicts. Some parts of the cell wall exhibit a pitted pattern, which upon higher magnification can be resolved as an area with a regular, repeating shape (Fig. 23, circled areas). The center-to-center distance of these subunits is approximately 10 nm. Freeze-etch preparations are needed to show such structures to advantage

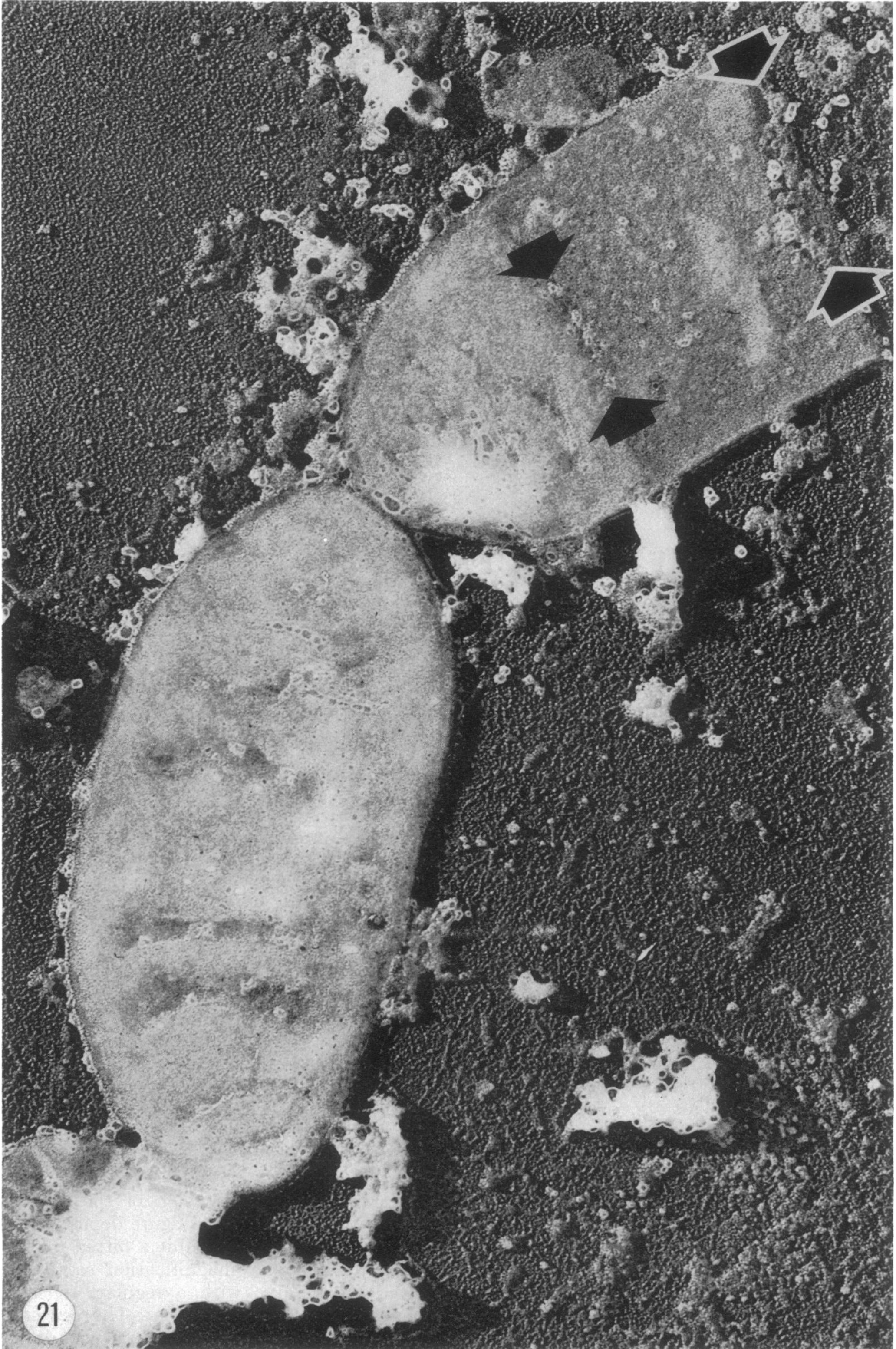


FIG. 21. *Ruptured cells of Selenomonas ruminantium HD 4 after sonic treatment. Note lines of fracture (empty arrows) resulting in clean breaks along the cell wall. Some escaped cytoplasmic material (full arrows) is still present and is evident in the partially empty organism. Platinum shadowed. ×51,000.*

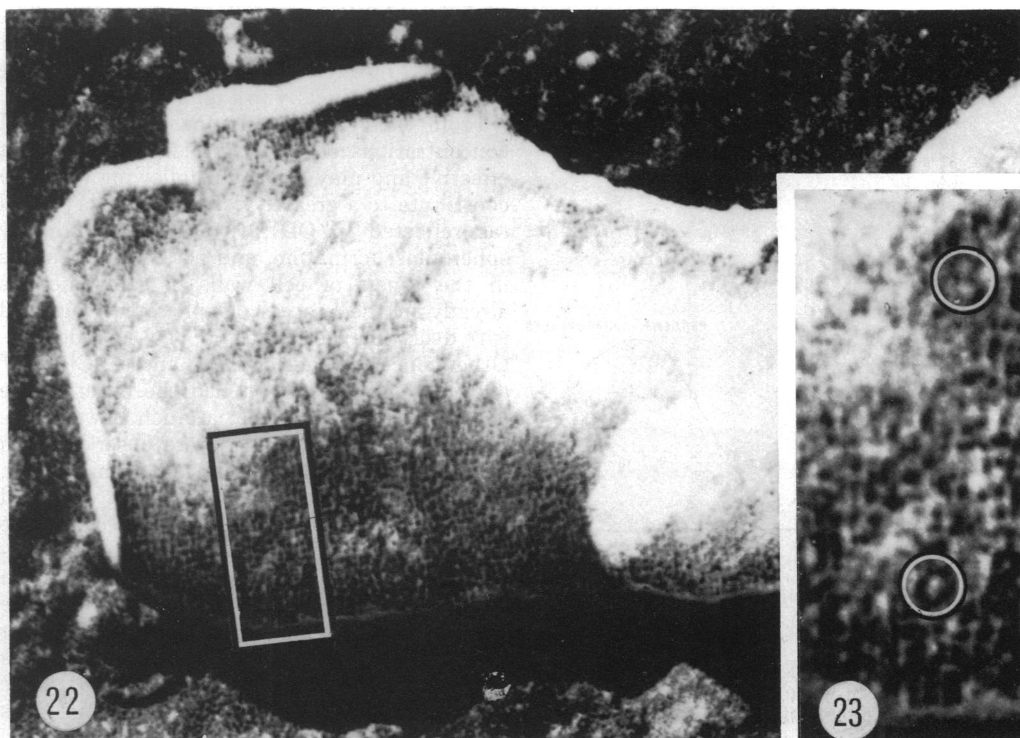


FIG. 22 and 23. *Cell wall of Selenomonas ruminantium HD 1.*

FIG. 22. *Part of a cell wall from a growing cell exposed to Ballotini beads (see text). The cell wall fragment appears angular, possibly indicating lines of weakness in the overall construction. White rectangular area is depicted at a higher magnification in Fig. 23. Palladium shadowed. ×125,000.*

FIG. 23. *A higher magnification of cell wall areas delineated in Fig. 22. Circles point to a regular pattern of subunits in some areas. Palladium shadowed. ×195,000.*

and in proper spatial relationship to other components of the cell wall.

Chemical characterization of murein. Using the method of Gunetileke and Anwar (31), preparations of cell wall hydrolysate were subjected to electrophoresis for a preliminary analysis. Standard muramic acid (Sigma Chemical Co., St. Louis, Mo.) was spotted side by side with the cell wall hydrolysate of *Selenomonas ruminantium* strain HD 1. Both had the same mobility and occurred 5.5 cm from the origin. These results are in excellent agreement with those for other organisms published by Gunetileke and Anwar (31). Paper chromatography of cell wall hydrolysates was performed for better resolution. The developed chromatogram showed the following amino acids to be present; asparagine, glutamine, alanine, glutamic acid, valine, tyrosine, phenylalanine, and isoleucine. Significantly, muramic acid was also present. The presence of such a component indeed demonstrates that *Selenomonas ruminantium* is a bacterium and not a protozoon. Hydrolysates were not prepared of any of the oral selenomonad strains. It was, of course, impossible to investigate *S. palpitans* for the reasons outlined above.

Natural lysis of ruminant selenomonads. When Hobson (32) grew *Selenomonas ruminantium* in continuous culture, he noted that, after maximum growth had been attained, the selenomonads tended to lyse fairly rapidly, and turbidity readings decreased. Similar observations were made with all ruminant strains of selenomonads in our investigation. This effect, however, was not observed with the oral strains.

There is no prolonged stationary phase in the growth curve of *Selenomonas ruminantium* (HD 1) (see Fig. 1). The decline phase sets in almost immediately after the peak of growth has been reached. Phase contrast observations show an increasing number of oval and round cells, some of which are still sluggishly motile. Quantitative data of these observations are compared graphically in Fig. 24, and show that *Selenomonas ruminantium* spheroplasts begin to appear under these conditions of cultivation

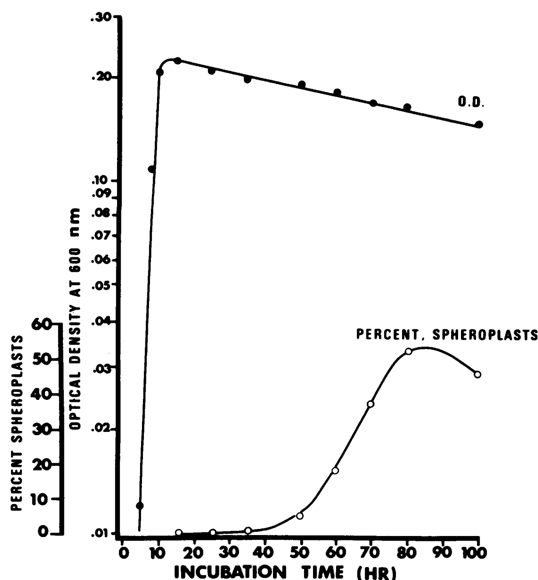


FIG. 24. *Appearance of spheroplasts in cultures of Selenomonas ruminantium HD 1 growing in MPB broth (see text). Note that the percentage of spheroplasts in the culture begins to increase immediately after the onset of the stationary and decline phases. Almost 50% of the cells have spheroplasted after 80 h of incubation.*

approximately during the 3rd day of incubation and that their number increases gradually. At 80 h of incubation, about half of the population had spheroplasted. Another set of experiments, employing a 10-fold increase in inoculum size, showed that spheroplasts appeared after 15 h of incubation. In each case the appearance of the spheroplasts coincided with the time after which maximum growth had been achieved, and where one would normally encounter the stationary phase in most other bacteria. No such population equilibrium has been demonstrated in *Selenomonas ruminantium*. It is interesting to note that, after the number of spheroplasts in the culture reaches its peak, a decline in the number of such cells follows.

Murray (30) quoted Glaister's work which showed that *Spirillum* lyses rapidly when the stationary phase is reached coinciding with the depletion of Ca^{2+}, but not of Mg^{2+}. For this reason we assumed that a similar lack of some divalent cations in the medium could be responsible for the weakening of cell walls in selenomonads as well as for the diminished activity of the enzymes involved in the synthesis of the layers of the cell wall, particularly murein. Four divalent cations in MPB broth have been tested for. These were Ca^{2+} (as $CaCl_2$), Mn^{2+} (as $MnCl_2$), Mg^{2+} (as $MgCl_2$), and Zn^{2+} (as $ZnCl_2$). The complete medium served as the control, but it contained no excess of cations. The results are presented graphically in Fig. 25.

From these results it appears that 10^{-2} M concentrations of $CaCl_2$ and $MnCl_2$ are most effective and may act in the following ways: (i) contribute to a greater yield of bacteria per ml (as reflected in OD); (ii) delay temporarily spheroplast formation, and possibly play a role in the repair of cell walls of selenomonads already in the process of spheroplasting; and (iii) delay the onset of the phase of decline. Other concentrations of $CaCl_2$ and $MnCl_2$ (10^{-3} and 10^{-4} M) also postponed the onset of the decline phase, although at a much slower pace. No such dramatic results were observed with $MgCl_2$ or $ZnCl_2$. At a concentration of 10^{-2}, however, $ZnCl_2$ did noticeably affect cell yield as reflected in OD and in the presence of a true stationary phase. The Zn^{2+} at a lower concentration (10^{-4} M) results in heavier growth than is achieved by the 10^{-2} M concentration. On the other hand, there is a rapid decline in OD after the peak of growth has been reached, and this would indicate lysis of organisms in the culture medium. When compared to the control, lysis appears much later in the growth cycle. Although Burger and Glaser (13) found that high levels of Ca^{2+} are required for teichoic acid synthesis, so far this cation has not been implicated in any of the reactions of murein synthe-

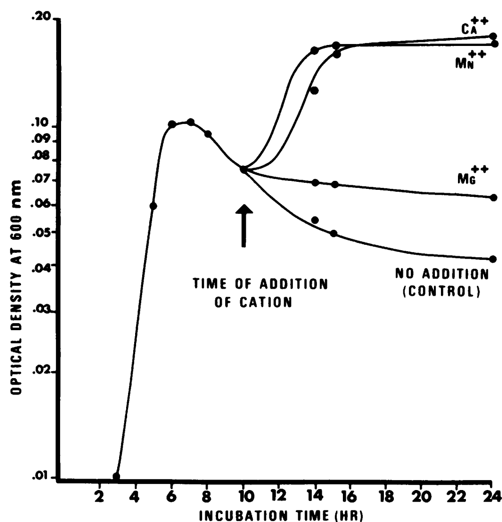


FIG. 25. *Effect of addition of various cations on OD in growing cultures of Selenomonas ruminantium (see text). It is of interest to note that the number of spheroplasted cells was minimal in cultures containing additional* Ca^{2+} and Mn^{2+}.

sis. The cation is involved in some way in cell division and possibly in cell wall formation. Abramson et al. (2) suggested that calcium binds very strongly to phospholipid micelles.

On the other hand, Mn^{2+} is required by the enzyme which adds D-alanine to mucopeptide (42). Similarly, Ca^{2+} and Mn^{2+} protect and stabilize the plasma membrane. That Mg^{2+} and possibly Zn^{2+} may play similar roles in cell wall synthesis can be partially inferred from the results of these experiments, although the latter two divalent cations are not as effective as Ca^{2+} or Mn^{2+}.

Selenomonads form spheroplasts either spontaneously (see above) or by controlled exposure in MPB medium to 5,000 IU of penicillin per ml with 20% (wt/vol) sucrose and 0.2% $MgSO_4$, or to 70 μg of crystalline lysozyme (EC 3.2.1.17) per ml, with a final concentration of 134 μg of ethylenediaminetetraacetate (EDTA) in an osmotically stable medium with 10% (wt/vol) sucrose. Our results indicate that, although such spheroplasts differ from each other in ultrastructure, the individual process of lysis seems to be similar to that observed in other bacteria. During the process of spheroplast formation, some parts of the outer cell wall layers appear to be stretchable, whereas other parts seem fairly non-elastic, possibly due to the condition of the underlying rigid layer. Hydrolyzed murein portions of the cell wall cannot offer the rigid, corset-like protection to the ballooning cytoplasm. This is evident in the lower cell in Fig. 26. The left part of the murein layer in the cell is in an advanced state of hydrolysis, whereas the right portion of the cell still exhibits a well-defined rigid layer, albeit with some breaks in it. This leads to the assumption that a general weakening of the rigid cell wall layer (due to multiple-point hydrolysis of the murein) does not favor any "single-point" escape of the spheroplast, and may mean that the murein synthesis in *Selenomonas ruminantium* HD 1 takes place at many points simultaneously as was observed by Cole (20) in other gram-negative cells.

It was interesting to note that there was not a single lysing or lysed cell (whether spontaneously produced, or induced by lysozyme or by penicillin) which showed any intracytoplasmic membranous intrusions of the mesosome type. This observation is in accord with a number of published results by Ryter and Landman (93), Fitz-James (25, 26), Kushnarev and Pereverzev (52), Ghosh and Murray (29), and Ryter (92).

A Note on the Cell Wall of S. palpitans

S. palpitans has never been grown in pure culture. For this reason it was impossible to perform controlled experiments of the type carried out with the other two selenomonads. Likewise it was not possible to obtain final proof whether this particular organism is a protozoon or a bacterium. However, one experiment in which large concentrations of lysozyme were added to a diluted, fresh preparation of guinea pig cecal matter was designed to yield at least some information as to the basic nature of this elusive organism, especially regarding the composition of its cell wall. It was reasoned that, if lysozyme lysed bacterial cells in the cecal fluid, then partial evidence supporting classification of the organism as a bacterium would be obtained. Figure 27 shows a negatively stained cell of *S. palpitans* after 2 h of treatment with lysozyme (approximately 200 μg for each ml of cecal matter). The surface of the cell is irregular, indistinct, and obviously disrupted. Two flagellar tufts, having 24 flagella each, are well separated from one another, signifying that the cell was either in the process of division, or had already divided. The action of lysozyme on the cell is quite devastating, showing that the whole of the cell surface was affected. This observation is cautiously taken as positive evidence that the organism possesses a murein-like component in its cell wall.

Plasma Membrane and Its Variations

Membranous intrusions in the form of mesosomes are known to occur in gram-positive bacteria more frequently than in gram-negative bacteria, or at least are more easily demonstrated in the former than in the latter (24, 41, 78, 91). Such an internal membrane system is also present in the selenomonads.

Certain types of membranous intrusions appear to predominate in selenomonads during certain stages of growth. As in other bacteria (11) such membranes are often found in close association with a morphologically distinct entity, such as the developing cell septum, flagellar region, etc.

Membranous intrusions. When a growing culture of *Selenomonas ruminantium* is sampled at intervals and prepared for thin sectioning, the cells at first show a scarcity of mesosomes or membranous intrusions, or both. During further growth, the number and size of mesosomes increase, and, as shown by Burdett and Rogers (12) for *Bacillus licheniformis*, the general appearance of these structures depends on the preparative techniques used prior to sectioning. Mesosomes and other types of membranous intrusions in selenomonads are shown in Fig. 28 and 29. Generally speaking, the mesosomes conform in their appearance to membranous structures observed in other bac-

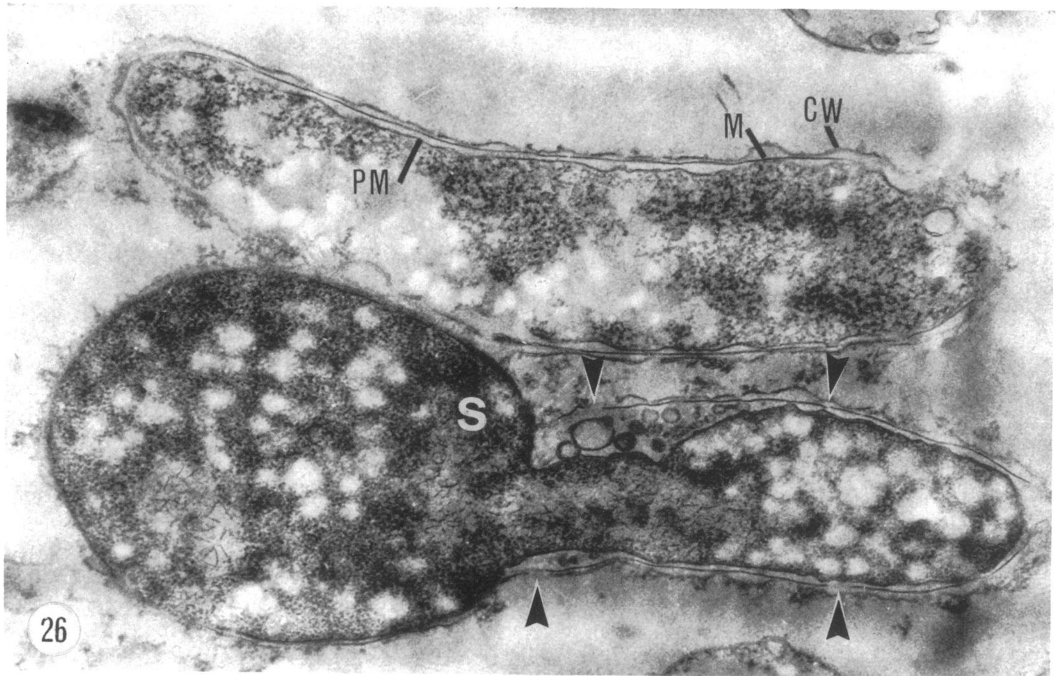


FIG. 26. *Thin section of S. ruminantium HD 1 grown in penicillin. The lower cell is a well-defined spheroplast (S) in the process of emerging from the still fairly rigid cell wall remnant. Note multiple breaks in glycopeptide layer (pointers) represented by dense, fine line. The upper cell denotes some of the structures in thin sectioned selenomonads. Abbreviations: CW, cell wall layers; M, murein component of cell wall; PM, plasma membrane. R-K fixation.* ×*39,150.*

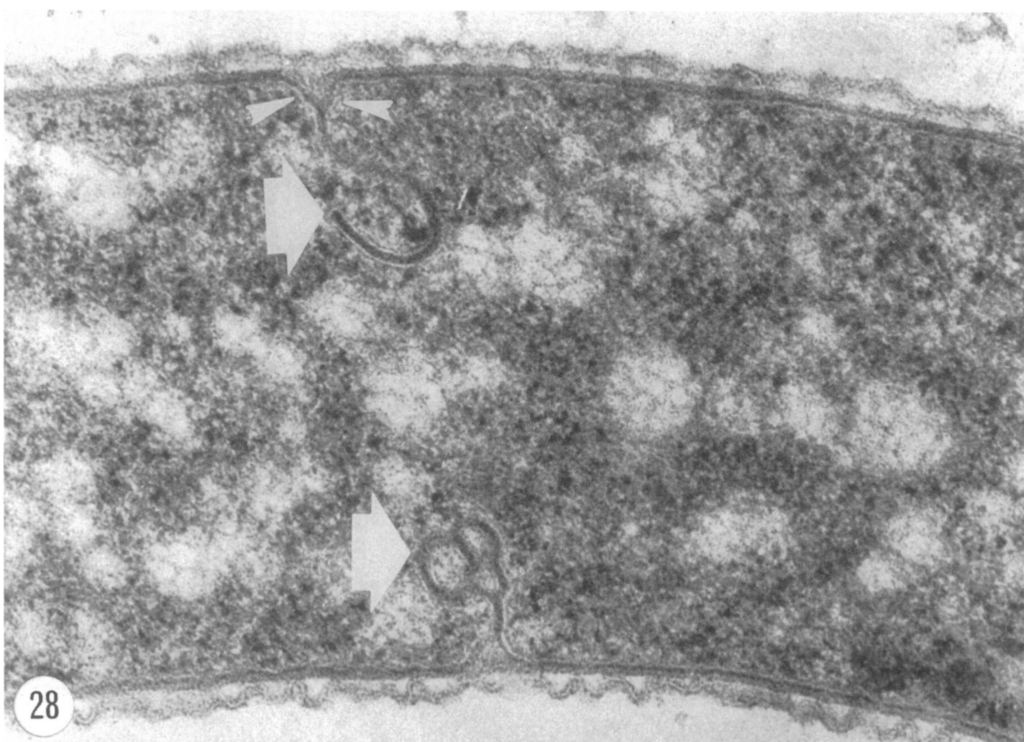
teria by a number of workers. It is interesting to note that the diameter of the membranous intrusions seen in these thin sections varies. The diameter of the membrane vesicles is approximately twice as thick as the lamellar type, and also much more dense. This difference in density corresponds with evidence presented by Silva (96) for some *Bacillus* species. In Fig. 28, both the murein layer and the plasma membrane are seen to turn off into the growing cell. There is a suggestion that the murein layers join at the site of future septum formation. Also, the relative densities of the murein-plasma membrane layers already present are identical to those that are forming.

In selenomonads the lamellar and the vesicular types of membranous intrusion apparently arise from the tubular type, as can be seen in a longitudinal section of *S. sputigena* (Fig. 30) where the membranous intrusion emerges as a long tube approximately 0.8 μm long, extends along the wall of a part of the cell, and terminates abruptly. The thickness of the two layers together is approximately 16 nm. Generally, tubular mesosomes were seen in close association with the concave side of selenomonad cells, but it was not always clear that actual tubules were involved. In some cases such membranes appeared stacked on top of one another, presenting multiple layers (Fig. 31), and apparently not immediately connected with the plasma membrane. This, of course, could be due to the fact that the plane of sectioning was such that no connections or continuities with the plasma membrane were revealed.

A series of observations of mesosomes in the immediate vicinity of the developing cell septum of *Selenomonas ruminantium* is presented in Fig. 32 showing thin-sectioned cells sampled at various stages in their growth cycle. The ingrowing septum-mesosome complex must be interpreted three-dimensionally in the form of a constricting "doughnut."

The initial stages in the formation of the septum are accompanied by the development of convoluted tubular mesosomes (Fig. 32a). The membranes grow inward, at first in a straight manner, but later, by bending, they form membrane vesicles (Fig. 32b). Further inward growth of the apposing mesosomes brings the opposing surfaces in contact (Fig. 32c). A septum is formed consisting of the plasma membrane and

FIG. 27. *A lysozyme-treated cell of Selenomonas palpitans. The cell exhibits two prominent tufts of approximately 22 flagella each. The organism is considered to be in the process of division as usually only one flagellar tuft is present. The surface of the cell appears disrupted due to lysozyme (compare to Fig. 19). Phosphotungstate.* ×*38,000.*



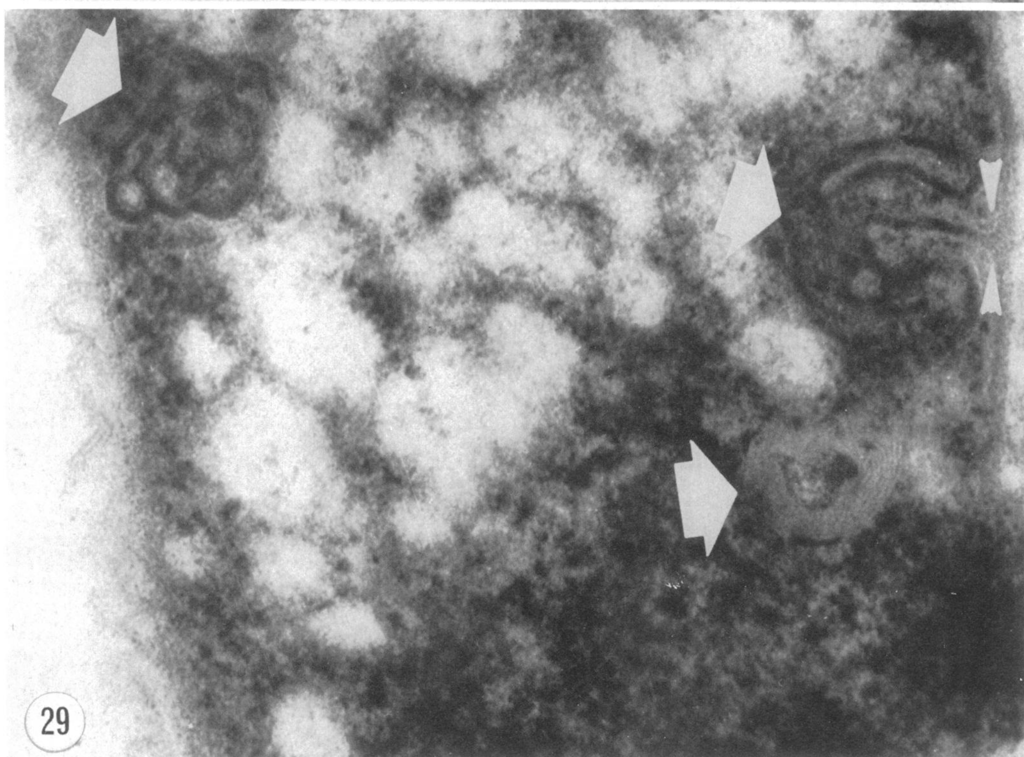


FIG. 28 and 29. *Membranous intrusions in selenomonads.*

FIG. 28. *Thin section of two developing mesosomes (arrows) in Selenomonas ruminantium HD 1. Note that the mesosomes are opposite each other as the cell prepares to divide. At the future site of the septum the plasma membrane and the murein layer have invaginated on both sides of the cell (pointers). Glutaraldehyde fixation. R-K postfixation.* ×*100,000.*

FIG. 29. *Presence of various types of mesosomes in dividing Selenomonas ruminantium HD 1. Thin section of membrane vesicles, membrane tubules, and membrane lamellae (arrows)—all are present at once. Murein layer and plasma membrane invagination are seen at right (pointers) turning off into the cell. Glutaraldehyde fixation. R-K postfixation.* ×*146,000.*

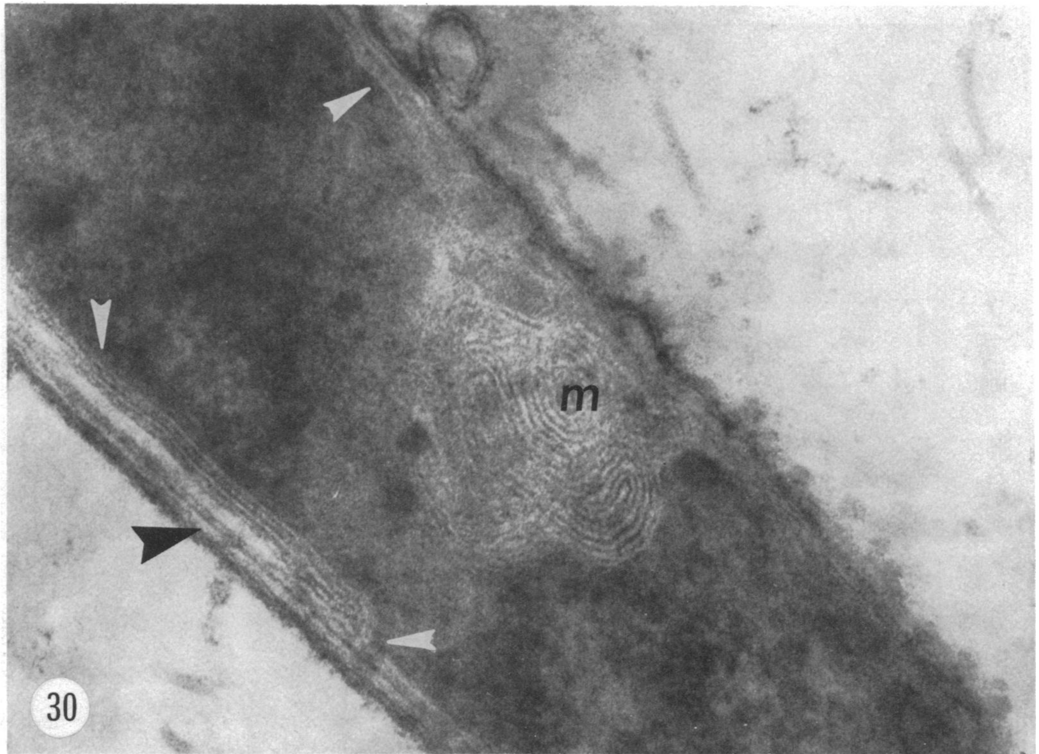


FIG. 30. *Thin section of an oral selenomonad. A large mesosome (m) occupies part of the cytoplasm of S. sputigena. White pointers show the extent of membranous intrusions. The plasma membrane is closely apposed to the inside of the cell wall layers (black pointer). R-K fixation. ×117,000.*

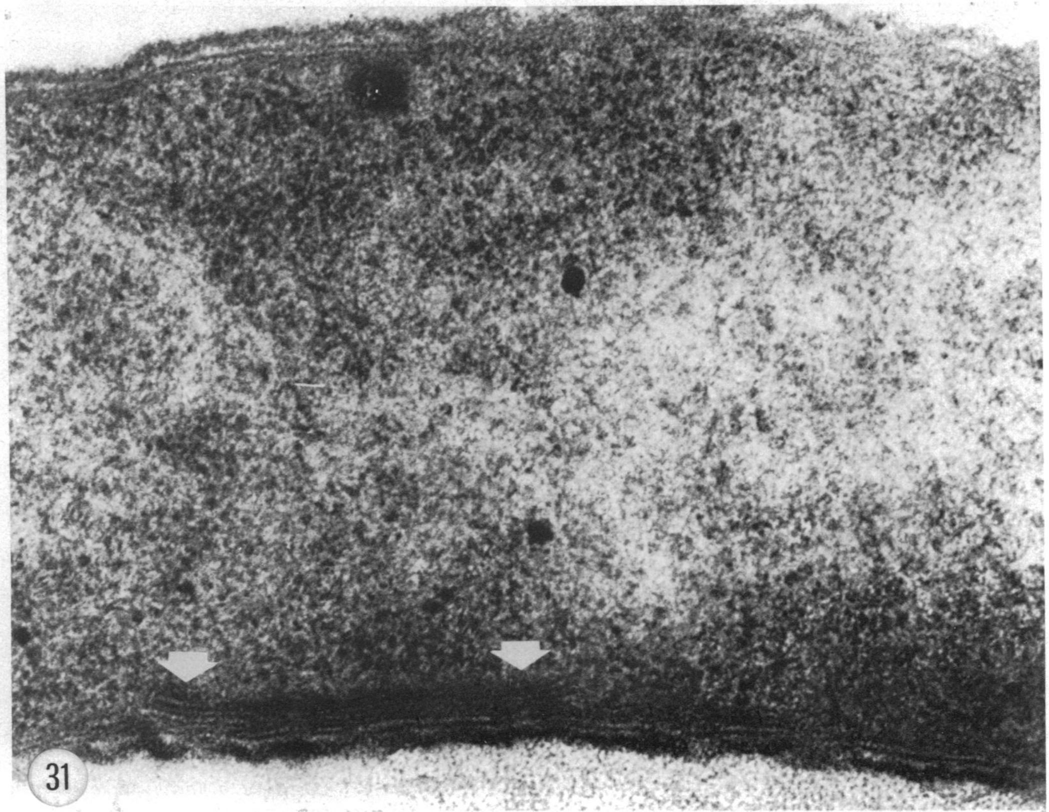


FIG. 31. *Membranous intrusions of selenomonads. A thin section of Selenomonas ruminantium showing membrane lamellae (arrows) closely associated with the cell wall. No connections of the lamellae to the plasma membrane can be seen in this section. Glutaraldehyde fixation. R-K postfixation. ×119,000.*

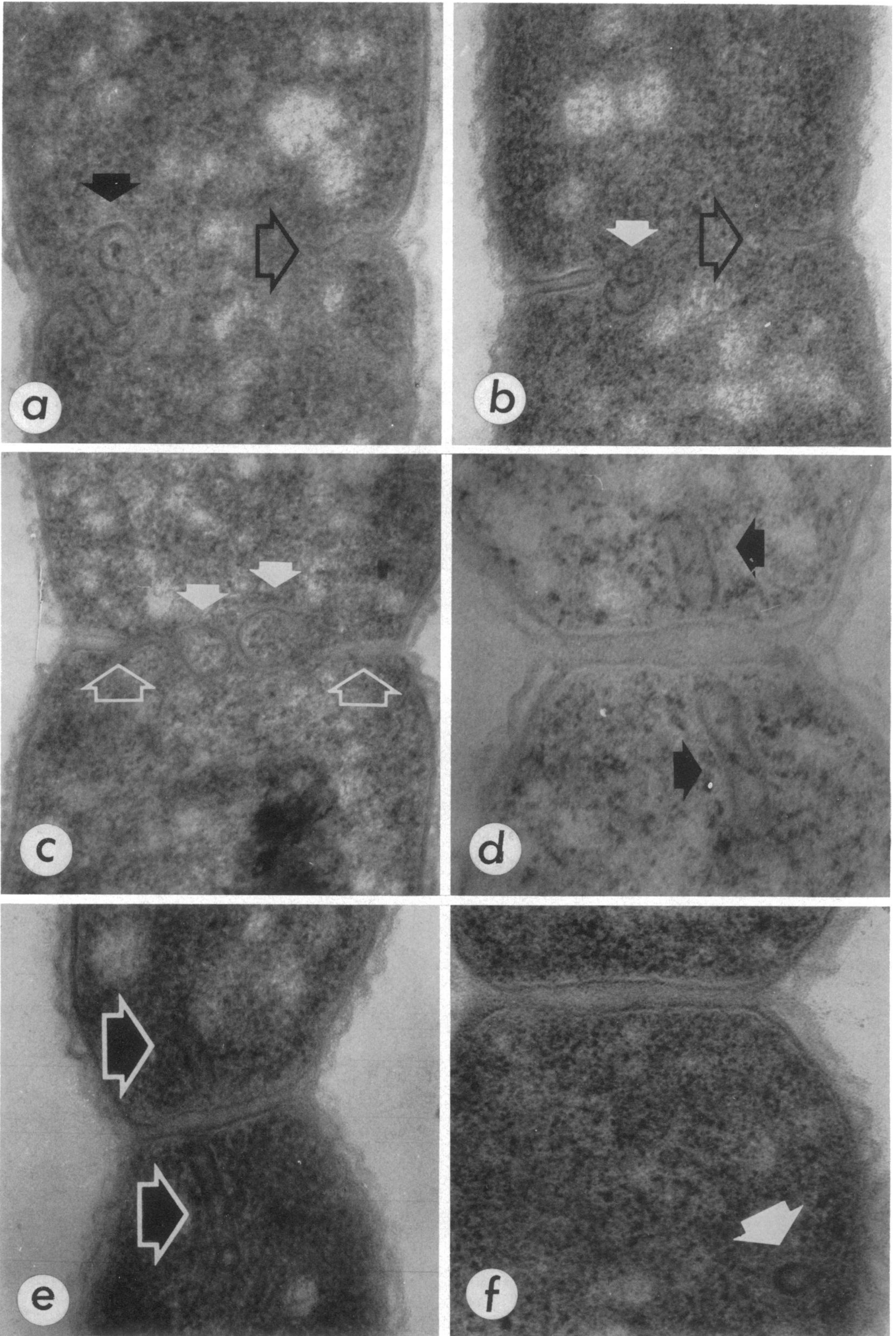


FIG. 32. *A postulated sequence of events in thin-sectioned mesosomes associated with cell septum formation in Selenomonas ruminantium HD 1. All are glutaraldehyde fixed and R-K postfixed. a, Initial stage in septum formation. Full arrow points to a developing mesosome. Empty arrow indicates a section of a septum that has already been synthesized.* ×100,000. *b, Formation of a tubular (empty arrow) and vesicular (full arrow) mesosomes.* ×88,000. *c, Inward growth of opposing mesosomes (full arrows). Cell septum (empty arrows)*

parts of the rigid murein layer (Fig. 32d). The mesosomes then appear to be drawn out into tubules (Fig. 32e) and grow away from the septum (Fig. 32f), or may be dispensed with.

Results obtained with thin sections of oral selenomonads were similar to those described above, except that in some cases tremendously convoluted mesosomes were observed (see Fig. 30) with no evidence of septum formation. Other sections, notably cross sections, revealed some mesosomes to be immediately adjacent to the inner circumference of the plasma membrane encircling not only the cytoplasm but also several lamellar mesosomes within it. Whether such structures extend from one end of the cell to the other is not known. Depending on structure, such circumferential mesosomes would appear in longitudinal sections as long tubules stretching the length of the wall. Some cross sections show the true tubular nature of vesicular mesosomes when the same mesosome is cut as is the case in Fig. 33. (Compare with diagrams in Burdett and Rogers [12].) (Here most of the tubules making up the mesosome were sectioned crosswise, whereas only one, situated on the periphery, was cut longitudinally [arrow, Fig. 33].)

Occasionally, membrane-like structures were observed in thin sections of *Selenomonas ruminantium* that did not resemble the membranous intrusions referred to as mesosomes (Fig. 34). These structures are strongly reminiscent of the "polar membrane" of *Spirillum serpens* (74), the "polar cap" in *Rhodospirillum rubrum* (18), the "complex cytoplasmic membrane" in *Vibrio fetus* (86), the "septate membrane" of *E. coli* grown in a magnesium-deficient medium (70), the "electron-dense strands" in *Halobacterium halobium* (17), and the "polar plate" of *Vibrio metchnikovii* (106). Such membranes suggest by their location and structure the flagellar membrane of *Rhodospirillum* (19).

The thin section of a selenomonad in Fig. 34 shows such a flagellar membrane. The organism has been sectioned at an oblique angle and the membranous structure is not sharply defined. Other thin sections in which the cell wall, membranes, and the nucleoplasm are sharply delineated did not, however, show the flagellar membrane to better advantage. Figure 35 shows clearly the various layers of the cell wall, plasma membrane, and flagellar membrane, the latter always appearing on the concave side of the cell as a diffuse strip, usually well separated from the plasma membrane and indicating some sort of septation.

Such flagellar membranes were never observed in dividing selenomonad cells, and in fact were found most frequently in those cells which were not undergoing fission. In no case were mesosomes and flagellar membranes seen together in the same cell.

Observation of a large number of micrographs of longitudinal sections of selenomonads suggests that the flagellar membrane is located on the concave side of the organism, either adjoining the plasma membrane or being part of it and not extending beyond the central region of the cell. Serial cross sections, however, indicate that the membrane is discontinuous at the point where flagella are expected to be located. Figure 36 represents one of such sections.

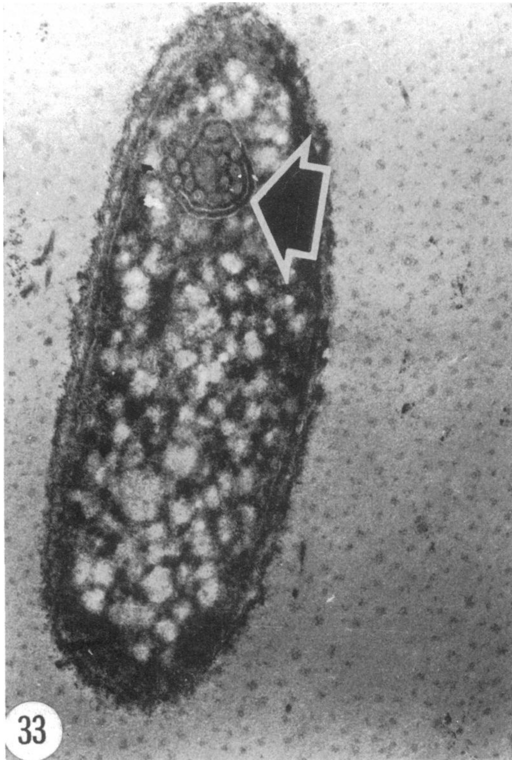
A three-dimensional topographical model shows (Fig. 37) that the membrane encircles the area of flagellar insertion and does not extend beyond the immediate vicinity of this region. Similar conclusions were first reached by Murray and Birch-Andersen (74) with *S. serpens*.

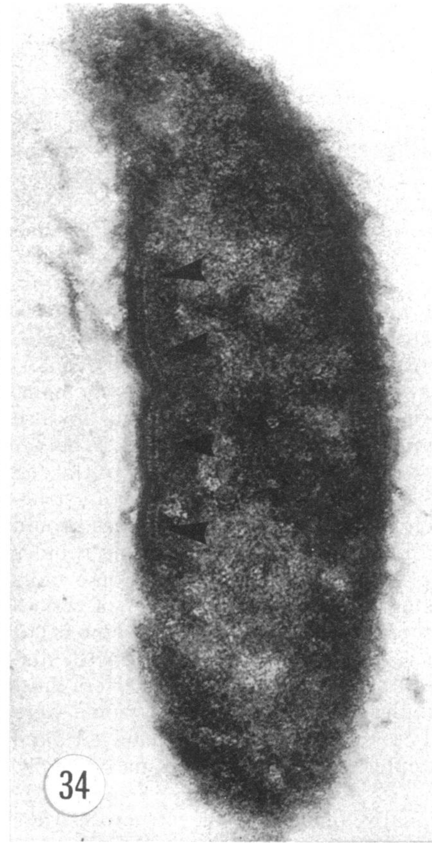
No comparable structures were ever observed in thin sections of *S. sputigena*.

CLASSIFICATION

A number of workers have insisted that *Selenomonas* is actually a protozoon, and not a bacterium (43, 109). However, it was suggested by Lessel and Breed (56) that *Selenomonas* be included in the family *Spirillaceae*, and the present edition of *Bergy's Manual* (6) lists three different species within the genus *Selenomonas*, all being differentiated on the basis of habitat alone. Thus *S. palpitans* has as its habitat the guinea pig cecum, *Selenomonas ruminantium* inhabits the rumen of herbivores, and *S. sputigena* is found in the oral cavity of man. However, M. P. Bryant (personal communication 1970), in rewriting the section on the genus *Selenomonas*, mentions only two species within the genus, namely *S. sputigena* and *S. ruminantium*, with the former as the type species, especially since *S. palpitans* "has not been obtained in pure culture and might be identical with" one of the other species.

appears to be synthesized between apposing mesosomal membranes. ×85,000. d, After the completion of septum formation each mesosome (arrows) appears to be directed towards the center of each new cell. ×110,000. e, Further removal of mesosomes (arrows) from the formed cell septum. ×90,000. f, A portion of a mesosome (arrow) is now visible only at some distance away from the formed septum. New cell wall material appears to segregate the two separate dense bands of glycopeptide between the daughter cells. ×120,000.





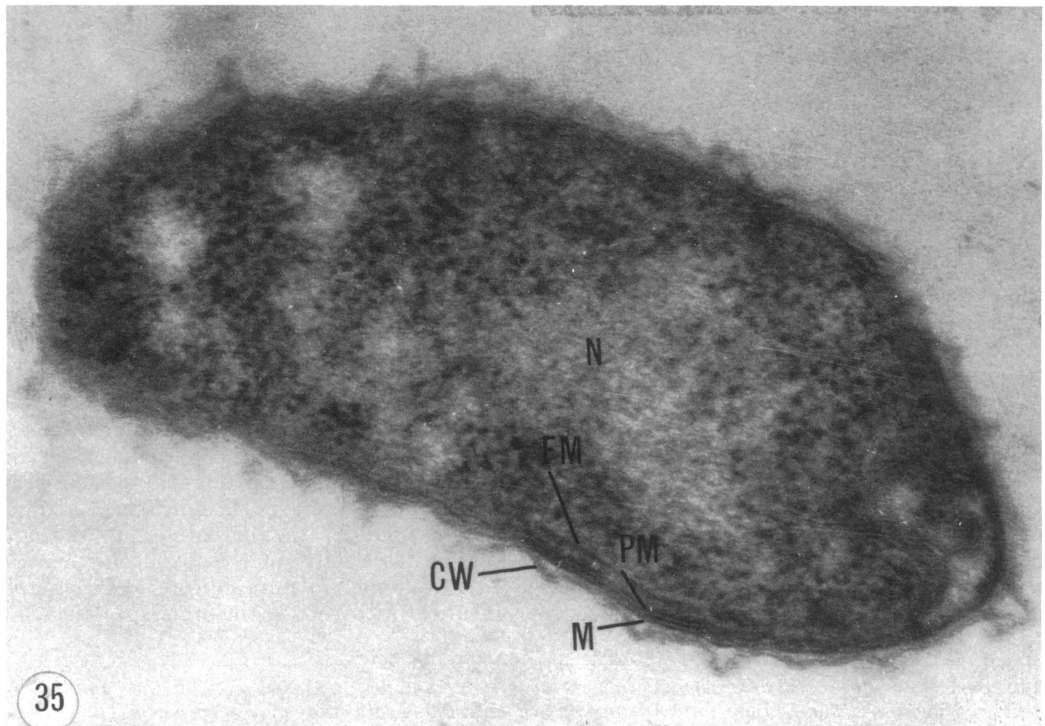


FIG. 33. *Membranous intrusions in selenomonads. A thin section of S. sputigena F 2 showing the presence of a prominent vesiculo-tubular mesosome (arrow). Glutaraldehyde fixation. R-K postfixation. ×62,500.*

FIG. 34. *Flagellar membrane in Selenomonas ruminantium. An oblique thin section of S. ruminantium no. 17 showing the presence of an extensive membranous complex (pointers). Note that the structure appears only on the concave side of the organism—the side on which the flagellar tuft is inserted (compare to Fig. 11). It is*

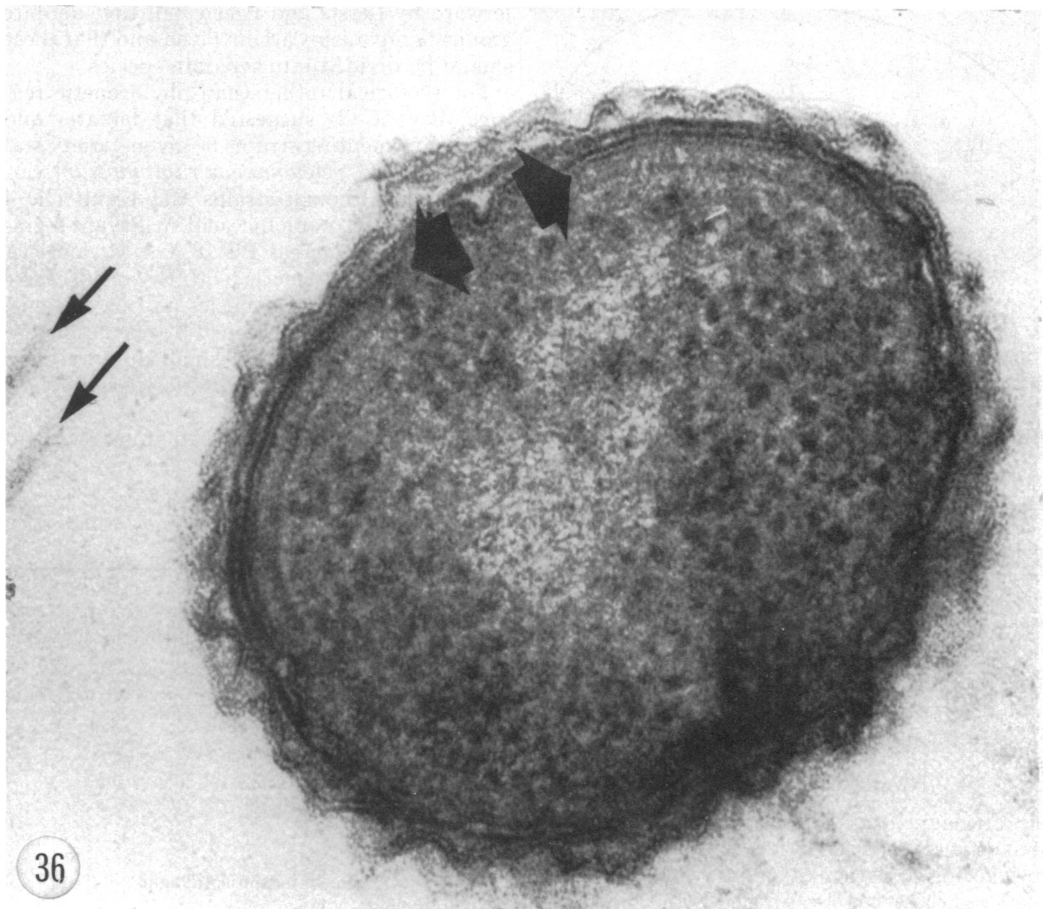


FIG. 36. *Flagellar membrane in Selenomonas ruminantium. This is a cross section of S. ruminantium no. 17 showing the presence of a discontinuous flagellar membrane (large arrows). Small arrows point to two flagella. R-K fixation. ×90,500.*

A modern taxonomic study of *S. palpitans* is greatly hindered by the fact that the organism is not available in pure culture, since all attempts to isolate and grow it in pure culture have proven futile (5, 61). MacDonald (60) and Bryant (8) classified *Selenomonas ruminantium* and *S. sputigena* separately on two different occasions. However, these characterizations were carried out independently, so that no clear taxonomically valid groups have been established. Bryant (8) at that time accepted the classification of Lessel and Breed (56), but considered the criteria of differentiation between the species rather superficial, albeit workable till such time as results from a comparative investigation should become available. Obviously, such results would have to incorporate the data of as many biological criteria as possible, including physiological, serological, biochemical, and deoxyribonucleic acid (DNA) characteristics. It was for this reason that a more thorough comparative study of the classification of *Selenomonas* was performed here. A large number of the reactions of ruminant selenomonads had previously been reported by Bryant (8) and by Hobson et al. (34); however, comparative studies of the strains from different habitats were not performed then. Many criteria in our studies were investigated for the first time, and many of

postulated from such sections that the structure represents a flagellar membrane. R-K fixation. ×50,000.

FIG. 35. *A thin section of Selenomonas ruminantium no. 17 showing some structural components. Abbreviations: N, nucleus; CW, outer cell wall layers; M, murein (glycopeptide) layer; PM, plasma membrane; FM, flagellar membrane. R-K fixation. ×100,000.*

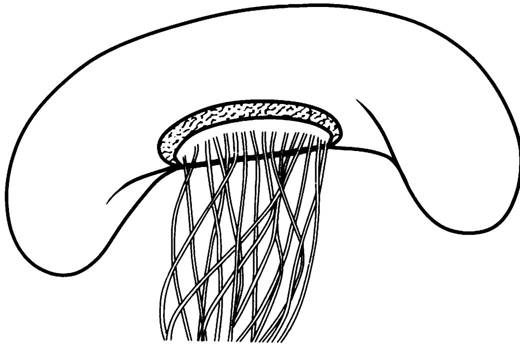


FIG. 37. *A topographical reconstruction of the extent of the flagellar membrane in Selenomonas ruminantium (see text). This drawing is based on a number of serial sections of ruminant selenomonads and illustrates not only the overall morphology of the ruminant organism, but also the position of the flagellar tuft, its mode and site of insertion into the cell, and its relationship to the underlying flagellar membrane. The flagellar membrane is not obvious, nor can it be seen from the outside of the cell as is represented here. Also, other structural detail has been omitted here for purposes of clarity. Approximately ×25,000.*

those of Bryant (8) and Hobson et al. (34) were repeated for purposes of uniformity.

Numerical Taxonomy

In all, eight ruminant and three oral selenomonads were characterized and analyzed by methods for numerical taxonomy established by Sokal and Sneath (100). An electronic computer (IBM 7094-II) was used to process the data. A total of 70 characters was scored for each organism tested; these consisted of morphological, physiological, biochemical, and some cultural properties, as listed in Table 2. Most of the properties were scored as either present or absent. Some characteristics were not determined for some organisms and were scored as such, mainly as applied to the related organisms *S. serpens* and *Vibrio comma*. For these bacteria, data were taken from the published literature (107, 110). All reactions of selenomonad strains were scored from our own experimental data with the exception of fermentation products, the results of which were taken from Bryant (8) and Loesche and Gibbons (57), and incorporated into the final description. The organisms employed in these studies are listed in Table 3. The results of tests are given in Table 4, and are presented as a dendrogram in Fig. 38. It is apparent that *Selenomonas ruminantium* and *S. sputigena* form two distinct clusters with a level of association of 78%, and as such tend to confirm the suggestions put forward by Lessel and Breed (56) that definite groups of organisms are involved and that these should be divided into separate species.

For ecological rather than phylogenetic reasons, Bryant (8) suggested that lactate- and glycerol-fermenting strains be given variety status and called *Selenomonas ruminantium* var. *lactilytica*. Although strains HD 1 and HD 4 formed mainly propionic acid in Bryant's glucose medium, whereas PC 18, GA 31, and GA 192 formed mainly lactic acid, Bryant (8) hesitated to subdivide the species on this account. His reasoning is strengthened by our numerical results which show rather small differences in overall similarity to be considered significant (Fig. 38).

The two organisms obtained from Scotland (no. 6 and no. 17) do not form a separate cluster,

TABLE 2. *Properties of selenomonads scored for numerical taxonomy*

Morphological:
- Shape: vibrioid
- Cell arrangement: single, double, short chains
- Clumps formed
- Irregular forms present
- Length: up to 3.5 μm; between 3.5 and 4.0 μm; over 4.0 μm
- Presence of flagella
- Capsule
- Metachromatic granules

Cultural:
- Motility: in broth; in semisolid agar
- Chromogenesis absent
- Anaerobic
- Colony: convex, margin entire, color light tan, size 1 mm or less

Physiological:
- Gram-negative
- pH in glucose: 4.5, 4.6 to 5.0, over 5.0
- Catalase not produced
- Growth at 22, 30, 37, 45, 50 C
- Cellulose hydrolysis
- Starch hydrolysis
- Gelatin liquefied
- Casein digestion
- Nitrate reduction
- Indole
- H_2S production
- Methyl red test
- Voges-Proskauer reaction
- Fermentation of lactate
- Acid from: arabinose, cellobiose, dextrin, dulcitol, esculin, fructose, galactose, glucose, glycerol, gum arabic, inositol, inulin, lactose, maltose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose, xylan, xylose
- Production of: acetic, butyric, formic, lactic, propionic acids

TABLE 3. *Organisms employed in this study and their sources*

Name	Strain	Source and appropriate original reference
Selenomonas ruminantium	HD 1, HD 4	M. P. Bryant, Univ. of Illinois, Urbana; (originally from L. Gall, see ref. 8).
	PC 18, GA 31, GA 192	M. P. Bryant, Univ. of Illinois, Urbana (see ref. 8).
	no. 6, no. 17	P. N. Hobson, Rowett Research Institute, Scotland (see ref. 33).
	CHR	Original isolate from bovine rumen juice, Toronto (see Materials and Methods).
Selenomonas sputigena	F 2	E. Fabenyi, Faculty of Dentistry, Univ. of Toronto, Toronto, Ontario. (For isolation method see ref. 62.)
	B	E. M. Madlener, Faculty of Dentistry, Univ. of Toronto, Toronto, Ontario. (For isolation method see ref. 62.)
	ITK	Original isolate from a 4-year-old boy, Toronto, Ontario. (For isolation method see ref. 62.)
Spirillum serpens[a]	VH	R. G. E. Murray, Univ. of Western Ontario, London, Ontario.
Vibrio comma[b]	9168	American Type Culture Collection, Rockville, Md.

[a] Used in comparison of DNA base ratios and for serological study.
[b] Employed in serological study only.

but fit in well with the other strains isolated in North America.

There are six clearly obvious differences in reactions or in characteristics between the rumen and the oral strains of the selenomonads examined: ruminant cells are vibrioid whereas oral selenomonads tend to be spiral in shape; cell clumping does not occur in ruminant species, but is frequently observed in pure cultures of oral strains; the production of H_2S, acid from cellobiose, dulcitol, and salicin occurs in the ruminant selenomonads, whereas these reactions are negative in the oral species.

Serology

Since the oral and the ruminant selenomonads are thought to be closely related, a relationship between the different groups could be clarified by serological testing. Using methods outlined by Kauffman (48), nonabsorbed rabbit antisera were therefore prepared against one ruminant strain (HD 1) and one oral strain (F 2); antisera were made for both the antisomatic (O) and the antiflagellar (H) antigens.

Slide agglutination tests proved unsatisfactory due to an apparent autoagglutination of the organisms. When employing methods described by Campbell et al. (14), tube agglutination results showed the anti-O antisera to have a very low titer (1:10) in both cases when ruminant and oral selenomonads provided the homologous antigens. With heterologous antigens, i.e., all the other strains of selenomonads, no agglutination could be demonstrated.

The anti-H (antiflagellar) antisera had a much higher titer, yet were 5 to 10 times weaker than comparable antisera from other bacteria. A typical agglutination titer of HD 1 antiflagellar antiserum against the other selenomonads and against related genera *S. serpens* and *V. comma* is shown in Table 5. No agglutination could be observed in tubes containing *V. comma* antigen, although *S. serpens* showed a fairly good agglutination titer of approximately 1:160.

A similar series of tests was set up with the oral selenomonad strain F 2 antiflagellar antiserum against the other organism. Again, no agglutination could be demonstrated between the two main groups of selenomonads tested, i.e., ruminant and oral, whereas *S. serpens* and *V. comma* showed some cross-reactions. The results of these tests are listed in Table 6.

To eliminate the possibility that soluble antigens were present, an Ouchterlony plate precipitation test (14) was prepared by use of anti-O and anti-H antisera, against a variety of antigens obtained from the corresponding selenomonads, and by including in one of the wells the supernatant of the growth medium (MPB). Again, no reactions with the anti-O antiserum were observed. The anti-H antisera, however, showed some weak precipitation lines (as diagrammed from original Ouchterlony plates in

TABLE 4. *Results of morphological, physiological, biochemical, and cultural tests on 11 strains of selenomonads*[a]

Scorable character or reaction	Ruminant strains								Oral strains		
	HD 1	HD 4	PC 18	GA 31	GA 192	no. 6	no. 17	CHR	F 2	B	ITK
Shape: vibrioid	+	+	+	+	+	+	+	+	−	−	−
Cell length[b]	2	2	1	1	3	3	1	1	2	2	2
pH in glucose[c]	1	1	1	1	1	2	2	1	2	2	3
Growth at 45°C	+	+	−	−	−	−	−	−	−	−	−
Acid from:											
cellobiose	+	+	+	+	+	+	+	+	−	−	−
trehalose	+	−	−	+	+	−	−	+	+	−	−
raffinose	+	+	+	+	+	+	+	+	+	+	−
dextrin	+	+	+	+	+	+	−	+	+	+	+
glycerol	−	+	+	+	−	+	+	−	+	+	+
sorbitol	−	−	+	+	+	+	+	−	−	−	−
inositol	+	+	−	+	+	−	+	+	−	−	−
dulcitol	+	+	+	+	+	+	+	+	−	−	−
salicin	+	+	+	+	+	+	+	+	−	−	−
gum arabic	−	−	−	−	−	?[d]	?	?	?	?	?
fructose	+	+	+	+	+	+	+	?	?	?	?
H_2S production	+	+	+	+	+	+	+	+	−	−	−
Casein digestion	−	−	+	−	−	?	?	?	−	−	−
Starch hydrolysis	+	+	−	+	−	+	−	+	+	+	+
Nitrate reduction	+	+	−	−	−	+	−	+	+	+	+

[a] The following reactions were positive in all strains: motile in broth and in semisolid agar; gram-negative; colonies punctiform, convex with entire margin; anaerobic; good growth at 30 and 37 C; acid produced from esculin, galactose, arabinose, xylose, glucose, lactose, sucrose, maltose, inulin, and mannitol. All tested strains lacked capsules; chromogenesis; catalase. No growth occurred at 22 or 50 C; no acid was produced from xylan; cellulose was not hydrolyzed; no indole was produced; MR-VP tests were negative; gelatin was not liquefied; and no metachromatic granules were present. Note: Methods relating to the above results were taken from *Manual of Microbiological Methods*, Society of American Bacteriologists, McGraw-Hill, 1957.

[b] 1 = 3.5 μm or smaller; 2 = 3.6 to 4.0 μm; 3 = larger than 4.0 μm.

[c] 1 = pH 4.5; 2 = pH 4.6 to 5.0; 3 = pH over 5.0.

[d] ? = reactions were ambiguous and/or inconsistent.

Fig. 39a, 39b) and suggest that some soluble antigens are indeed present not only in growing selenomonad cultures, but in cultures that have been formalinized (wells 4 and 3, respectively). No visible lines of precipitation were seen between the anti-H antiserum (center well) and the supernatant from the overnight culture (well 1), indicating a possible breakdown of soluble antigenic components, or, what appears to be less likely, the absence of such antigenic components.

If a selenomonad culture is spun down, then whole cells (cell body and attached flagella) comprise the pellet. This is the case in well 4 where there are at least two distinct lines formed against the anti-H antiserum. This would indicate that there are at least two distinct antigen-antibody systems present. However, if such a culture is sonically disrupted and then centrifuged, the pellet consists of whole cells and large cell fragments, but will usually not contain flagella or flagellar fragments since these are retained in the supernatant. A sonically disrupted culture treated in this manner was placed in well 5. Since the antiserum here is the anti-H antiserum, no bands are expected between the center and no. 5 wells, yet a distinct precipitation band can be seen in the plate (Fig. 39a). This discrepancy could be resolved if one assumes that: (i) whole cells and fragments of cells forming the pellet contain portions of flagella, which are responsible for the precipitation bands, and/or (ii) the anti-H antiserum is, in effect, also an anti-O antiserum. The latter possibility suggests either that the preparation of the anti-H antiserum was faulty, or that each anti-H antiserum contains some somatic antibodies, which should be absorbed before crucial tests are made.

No comparable results were observed with the F 2 anti-H antiserum (Fig. 39b).

Similar tests were performed with *S. serpens* and with *V. comma* serving as antigens in wells 1 to 6, against HD 1 and F 2 anti-H antisera. No precipitation could be demonstrated.

In all, it appears that the antibodies may be

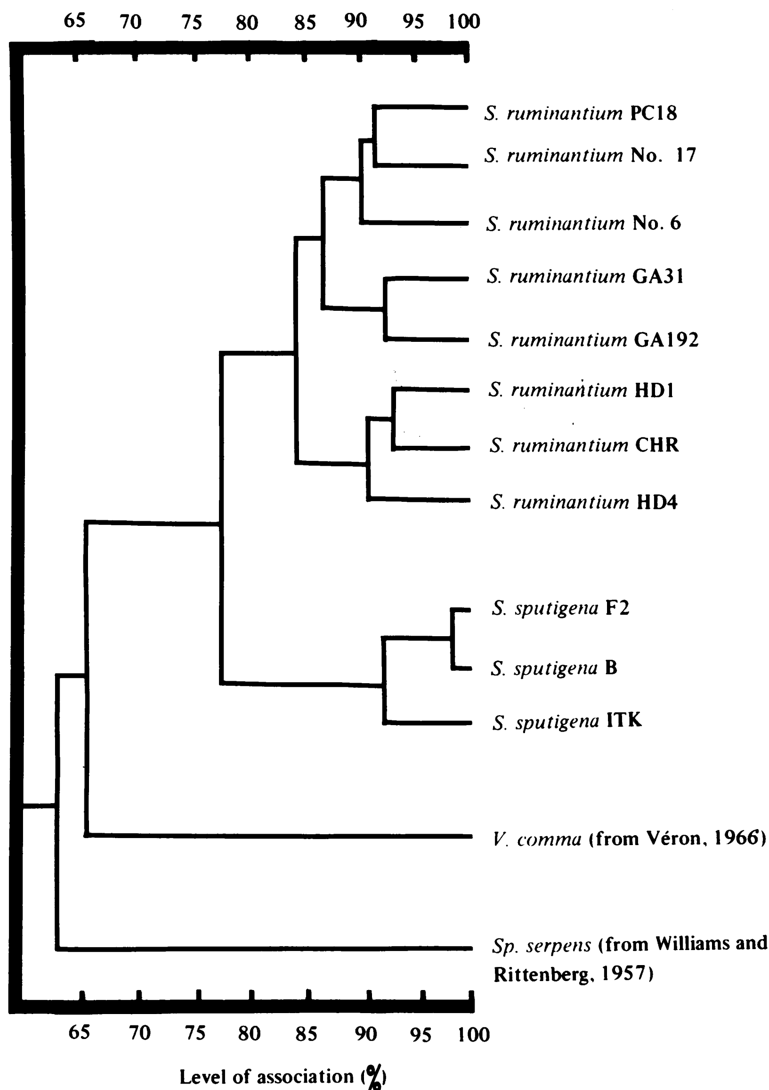


FIG. 38. *Dendrogram of tested selenomonads and their relationship to Vibrio comma and Spirillum serpens.*

strain specific, and although a serological relationship could not be demonstrated between the ruminant and oral selenomonads, there may exist some flagellar relationship between the ruminant selenomonads and *S. serpens*, and among the oral selenomonads, *S. serpens*, and *V. comma*. It is of interest to note at this point that certain serological results of Hobson et al. (34) suggested that there was a difference in the O antigen of ruminant selenomonads of different varieties, and considerable difference in the H antigens of selenomonad strains.

DNA Base Ratios

Determinations of the base composition of DNA in bacteria were employed to supplement taxonomical data. The method of Marmur (64) was followed for the extraction of DNA from selenomonads. The relationship between mole percent guanine plus cytosine (GC) and the buoyant density of DNA in a CsCl density gradient was calculated by using the formula of Schildkraut et al. (95).

Two ruminant strains (HD 1 and HD 4) and two oral strains (F 2 and B) of selenomonads were employed as arbitrary representatives of their respective groups to give additional information. The reference sample of DNA was prepared by the method of Kung and Williams (51) from broad bean chloroplast of density of 1.696 g cm^{-3}, having been previously standardized against *E. coli* DNA of density 1.710 g

TABLE 5. *Presence or absence of tube agglutination of HD 1 antiflagellar antiserum against other selenomonads and some related organisms*

Organisms	Tube agglutination[a]								
	1:10[b]	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Selenomonas ruminantium									
HD 1	+	+	+	+	+	+	+	±[c]	–
HD 4	+	+	+	+	+	+	–	–	–
PC 18	+	+	+	+	+	+	–	–	–
GA 31	+	+	+	–	–	–	–	–	–
GA 192	+	±[c]	–	–	–	–	–	–	–
no. 6	+	+	+	+	–	–	–	–	–
no. 17	+	±[c]	–	–	–	–	–	–	–
CHR	+	+	–	–	–	–	–	–	–
Selenomonas sputigena									
F 2	±[c]	–	–	–	–	–	–	–	–
B	±[c]	–	–	–	–	–	–	–	–
ITK	±[c]	–	–	–	–	–	–	–	–
Spirillum serpens VH	+	+	+	+	±[c]	–	–	–	–
Vibrio comma ATCC 9168	–	–	–	–	–	–	–	–	–

[a] Presence (+) or absence (–).
[b] Antiserum titer.
[c] One day later these results showed no agglutination.

TABLE 6. *Presence or absence of tube agglutination of F 2 antiflagellar antiserum against other selenomonads and some related organisms*

Organisms	Tube agglutination[a]								
	1:10[b]	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Selenomonas ruminantium									
HD 1	–	–	–	–	–	–	–	–	–
HD 4	–	–	–	–	–	–	–	–	–
PC 18	–	–	–	–	–	–	–	–	–
GA 31	–	–	–	–	–	–	–	–	–
GA 192	–	–	–	–	–	–	–	–	–
no. 6	–	–	–	–	–	–	–	–	–
no. 17	–	–	–	–	–	–	–	–	–
CHR	–	–	–	–	–	–	–	–	–
Selenomonas sputigena									
F 2	+	+	+	+	+	+	+	±[c]	–
B	+	+	+	+	+	+	+	+	–
ITK	+	+	+	+	+	+	+	–	–
Spirillum serpens VH	+	+	–	–	–	–	–	–	–
Vibrio comma ATCC9168	+	+	+	±[c]	–	–	–	–	–

[a] Presence (+) or absence (–).
[b] Antiserum titer.
[c] One day later these results showed no agglutination.

cm^{-3}. All selenomonad DNA determinations were performed in triplicate, and *S. serpens* strain VH was run concurrently. The GC base ratio results are shown in Table 7, together with the standard error for each mean. Typical microdensitometer tracings of the ultraviolet absorbing bands of DNA purified from the representative groups of selenomonads are reproduced in Fig. 40.

The results demonstrate the heterogeneity of the group of bacteria known as selenomonads and complement the results obtained by numer-

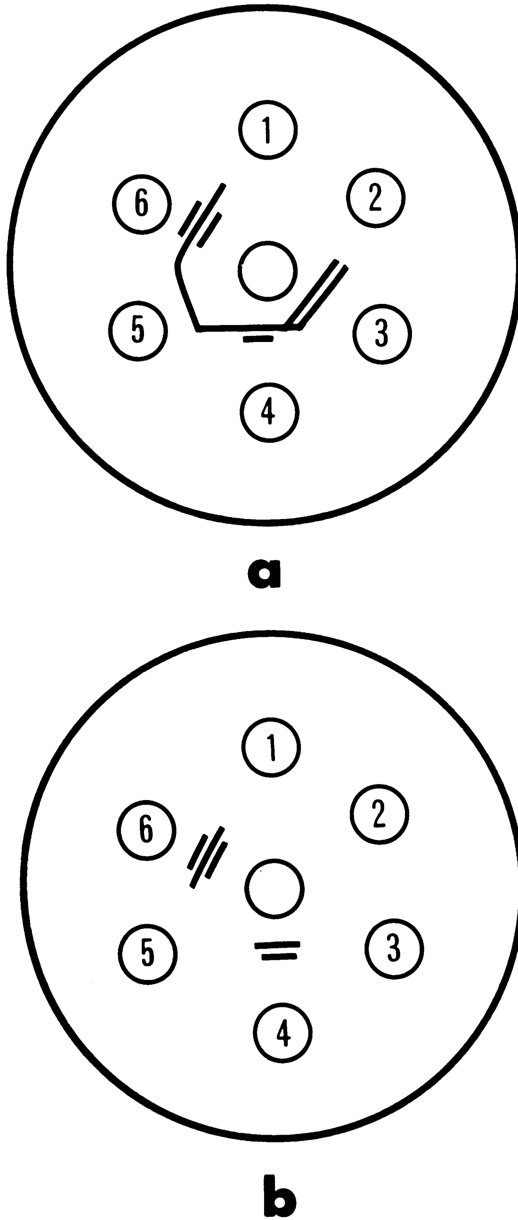


FIG. 39. *Diagrams of precipitation lines of anti-H antisera of selenomonads against various antigen preparations. a, Selenomonas ruminantium HD 1 anti-H-antiserum (center well). Well 1: supernatant from overnight culture of S. ruminantium HD 1. Well 2; normal serum. Well 3: formalinized suspension of overnight S. ruminantium HD 1 cells. Well 4: centrifuged pellet from overnight culture of S. ruminantium HD 1. Well 5: centrifuged pellet from sonically disrupted culture of S. ruminantium HD 1. Well 6: supernatant from sonically disrupted culture of overnight cells of S. ruminantium HD 1. b, Selenomonas sputigena F 2 anti-H-antiserum (center well). Well 1: supernatant from overnight culture of S. sputigena F 2. Well 2: normal serum. Well 3: formalinized suspension of overnight culture of S. sputigena F 2. Well 4: centrifuged pellet from overnight culture of S. sputigena F 2. Well 5: centrifuged pellet from sonically disrupted overnight culture of S. sputigena F 2. Well 6: supernatant from sonically treated overnight culture of S. sputigena F 2.*

TABLE 7. *GC content (mol %) of some members of the family Spirillaceae*

Organism	Strain	GC content + SE	Group avg
Selenomonas ruminantium	HD 1 HD 4	54.5 ± 0.6 53.5 ± 0.7	54.0 ± 0.8
Selenomonas sputigena	F 2 B	60.2 ± 1.1 61.0 ± 0.4	60.6 ± 0.8
Spirillum serpens	VH	58.1 ± 0.5[a]	
Vibrio comma	CIP 6215	48.7[b]	

[a] Véron (107) quotes a mean value of 55.2 ± 1.9 GC mol % for spirilla, but says that a spread of 49 to 61 GC mol % exists for this group. Primrose (80) recently quoted an average of 61 GC mol % for *Spirillum* SP5.

[b] This value was obtained from Véron (107). A spread of 44 to 49 GC mol % exists for the vibrios.

ical taxonomy. The latter, in turn, were supported by serological data which indicated that the selenomonads comprise two distinct groups of bacteria, albeit related to one another. This distinction exists not only between the selenomonads, but also among the individual selenomonads and *S. serpens*, and is justified on the basis of statistical analysis, since they are significantly different at the 95% confidence level.

INTERPRETATIONS

Although our investigations have confirmed a number of observations of previous workers concerning the general morphology, cytology, and classification of *Selenomonas*, many additional findings, especially in the area of fine structure, serology, and DNA base ratios have revealed aspects of the selenomonads which were either totally unknown, or at best suspected. There are several main points emerging from this review which facilitate a more precise definition of this group of microorganisms and give insight into certain aspects of their ultrastructure.

Selenomonads are unusual bacteria as regards the location of their flagella, for the flagellar tuft is situated exactly in the region of binary fission. *Spirillum* with its bipolar tufts of flagella is in a somewhat different position. In

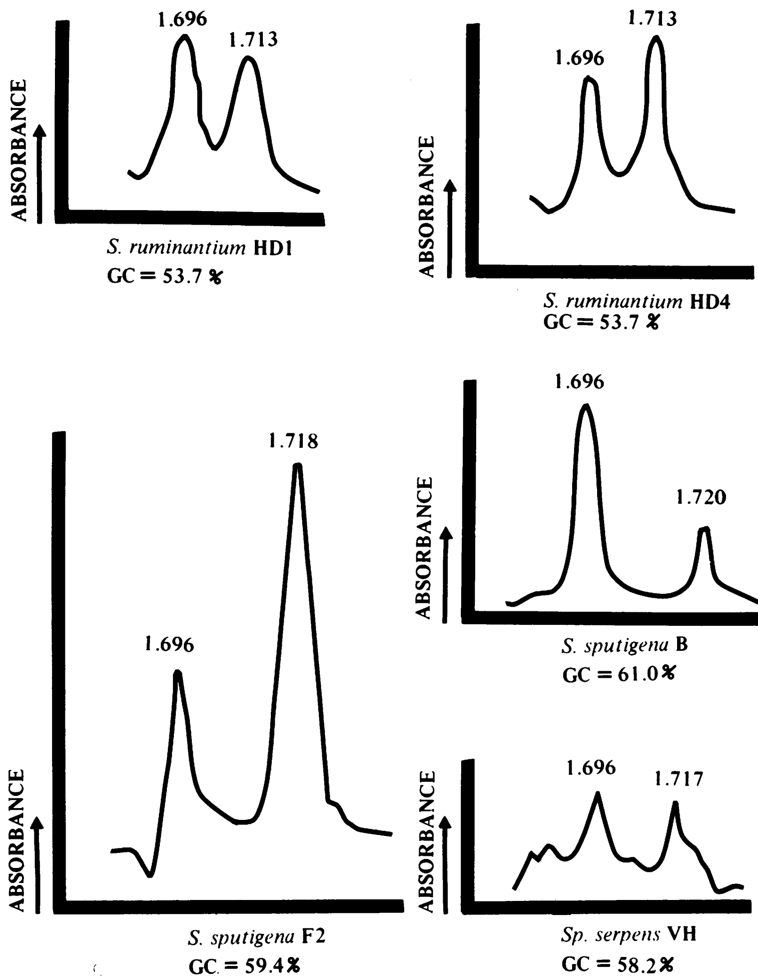


FIG. 40. *Microdensitometer tracings of ultraviolet absorbing bands of DNA comparing some selenomonads with S. serpens VH. GC content is expressed in terms of GC mol %.*

Spirillum, only one of the tufts of flagella must be synthesized anew in each of the daughter organisms. In the spirilla there is no reason for speculation regarding tuft formation, since a new tuft of flagella must be formed anew in both cells after separation is complete. Further, each new cell always possesses one set of flagella, even during the division process (64). In selenomonads, however, three distinct theoretical possibilities exist as to the disposition of flagella during fission (Fig. 41).

Recent observations by Ingram and Kingsley (Abstr. Proc. Can. Fed. Bio. Soc., no 135, 1972) suggest that changes in the number of flagella per cell during the growth cycle favor situation (a) diagrammed in Fig. 41. Evidence was derived from motility characteristics of cell populations and statistical models coupled with experimental data. When flagellar distribution occurs concurrently with rapid division, then it is conceivable that the flagella will be "diluted out": the less motility, the smaller the number of flagella per cell, and vice versa. This could be interpreted to mean that a certain number of flagella must be present for a cell to be scored as motile in such a test or it could imply the presence of nonfunctional flagella. On the other hand, the beating flagella may not provide sufficient thrust for the cell to move. If one assumes that most of the population is nonmotile in the latter part of the logarithmic phase of growth (see Fig. 7), then from Fig. 8 it can be assumed that this nonmotility can be scored when selenomonad cells have three to four flagella per cell. This constitutes approximately one-fifth to one-fourth of the usual complement of 16 flagella observed in the selenomonad cell from fresh rumen contents. Leifson

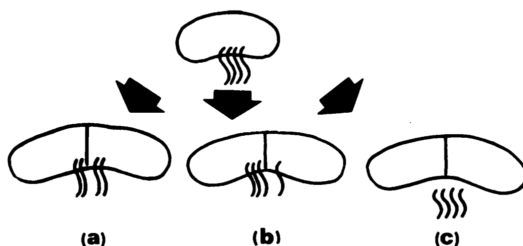


FIG. 41. *Theoretical possibilities of flagellar distribution patterns during growth in selenomonads. The uppermost diagram represents a cell with one set of flagella. For ease of interpretation only four flagella per tuft are shown. When selenomonads divide, the existing flagella may be numerically separated equally between the two forming cells (a). In (b) an unequal numerical distribution of flagella is depicted, with one cell receiving the majority of these organelles, while the other daughter cell ends up with a very small number of flagella. Diagram (c) emphasizes yet another possibility consistent with our observations, namely that prior to or during the division process most selenomonad flagella may be shed or may become nonfunctional.*

(55) found that germinating *Bacillus* spores become motile after the formation of two or three short flagella. Similarly, Stocker and Campbell (101) suggest that, in *Salmonella typhimurium*, a flagellum shorter than 0.3 μm can cause rotational, but not translational, motility.

Further support for situation (a) in Fig. 41 is derived from the data showing percentage motility (see Fig. 7). A cyclic phenomenon is operative here in which the reduction in motility is halved with each cycle and corresponds approximately with the generation time of this bacterium, in MPB medium, namely 45 min. Similarly (interpreting Fig. 7), it appears that the number of flagella per cell in the exponential phase of growth is approximately halved with each division, an observation which can be taken as further evidence that approximately one-half of the flagella pass to each new daughter cell. Situation (a) of Fig. 41, therefore, offers the most plausible explanation of our experimental data.

Kerridge (50) reported that *S. typhimurium* could regenerate flagella under conditions when protein or ribonucleic acid synthesis, or both, were inhibited. Martinez and Gordee (65), working with *S. serpens* and *Bacillus subtilis*, suggested that these bacteria possess a pool of preformed molecules of flagellin protein which serves as a precursor for the ordered structure of flagella, and flagella continue to be synthesized, independently of any inhibitors, as long as a pool of flagellin is present in the cell. The initial rise in motility and number of flagella per cell in the ruminant selenomonad (see Fig. 7 and 8) can therefore be attributed to the rapid utilization of the flagellin molecules within the pool.

The flagellin pool probably must be spatially distributed within a narrow area of the selenomonad cell and precisely within the region coinciding with the area where convoluted membranous systems necessary for septum formation appear and fission takes place. This situation may lead to unnecessary complications in the simultaneous elaboration of new structures. It is apparently of advantage to the selenomonad cell to dispense temporarily with such organelles as flagella, and then regenerate them when division has ceased.

Genetically, it would not be too difficult to envisage the situation in which a genetic locus controlled the presence or absence of the flagellin pool, and be under the functional influence of a neighboring genetic locus controlling the divisional process in the selenomonad cell. It is quite conceivable that the repressor shuttles back and forth between the loci responsible for flagelling synthesis and for divisional processes—as one locus is repressed, the other is automatically derepressed.

Vaituzis and Doetsch (104) showed that the formation of flagella in *S. typhimurium* was dependent upon prior cell wall synthesis. Later, the same workers (105) extended their findings to include *E. coli* and concluded that the mechanism for synthesizing flagella is closely associated with the cytoplasmic membrane, and that the system is inactivated when the membrane is deformed.

Of interest in this connection are the "long forms" of selenomonads grown in *n*-valerate-deficient medium which possess a large number of flagellar tufts on alternate sides; in fact, the number corresponds to the number of expected "single cells." According to Kanegasaki and Takahashi (47) these long forms appear to be nutritionally deficient and are devoid of cell septa. These separate observations indicate that the two processes in selenomonads, the synthesis of flagella and of cell septa, may be functionally related.

In *Pasteurella pseudotuberculosis*, Preston and Maitland (79) found that the formation of flagella can occur in the absence of cell division. When Kerridge (50) exposed *S. typhimurium* to penicillin, it was impossible to detect flagellar synthesis, and " . . . it may be that, although penicillin does not prevent the formation of individual flagellin molecules, the disorganization of the cell wall . . . in some way prevents the formation of flagella." Similarly, in the developmental sequence of *Proteus mirabilis*

which results in the formation of swarmers, the whole physiological organization of the bacterium changes. Hoeniger (35) ascribes such change to the inhibition of cellular division without a concurrent inhibition of flagellin synthesis. This suggests a curtailment in the production of some cellular protein and an increased production of flagellar protein.

Membranous complexes in *Selenomonas ruminantium* are to be found in a functionally equivalent position to the polar membrane of *S. serpens* described by Murray and Birch-Andersen (74). It may therefore be assumed that such membranes perform similar functions, i.e., possibly the synthesis of flagellar precursors, aggregation of such precursors into morphologically distinct organelles, and supply of energy to the flagella. This assumption is reinforced by the observation that no such flagellar membranes were observed during divisional processes in selenomonads, but only after division was complete. In *S. serpens*, Murray and Birch-Andersen (74) made no distinction between the old (maternal) pole and its flagella and the new (bare) pole at which flagella are emerging. For this reason it is suggested here that the polar membrane in spirilla (74) appears only in either deflagellated organisms or in new polar regions of recently divided cells.

This leads to a speculative interpretation of the structure and function of the flagellar membrane observed in selenomonads. In our studies, no truly sharp micrographs of the flagellar membrane could be obtained, although adjacently placed delicate structures, such as the dark-light-dark profile of the plasma membrane, were well resolved. It is conceivable that the flagellar membrane in *Selenomonas* does not represent a true "membranous" structure, but possibly an orderly accumulation of some material, such as molecules of flagellin. Functionally, there is as yet no completely acceptable evidence for the activation of bacterial flagella, although a theory by Vaituzis and Doetsch (106) appears to correlate available data into a workable postulate. They state that the flagellar filament is an essentially inert structure activated by contractions of the flagellar membrane. The motion is propagated via the basal body and the rigid hook portion of the flagellum to the filament, which, due to its helical structure, imparts motion to the cell.

No flagellar membranes were observed in either induced or spontaneous spheroplasts of selenomonads, and one is reminded of the suggestion by Vaituzis and Doetsch (105) that there exists some connection between the disarranged plasma membrane, cell wall synthesis, and the flagellar synthesizing mechanism. It appears that the processes of fission and of the formation of flagella are mutually exclusive in selenomonads.

Sluggishly motile spheroplasts were frequently observed in all cultures of ruminant selenomonads. The presence of such lysing cells had been noted even in continuous cultures by Hobson (32), where they contributed to the decrease in turbidity.

Williams and Rittenberg (110) have observed spheroplasts in other *Spirillaceae*, but called them "microcysts." These structures, however, do not differ from phase-contrast or from Giemsa-stained preparations of spheroplasted selenomonads. Similar "cysts" and "resting stages" in *S. palpitans* were observed by Boskamp (5) and even earlier by von Prowazek (82). We speculate, in view of our studies, that such cysts were spontaneously formed spheroplasts.

In the present studies it was shown that spheroplasting in ruminant selenomonads may temporarily be prevented by the addition of divalent cations, specifically Ca^{2+} and Mn^{2+}. Similar requirements are shown by *S. serpens* by Murray (73). On similar lines, Strominger (102) reported that Mn^{2+} is required in at least four different sites in the biosynthesis of murein, together with adenosine 5'-triphosphate, for the addition of alanine, glutamic acid, and lysine successively to uridine 5'-diphosphate-muramic acid.

It must therefore be assumed that when Ca^{2+} is depleted, some additional mechanism is involved in the lysis of selenomonads, since the addition of Ca^{2+} to the growth medium prevented spheroplast formation. It is of interest to point out that dipicolinic acid may be chelated to calcium and figures prominently in the biosynthesis of diaminopimelic acid (DAP), also necessary for cell wall structure. It should be noted here that DAP was not found in our investigation of cell wall composition. Similarly, Purser and Buchler (84) did not find DAP in whole cells of *S. ruminantium* GA192. DAP-deprived cells of *E. coli* differ in the process of spheroplast formation in that no "rabbit-eared" stage was present (105). Ruminant selenomonads that undergo such spontaneous formation of spheroplasts do not show these stages either (Kingsley and Mendes, unpublished data), an observation which would tend to indicate that a generalized weakening of the cell wall of selenomonads takes place, and that the remainder of the cell wall is elastic and sufficiently stable to be able to contain temporarily the osmotically enlarging spheroplast.

From the point of view of classification, it can

now be unequivocally stated that selenomonads should be placed among the procaryotes together with other *Eubacteria* and that their classification, both within the family *Spirillaceae* and within the genus *Selenomonas* (M. P. Bryant, personal communication, 1970), appears justified.

The taxonomical results of our studies support the separation of the two species of selenomonads, *Selenomonas ruminantium* and *S. sputigena*, and thus confirm the suggestion of Lessell and Breed (56) that these organisms be classified by habitat. In this regard, no changes should be made in future editions of *Bergey's Manual* since the present primary differentiation into habitat groups seems quite adequate. Except for *S. palpitans*, this separation is substantiated by results obtained in areas of structure, physiology, biochemistry, and serology. Furthermore, the difference in DNA base ratios between the two groups of selenomonads supports the conclusion that two heterogeneous groups of organisms are involved. Thus the genus *Selenomonas* includes species whose GC content ranges from 54 to 61 mol%. It should be noted that the DNA base composition of selenomonads investigated here fit well within the group of organisms generally known as spirilla and described first by Véron (107) and recently by Primrose (80).

CONCLUSIONS

The two species of selenomonads which have been investigated can now be described as follows.

(i) *Selenomonas sputigena* (Flügge, 1886) Boskamp, 1922. Gram negative, anaerobic, rigid, vibrioid to spiral-shaped cells, measuring 0.9 to 1.1 by 3.0 to 5.5 μm. Cells occur singly, in pairs, and short chains but also in groups. No capsules, no metachromatic granules. Approximately 16 flagella are present as a tuft in the center of the concave side of the cell. Cells in the process of division may be nonmotile. Catalase is not produced. In thioglycolate medium, growth is cloudy but confined to lower areas of the tube. Growth is not apparent until after overnight incubation. In MPB broth, more rapid growth occurs with overall turbidity, later forming a granular deposit. Growth is best in thioglycolate media. Blood agar is a poor substitute, with the medium showing a brown discoloration after several days of incubation or storage. Subcultures (from blood to blood media) grow poorly. Abundant growth, however, is obtained when transfer is made from blood to thioglycolate media. Growth is also good on special media (e.g., MPB) after three to four transfers from blood or from thioglycolate media.

Gelatin stab: no liquefaction. Blood agar colonies: gray, convex, margin entire. Blood agar slants: growth scanty, beaded, no chromogenesis. MPB broth: turbid with settling, often granular. Growth at 30 and 37 C, but no growth at 22, 45, or 50 C. Minimum pH in glucose-supplemented medium: 5.0, but occasionally higher. No acid from cellobiose, dulcitol, inositol, salicin, sorbitol, and xylan. Acid from arabinose, esculin, galactose, glucose, inulin, lactose, maltose, mannitol, raffinose, sucrose, and xylose. Incubation in fructose gave ambiguous results. Glucose is fermented with the production of propionate and acetate. Whether lactate or carbon dioxide are produced has not been adequately studied. Whether amino acids are fermented has not been adequately studied.

No H_2S produced. Casein not digested. Starch hydrolyzed. Nitrate reduced to nitrite. Cellulose not hydrolyzed. No indole produced. MR-VP tests negative. GC content: 60.6 ± 0.8 mol %.

Main distinguishing characters from *Selenomonas ruminantium*: H_2S not produced; no acid from cellobiose; no acid from salicin; no acid from dulcitol. Habitat: buccal cavity of man.

(ii) *Selenomonas ruminantium* (Certes, 1889) Wenyon, 1926. Gram negative, anaerobic, rigid, crescent- to vibrioid-shaped cells, measuring 0.9 to 1.1 by 3.5 to 6.0 μm, occurring singly, in pairs, and short chains. No capsule, no metachromatic granules. A tuft of approximately 16 flagella arises from the center of the concave side of the cell. The length of the flagella is approximately 1 to 1.5 times the length of the cell. The flagella are not thicker at the base than at the free end and no flagellar sheath is present. Some cells may be nonmotile, especially during binary fission. No catalase produced. Does not grow on ordinary media, except in well-reduced thioglycolate medium, in which growth and motility are both poor at high glucose concentration. Grows well in chemically defined medium containing B vitamins, glucose, minerals, carbon dioxide, acetate, and some volatile fatty acids, especially *n*-valerate.

Gelatin stab: no liquefaction. Agar colonies: tan, convex, margin entire. Agar slant: growth scanty, effuse, not chromogenic. Broth: turbid, with settling after 1 to 2 days of incubation, followed by clearing due to lysis of cells. Abundant growth in established cultures, especially at 37 C. Good growth also at 30 C; no growth at 22 and at 50 C; some strains may show re-

stricted growth for short periods at 45 C. Minimum pH in glucose-supplemented medium: 4.5 to 5.0. Acid produced from arabinose, esculin, galactose, glucose, inulin, lactose, maltose, mannitol, raffinose, sucrose, and xylose. No acid from xylan. Cellulose not hydrolyzed. No indole produced. MR-VP tests negative. Some strains varied in starch hydrolysis, production of nitrite from nitrate, and digestion of casein. Similarly, acid production varied with glycerol, inositol, sorbitol, and trehalose as substrates. Produces propionic and acetic acids, CO_2, and/or DL-lactic acid as major products of glucose fermentation. Some strains showed ambiguous reaction with casein digestion. GC content: 54.0 ± 0.8 mol %.

Main distinguishing characters from *S. sputigena*: H_2S produced; acid from cellobiose; acid from salicin; acid from dulcitol; habitat —rumen of herbivores.

ACKNOWLEDGMENTS

We are specifically indebted to M. P. Bryant, University of Illinois, for his most helpful suggestions and constructive criticisms during the preparation of the manuscript. Originally the study was supported by the Medical Research Council of Canada (grant to JFMH, MT-1040), but later by the National Research Council of Canada (grant to VVK, A6222).

LITERATURE CITED

1. Abou-Akkada, A. R. and T. H. Blackburn. 1963. Some observations on the nitrogen metabolism of rumen proteolytic bacteria. J. Gen. Microbiol. **31**:461-469.
2. Abramson, M. B., R. Katzman, C. E. Wilson, and H. P. Gregor. 1964. Ionic properties of aqueous dispersions of phosphatidic acid. J. Biol. Chem. **239**:4066-4072.
3. Barnes, E. M., and G. C. Burton. 1970. The effect of hibernation on the cecal flora of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). J. Appl. Bacteriol. **33**:505-514.
4. Bisset, K. A. 1955. The cytology and life history of bacteria. E. and S. Livingstone, Edinburgh.
5. Boskamp, E. 1922. Ueber Bau und Lebensweise und systematische Stellung von *Selenomonas palpitans* (Simons). Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **88**:58-73.
6. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore.
7. Bryant, M. P. 1951. Some characteristics of the different bacteria present in the rumen of cattle on a constant ration. J. Anim. Sci. **10**:1042.
8. Bryant, M. P. 1956. The characteristics of strains of *Selenomonas* isolated from bovine rumen contents. J. Bacteriol. **72**:162-167.
9. Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205-217.
10. Bryant, M. P., and I. M. Robinson. 1962. Some nutritional characteristics of predominant culturable ruminant bacteria. J. Bacteriol. **84**:605-614.
11. Burdett, I. D. J., and H. J. Rogers. 1970. Modification of the appearance of mesosomes in sections of *Bacillus licheniformis* according to the fixation procedure. J. Ultrastruct. Res. **30**:354-367.
12. Burdett, I. D. J., and H. J. Rogers. 1972. The structure and development of mesosomes studied in *Bacillus licheniformis* strain 6346. J. Ultrastruct. Res. **38**:113-133.
13. Burger, M. M., and L. Glaser. 1965. The biosynthesis of teichoic acids. I. Polyglycerophosphate. J. Biol. Chem. **239**:3168-3177.
14. Campbell, D. H., J. S. Garvey, N. F. Cremer, and D. H. Sussdorf. 1964. Methods in immunology. W. A. Benjamin Inc., New York.
15. Certes, A. 1889. Note sur les micro-organismes de la panse des ruminants. Bull. Soc. Zool. France, **14**:70-73.
16. Cheng, K. J., G. A. Jones, F. J. Simpson, and M. P. Bryant. 1969. Isolation and identification of rumen bacteria capable of anaerobic rutin degradation. Can. J. Microbiol. **15**:1365-1371.
17. Cho, K. Y., C. H. Doy, and E. H. Mercer. 1967. Ultrastructure of the obligate halophilic bacterium *Halobacterium halobium*. J. Bacteriol. **94**:196-201.
18. Cohen-Bazire, G., and R. Kunisawa. 1963. The fine structure of *Rhodospirillum rubrum*. J. Cell Biol. **16**:401-419.
19. Cohen-Bazire, G., and J. London. 1967. Basal organelles of bacterial flagella. J. Bacteriol. **94**:458-465.
20. Cole, R. M. 1965. Cell wall replication in *Salmonella typhosa*. Science **143**:820-822.
21. Cunha, A. M. da, 1915. Sobre a presenca de "Selenomonas" no coecum dos roedores. Brazil Medico **29**:33.
22. Doetsch, R. N. 1971. Functional aspects of bacterial flagellar motility. Crit. Rev. Microbiol. **1**:73-103.
23. Dobell, C. 1932. Antony van Leeuwenhoek and his "little animals." John Bale, Sons, and Danielson, Ltd. London.
24. Fitz-James, P. C. 1960. Participation of the cytoplasmic membrane in growth and spore formation of bacilli. J. Biophys. Biochem. Cytol. **8**:507-528.
25. Fitz-James, P. C. 1964. Fate of the mesosomes of *Bacillus megaterium* during protoplasting. J. Bacteriol. **87**:1483.
26. Fitz-James, P. C. 1968. The collection of mesosome vesicles extruded during protoplasting, p. 124-143. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts and L-forms. Williams and Wilkins Co., Baltimore.
27. Fonseca, O. O. R., da. 1916. Estudos sobre os flagellados parasitos dos mammiferos do Brazil. Mem. Inst. Oswaldo Cruz **8**:5.

28. Fulghum, R. A., and W. E. C. Moore. 1963. Isolation, enumeration, and characteristics of proteolytic ruminal bacteria. J. Bacteriol. **85**:808-815.
29. Ghosh, B. K., and R. G. E. Murray. 1969. Fractionation and characterization of the plasma and mesosome membrane of *Listeria monocytogenes*. J. Bacteriol. **97**:426-440.
30. Glaister, P. F. M. 1968. quoted by R. G. E. Murray (103) *In* L. B. Guze (ed.), Microbiol protoplasts, spheroplasts and L-forms, p. 1-18. Williams and Wilkins Co., Baltimore.
31. Gunetileke, K. G., and R. A. Anwar. 1966. Biosynthesis of uridine diphospho-N-acetyl muramic acid. J. Biol. Chem. **241**:5741-5743.
32. Hobson, P. N. 1965. Continuous culture of some anaerobic and facultatively anaerobic rumen bacteria. J. Gen. Microbiol. **38**:167-180.
33. Hobson, P. N., and S. O. Mann. 1961. The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of sheep. J. Gen. Microbiol. **25**:227-240.
34. Hobson, P. N., S. O. Mann, and W. Smith. 1962. Serological tests of a relationship between rumen selenomonads *in vitro* and *in vivo*. J. Gen. Microbiol. **29**:265-270.
35. Hoeniger, J. F. M. 1965. Development of flagella by *Proteus mirabilis*. J. Gen. Microbiol. **40**:29-42.
36. Hoffman, E., and S. von Prowazek. 1906. Untersuchungen über die Balanitis- und Mundspirochäten. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **41**:817-821.
37. Huhtanen, C. N., and L. S. Gall. 1953. Rumen organisms. I. Curved rods and related rod types. J. Bacteriol. **65**:548-553.
38. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1-49.
39. Hungate, R. E. 1969. A roll tube method for the cultivation of strict anaerobes, p. 117-132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press Inc., New York.
40. Iterson, W. van. 1954. Some thoughts on the possible relationship of bacterial flagella to cilia and flagella in other organisms, p. 602. *In* Proceedings of International Conference on Electron Microscopy, London.
41. Iterson, W. van. 1969. Some features of a remarkable organelle in *Bacillus subtilis*. J. Biophys. Biochem. Cytol. **9**:183-192.
42. Ito, E., and J. L. Strominger. 1962. Enzymatic synthesis of the peptide in bacterial uridine nucleotides. J. Biol. Chem. **237**:2689-2695.
43. Jeynes, M. H. 1955. Taxonomic position of the genus *Selenomonas* (von Prowazek). Nature (London) **176**:1077.
44. Jeynes, M. H., and K. A. Bisset. 1968. The flagella of *S. sputigena* in relation to cell wall growth and nuclear division of microorganisms. G. Microbiol. **16**:(1-4), 65-67.
45. Judicial Commission. 1958. Opinion 21. Conservation of the generic name *Selenomonas* von Prowazek. Int. Bull. Bacteriol. Nomen. Taxon. **8**:163-165.
46. Kamio, Y., H. Inagaki, and H. Takahashi. 1970. Possible occurrence of α-oxidation in phospholipid biosynthesis in *Selenomonas ruminantium*. J. Gen. Appl. Microbiol. **16**:463.
47. Kanegasaki, S., and H. Takahashi. 1967. Function of growth factors for rumen microorganisms. I. Nutritional characteristics of *Selenomonas ruminantium*. J. Bacteriol. **93**:456-463.
48. Kauffman, F. 1954. Enterobacteriaceae, 2nd ed. E. Munksgaard Publ., Copenhagen.
49. Kerandel, J. 1909. Sur quelques hématozaires observés au Congo. (Haute Sangha-Logone). Bull. Soc. Pathol. Exot. **2**:204-209.
50. Kerridge, D. 1960. The effect of inhibitors on the formation of flagella by *Salmonella typhimurium*. J. Gen. Microbiol. **23**:519-538.
51. Kung, S. D., and J. D. Williams. 1969. Chloroplast DNA from broad bean. Biochim. Biophys. Acta **195**:434-445.
52. Kushnarev, V. M., and N. A. Pereverzev. 1964. Sistema membran v kletkakh *E. coli*. Mikrobiologiya **33**:610-612.
53. Latham, M. J., and M. E. Sharpe. 1971. The isolation of anaerobic organisms from the bovine rumen, p. 133-147. *In* D. A. Shapton and R. G. Board (ed.), Isolation of anaerobes. Academic Press Inc., New York.
54. Lautrop, H., A. Reyn, and A. A. Birch-Andersen. 1964. A comparative electron microscope study of the cell walls of Gram-negative bacteria. Proc. XIV Scand. Congr. Pathol. Microbiol. Oslo.
55. Leifson, E. 1931. Development of flagella on germinating spores. J. Bacteriol. **21**:357.
56. Lessel, E. F., and R. S. Breed. 1954. *Selenomonas* Boskamp, 1922—a genus that includes species showing an unusual type of flagellation. Bacteriol. Rev. **18**:165-169.
57. Loesche, W. J., and R. J. Gibbons. 1965. A practical scheme for identification of the most numerous oral gram negative anaerobic rods. Arch. Oral Biol. **10**:723-725.
58. Loesche, W. J. 1969. Oxygen sensitivity of various anaerobic bacteria. Appl. Microbiol. **18**:723-727.
59. Loewenthal, W. 1906. Zur Kenntnis der Mundspirochäten. Med. Klin. **2**:278-281.
60. MacDonald, J. B. 1953. The motile nonsporulating anaerobic rods of the oral cavity. University of Toronto Press, Toronto.
61. MacDonald, J. B., and E. M. Madlener. 1955. Studies on *Spirillum sputigenum*. J. Dent. Res. **34**:709-714.
62. MacDonald, J. B., and E. M. Madlener. 1957. Studies on the isolation of *Spirillum sputigenum*. Can. J. Microbiol. **3**:679-686.
63. MacDonald, J. B., E. M. Madlener, and S. S. Socransky. 1959. Observations on *Spirillum sputigenum* and its relationship to *Selenomonas* species with special reference to flagellation. J. Bacteriol. **77**:559-565.
64. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208-218.

65. Martinez, R. J., and E. Z. Gordee. 1966. Formation of bacterial flagella. I. Demonstration of functional flagellin pool in *Spirillum serpens* and *Bacillus subtilis*. J. Bacteriol. **91**:870-875.
66. Mason, D. J., and D. M. Powelson. 1956. Nuclear division as observed in live bacteria by a new technique. J. Bacteriol. **71**:474-480.
67. Miller, W. D. 1887. Ueber pathogene Mundpilze. Inaugural Dissertation, Berlin.
68. Miller, W. D. 1890. The microorganisms of the human mouth. S. S. White, Dental Mfg. Co., Philadelphia.
69. Moir, R. J., and M. J. Masson. 1952. An illustrated scheme for the microscopic identification of the rumen microorganisms of the sheep. J. Pathol. Bacteriol. **64**:343-350.
70. Morgan, C., H. S. Rosenkranz, B. Chan, and H. M. Rose. 1966. Electron microscopy of magnesium-depleted bacteria. J. Bacteriol. **91**:891-895.
71. Mühlens, P. 1909. Ueber Züchtung von anaeroben Mikroorganismen der Mundhöhle (u.a. *Spirillum sputigenum*) Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **48**:523-528.
72. Munch-Peterson, E. 1964. A new culture medium for rumen bacteria. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **193**:353-362.
73. Murray, R. G. E. 1968. Bacterial cell wall anatomy in relation to the formation of spheroplasts and protoplasts, p. 1-18. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts, and L-forms. Williams and Wilkins Co., Baltimore.
74. Murray, R. G. E., and A. A. Birch-Andersen. 1963. Specialized structure in the region of the flagella tuft in *Spirillum serpens*. Can. J. Microbiol. **9**:393-402.
75. Murray, R. G. E., P. Steed, and H. E. Elson. 1965. The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other gram-negative bacteria. Can. J. Microbiol. **11**:547-560.
76. Ogimoto, K. 1972. Über Selenomonas aus dem Caecum von Ratten. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **221**:467-473.
77. Plaut, H. C. 1907. Ueber die Geisseln bei fusiformen Bacillen. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **44**:310-316.
78. Pontefract, R. D., and F. S. Thatcher. 1970. An electron microscope study of mesosomes in irradiation-resistant mutants of *Escherichia coli*. J. Ultrastruct. Res. **30**:78-86.
79. Preston, N. W., and H. B. Maitland. 1952. The influence of temperature of the motility of *Pasteurella pseudotuberculosis*, J. Gen. Microbiol. **7**:117-128.
80. Primrose, S. B. 1971. Studies on the deoxyribosenucleic acid from *Spirillum*. Biochim. Biophys. Acta **247**:29-37.
81. Prins, R. A. 1971. Isolation, culture, and fermentation characteristics of *Selenomonas ruminantium* var. *bryanti* var.n. from the rumen of the sheep. J. Bacteriol. **105**:820-825.
82. Prowazek, S. von. 1913. Zur Parasitologie von Westafrika. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **70**:32-36.
83. Purdom, M. R. 1963. Micromanipulation in the examination of rumen bacteria. Nature (London) **198**:307-308.
84. Purser, D. B., and S. M. Buchler. 1966. Amino acid composition of (cow) rumen organisms. J. Dairy Sci. **49**:81-84.
85. Remsen, C. C., S. W. Watson, J. B. Waterbury, and H. G. Trüper. 1968. Fine structure of *Ectothiorhodospira mobilis* Pelsh. J. Bacteriol. **95**:2374-2392.
86. Ritchie, A. E., R. F. Keeler, and J. H. Bryner. 1966. Anatomical features of *Vibrio fetus*: electron microscopic survey. J. Gen. Microbiol. **43**:427-438.
87. Robinow, C. F. 1942. A study of the nuclear apparatus of bacteria. Proc. Roy. Soc. Ser. B. **130**:299-324.
88. Robinow, C. F. 1944. Cytological observations on *Bact. coli*, *Proteus vulgaris* and various aerobic spore-forming bacteria, with special reference to the nuclear structure. J. Hyg. **43**:413-423.
89. Robinow, C. F. 1962. Morphology of the bacterial nucleus. Brit. Med. Bull. **18**:31-36.
90. Robinow, C. F. 1954. Addendum to: *Selenomonas* Boskamp, 1922—a genus that includes species showing an unusual type of flagellation, by E. F. Lessel and R. S. Breed. Bacteriol. Rev. **18**:169-170.
91. Ryter, A. 1968. Association of the nucleus and the membrane of bacteria: a morphological study. Bacteriol. Rev. **32**:39-54.
92. Ryter, A. 1969. Structure and functions of mesosomes of gram-positive bacteria. Curr. Top. Microbiol. Immunol. **49**:151-177.
93. Ryter, A., and O. E. Landman. 1964. Electron microscope study of the relationship between mesosome loss and the stable L state (or protoplast state) in *Bacillus subtilis*. J. Bacteriol. **88**:457-467.
94. Salton, M. R. J. 1964. The bacterial cell wall. Elsevier, Amsterdam.
95. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430-443.
96. Silva, M. T. 1967. Electron microscopic study on the effect of the oxidation of ultra-thin sections of *Bacillus cereus* and *Bacillus megaterium*. J. Ultrastruct. Res. **18**:345-353.
97. Simons, H. 1920. Eine saprophytische Oscillarie in Darm des Meerschweinchens. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **5**:356-364.
98. Simons, H. 1922. Ueber *Selenomonas palpitans* n. sp. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. **87**:50.
99. Smith, R. W., and H. Koffler. 1971. Bacterial flagella p. 219-339. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 6. Academic Press Inc., New York.

100. Sokal, R. R., and P. H. A. Sneath. 1963. Principles of numerical taxonomy. W. H. Freeman and Co., San Francisco.
101. Stocker, B. A. D., and J. C. Campbell. 1959. The effect of non-lethal deflagellation on bacterial motility and observation on flagellar regeneration. J. Gen. Microbiol. **20**:670–685.
102. Strominger, J. L. 1962. Biosynthesis of bacterial cell wall, p. 413. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 3. Academic Press Inc., New York.
103. Tiwari, A. D., M. P. Bryant, and R. S. Wolfe. 1969. Simple method for isolation of *Selenomonas ruminantium* and some nutritional characteristics of the species. J. Dairy Sci. **52**:2054–2056.
104. Vaituzis, Z., and R. N. Doetsch. 1965. Flagella of *Salmonella typhimurium* spheroplasts. J. Bacteriol. **89**:1586–1593.
105. Vaituzis, Z., and R. N. Doetsch. 1966. Flagella of *Escherichia coli* spheroplasts. J. Bacteriol. **91**:2103–2104.
106. Vaituzis, Z., and R. N. Doetsch. 1961. Relationship between cell wall, cytoplasmic membrane, and bacterial motility. J. Bacteriol. **100**:512–521.
107. Véron, M. 1966. Taxonomie numérique des vibrions et des certaines bactéries comparables. II. Correlation entre les similitudes phénétiques et la composition en bases de l'ADN. Ann. Inst. Pasteur **111**:671–709.
108. Wenyon, C. M. 1926. Protozoology, p. 311–312. Bailliere, Tindall, and Cox, London.
109. Wilson, G. S., and A. A. Miles. 1964. Topley and Wilson's principles of bacteriology and immunology, p. 648, 5th ed. Edward Arnold, London.
110. Williams, M. A., and S. C. Rittenberg. 1957. A taxonomic study of the genus *Spirillum* Ehrenberg. Int. Bull. Bacteriol. Nomen. Taxon. **7**:49–111.
111. Woodcock, H. M., and G. Lapage. 1913–1914. On a remarkable new type of a protistan parasite. Quart. J. Microsc. Sci. **59**:431–457.