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# Flagella of *Salmonella typhimurium* Spheroplasts

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ABSTRACT

VAITUZIS, Z. (University of Maryland, College Park), AND R. N. DOETSCH. Flagella of *Salmonella typhimurium* spheroplasts. J. Bacteriol. **89:**1586–1593. 1965.—The flagella of penicillin-induced spheroplasts of *Salmonella typhimurium* were examined by electron and light microscopy. The process of spheroplast formation was followed for a period of 20 hr from its inception. Flagella were found to be confined to those areas of the spheroplast where cell-wall fragments remained. Flagella disappeared as the spheroplasts aged. Spheroplasts produced from nonflagellated organisms were found incapable of synthesizing flagella. Upon inactivation of the penicillin, however, flagella again were synthesized by spheroplasts during reversion to their original rod form. Flagella formation, it is suggested, is dependent on prior synthesis of the normal cell wall. The morphology of the microorganisms at the time of appearance of new flagella is described.

When Weibull (1953) first produced protoplasts of *Bacillus megaterium*, he observed them to be nonmotile, although flagellated. Later, when penicillin-induced spheroplasts of *Salmonella typhimurium* were described (Lederberg and St. Clair, 1958), they, too, were found to be nonmotile and flagellated. Subsequent investigations have revealed that spheroplasts are capable of all physiological activities of intact whole organisms, including respiration, nutrient uptake, growth, formation of inducible enzymes, and the development of bacteriophage (McQuillen, 1960). Very little, however, has been reported on the synthesis of flagella in spheroplasts. Since the spheroplast is a result of continued protoplasmic synthesis without corresponding cell-wall synthesis, it would seem reasonable to expect that any flagella found on spheroplasts could be formed by them in the absence of the rigid cell-wall polymer. Kerridge (1960), however, suggested that the spheroplasts do not synthesize flagella.

The present study was initiated to determine the nature of flagellation on spheroplasts, and the conditions necessary for the synthesis of flagella by nonflagellated spheroplasts of *S. typhimurium.*

## MATERIALS AND METHODS

*Organism.* The organism studied was *S. typhimurium*, University of Maryland strain 45-4-A. It was cultured at 37 C, at which temperature it is flagellated and actively motile. When incubated at 44 C, this organism is nonmotile and without flagella, and thus behaves in a manner similar to the *Salmonella* strains studied by Quadling and Stocker (1956).

*Media.* The medium used was Trypticase Soy Broth (BBL). This was supplemented with 0.1% (w/v) $MgSO_4$ (anhydrous) and 12% (w/v) sucrose (Lederberg, 1956) for the formation and stabilization of spheroplasts and is hereafter referred to as "S medium." Penicillin G (potassium salt) was added to a final concentration of 700 IU/ml (hereafter referred to as "S-P medium"). The S and S-P media were sterilized by filtration through 0.45-$\mu$ cellulose filters (Millipore Filter Corp., Bedford, Mass.). The final *p*H of each medium was 6.8.

*Spheroplast formation.* To 1 volume of an actively growing culture of *S. typhimurium* containing approximately $10^7$ organisms per ml, 2 volumes of S-P medium were added. The same procedure was used for the flagellated (37 C) and nonflagellated (44 C) cultures. For optimal spheroplast production, the S-P medium had to be preheated to the same incubation temperature as that of the original culture source. Incubation was continued at the same temperature, and, within 3 hr, nearly all of the organisms had become spherical.

*Reversion of spheroplasts to rod-form cells.* The spheroplasts were centrifuged in sterile screw-cap tubes at 300 $\times$ *g* for 15 min. The supernatant S-P medium was discarded, and the spheroplasts were gently resuspended in an equal volume of preheated S medium containing 5,000 Kersey units of penicillinase (BBL) per ml to inactivate residual penicillin. The spheroplasts were incubated at the desired temperature (37 or 44 C) during the process of reversion to rod forms. For the determination of osmotic stability, the centrifuged spheroplasts were resuspended in an equal volume of distilled water. Decrease in turbidity was taken as evidence of lysis. The morphology of unlysed forms was determined by phase microscopy. The per cent of spheroplasts reverting to normal cell forms was determined from the arithmetic mean of per cent

reversion seen in 10 randomly selected microscopic fields.

*Microscopy and photography.* Light microscopic observations of the various morphological stages in the formation and reversion of spheroplasts were made on a drop of the culture placed on a slide and covered with a no. 1 cover slip. A Zeiss photomicroscope equipped with a Zeiss phase (no. 3, 100 ×) objective was used for observations and photography. A green, broad-band interference filter (maximal transmittance, 546 mμ) was used for all photographs of living organisms as well as for fixed preparations stained for flagella. An RCA electron microscope (EMU-3f) was used for observations of chromium-shadowed preparations. All cultures examined for flagella were fixed in 10% (v/v) buffered (*p*H 6.5) formalin and washed twice in distilled water.

## RESULTS

*Flagellation of newly formed spheroplasts.* Spheroplasts were formed by emerging as a bulging mass either from the middle of the bacterial body or from one of the ends. A third type of spheroplast formation was observed in which 2, 3, or, at times, even 4 units per organism emerged (Fig. 1). These three modes of spheroplast formation are considered important when the distribution of flagella on spheroplasts is studied. The majority of the newly formed spheroplasts were flagellated, the flagella seeming to occur in groups at one or two loci (Fig. 4, 5, and 6). Residual cell-wall fragments adhered to the surface of the spheroplast in one or two places, depending on

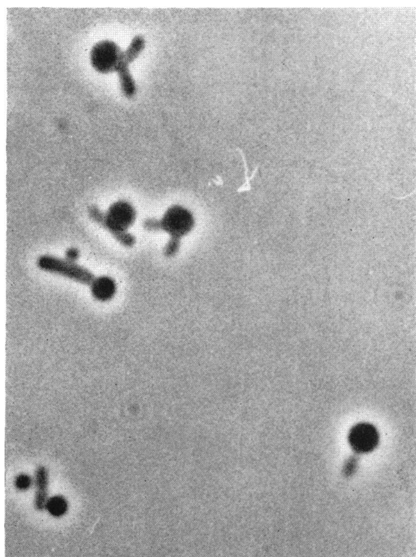

FIG. 1. *Modes of spheroplast formation in Salmonella typhimurium. Phase-contrast.* × *2,320.*

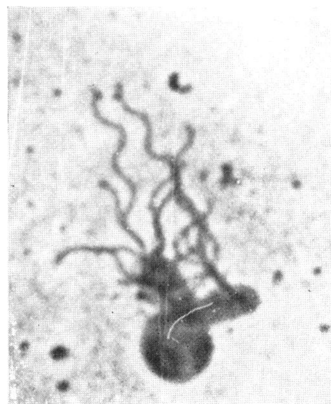

FIG. 2. *Flagella stain of a developing spheroplast, with flagella confined to the parent rod-shaped cell. Gray (1926) flagella stain.* × *2,920.*

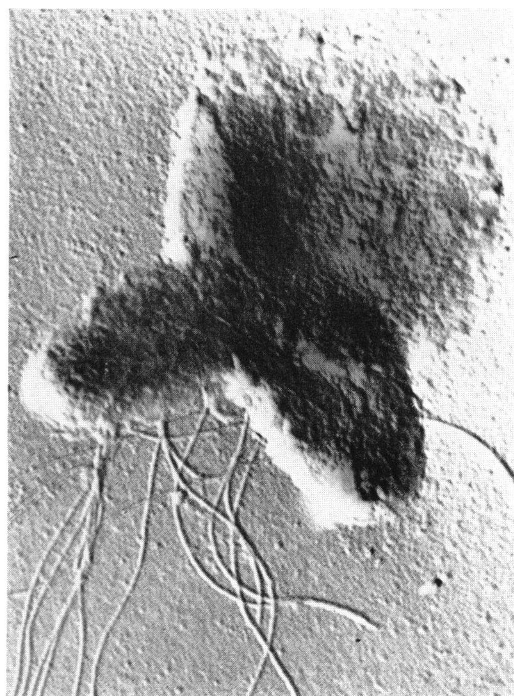

FIG. 3. *Chromium-shadowed electron micrograph of spheroplast shown in Fig. 2.* × *21,700.*

the type of spheroplast formation described above. The flagella in the residual cell wall remained localized in this area on the spheroplast (Fig. 2–6). The flagella observed on the newly formed spheroplasts, therefore, are not synthesized by them, but are, rather, the legacy of the same organism in its prespheroplast state. Lysis of a 3-hr-old spheroplast suspension illustrates the

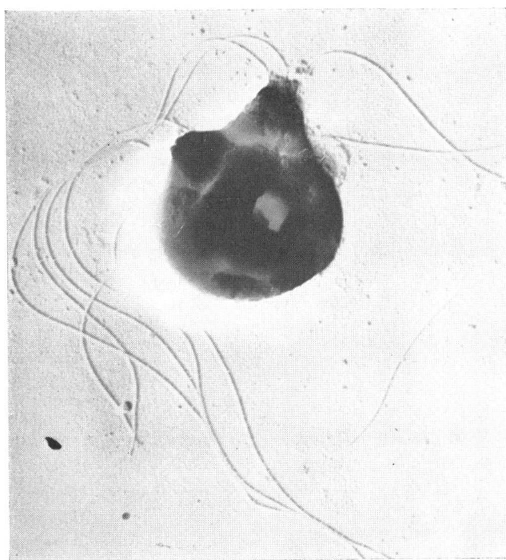

FIG. 4. *Spheroplast 1 hr after addition of penicillin. Flagella are confined to short, rod-form buds of parent cell.* × *16,200.*

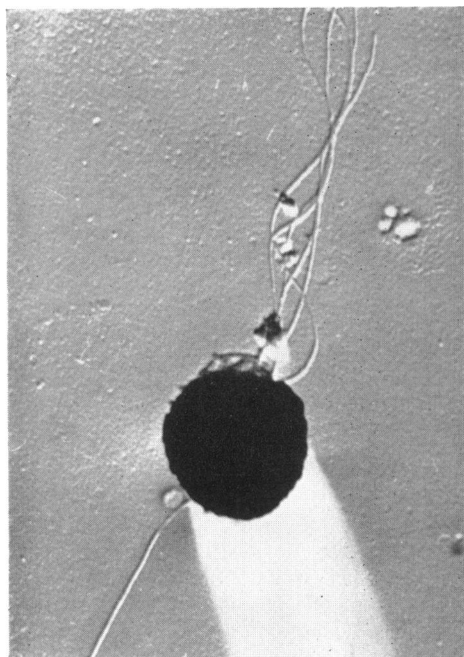

FIG. 6. *Chromium-shadowed electron micrograph of a spheroplast shown in Fig. 5.* × *8,800.*

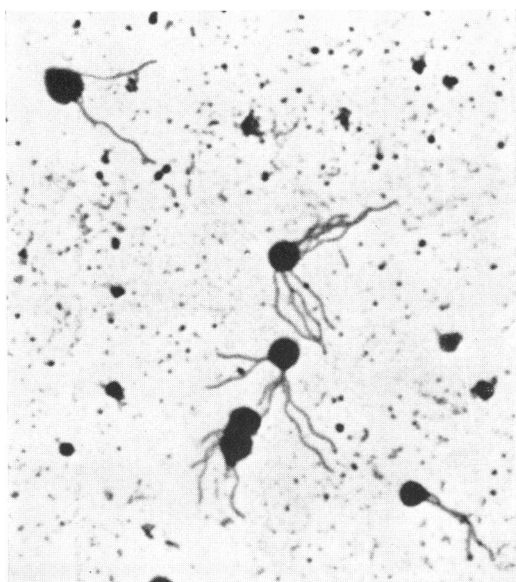

FIG. 5. *Flagella stain of 3-hr-old spheroplasts. Flagella appear in groups localized to one or two areas on the spheroplasts. Gray (1926) flagella stain.* × *2,320.*

adhering cell-wall fragments from which the flagella appear to originate (Fig. 7).

*Spheroplast motility.* The *S. typhimurium* culture forming the spheroplasts was fully motile

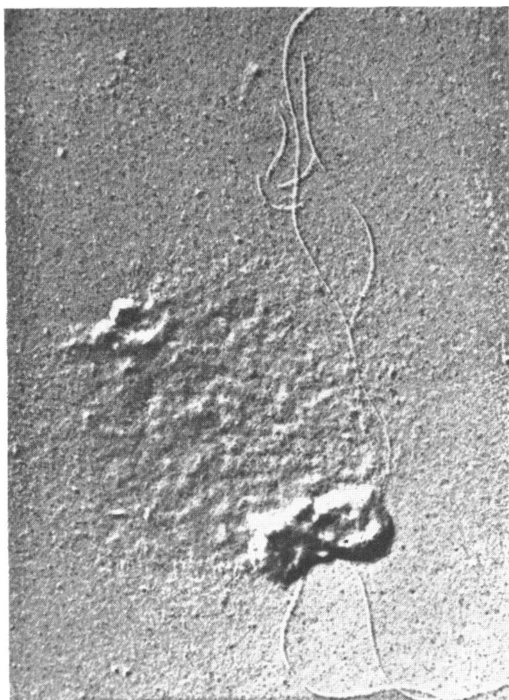

FIG. 7. *Lysed spheroplast with flagella originating from adhering parent cell wall. Chromium-shadowed electron micrograph.* × *10,800.*

throughout the various stages described above. In motile forms, the motility ceased within 30 min after the organisms became spherical. Addition of 10 μg of chloramphenicol per ml at this stage prevented the spheroplasts from increasing in volume, but they remained motile for up to 12 hr. These motile forms were osmotically unstable and lysed readily when the medium containing them was diluted with distilled water.

TABLE 1. *Relationship between age of spheroplast and degree of flagellation*

| Age | Avg no. of flagella per spheroplast | Flagellated spheroplasts | Avg spheroplast diameter |
|---|---|---|---|
| *hr* | | % | μ |
| 5 | 5 | 75 | 3 |
| 10 | 3 | 40 | 4 |
| 15 | 2 | 30 | 5 |
| 28 | 1 | 2 | 6 |

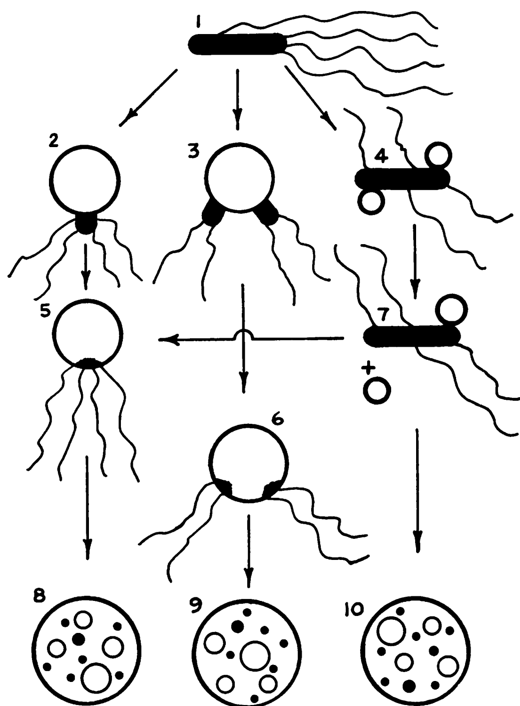


FIG. 8. *Mode of spheroplast formation in Salmonella typhimurium and the fate of their flagella. (1) Normal flagellated organism. (2, 3, 4) Various forms of spheroplast emergence (see text). (5) A terminally produced spheroplast possessing one group of flagella. (6) A centrally produced spheroplast possessing two groups of flagella. (7) An organism forming two or more spheroplasts. Some spheroplasts separate and are nonflagellated. The spheroplast remaining continues to enlarge and remain with one group of flagella. (8, 9, 10) Spheroplasts (24 hr or older) become large, vacuolated, and lose the adhering flagellated cell wall.*

TABLE 2. *Time for regeneration of flagella and motility by Salmonella typhimurium transferred from 44 to 37 C*

| Time at 37 C | Motility | Flagellated cells | Avg length of flagella | No. of flagella per cell* |
|---|---|---|---|---|
| *hr* | % | % | μ | |
| 1 | 1 | 5 | 1.5 | 5 |
| 2 | 5 | 40 | 2 | 7 |
| 3 | 10 | 60 | 3 | 8 |
| 4 | 40 | 80 | 4 | 8 |
| 5 | 70 | 99 | 5 | 8 |
| 6 | 70 | 99 | 5 | 8 |

* This culture grown at 37 C has approximately eight flagella per cell, each with an average length of 5 μ.

*Fate of flagella with aging.* As the spheroplasts aged, they increased in volume and became highly vacuolated. Flagella stains and electron micrographs revealed a gradual diminution in the number of flagella per organism with time (Table 1). These processes are illustrated schematically in Fig. 8.

*Formation of flagella in spheroplasts during reversion to rod-form cells.* When *S. typhimurium* is grown at 44 C through five or more serial transfers, it is nonmotile and nonflagellated, as determined by electron microscopy or by flagella-stained preparations. This actively growing nonflagellated culture formed flagella and regained motility within 1 hr when transferred to 37 C (Table 2). Spheroplasts were made from these nonflagellated *S. typhimurium* cultures. When 3-hr-old nonflagellated spheroplasts were transferred to 37 C and observed over a 48-hr period, flagellation was not apparent. Transfer to 25 C produced the same results. Kerridge (1960) observed that, when *S. typhimurium* flagella are sheared off mechanically and penicillin is added, the flagella are resynthesized during the time they are gradually being transformed into spheroplasts; thus, penicillin does not interfere with the process of flagella synthesis in bacteria still possessing a cell wall.

When penicillin was inactivated in a 3-hr-old nonflagellated spheroplast culture, a rigid cell wall was formed and the organisms reverted to the rod form. Such organisms then formed flagella and became motile. At 30 min after penicillin inactivation, the individual spheroplasts began to form numerous bulges, and, thereafter, one to three of these bulges elongated (Fig. 9); the spheroplasts

then lost their spherical form, and, in 1 hr, they became elongated forms (Fig. 10). Dilution with water at this time did not lyse these forms. Phase microscopic examination revealed that they maintain their shape, and, hence, it was inferred that they probably possessed a rigid cell wall. Further observation showed that these forms elongate into branched cells with lengths of 40 $\mu$ or more (Fig. 11). At this stage, flagella appeared, and these rather bizarre long branches became motile (Fig. 12). These motile branches then fragmented into individual organisms of normal dimensions, though some of them persisted as branched "Y" forms (Fig. 13 and 14). These "Y" forms disappeared in subsequent cell divisions.

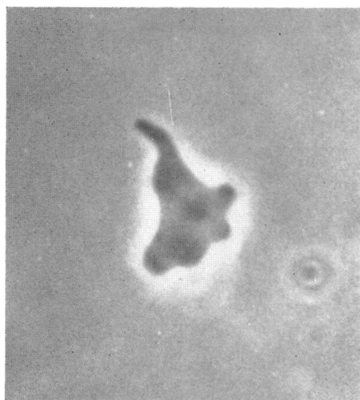

FIG. 9. *Spheroplast reverting to rod form 45 min after inactivation of penicillin.* × *2,970.*

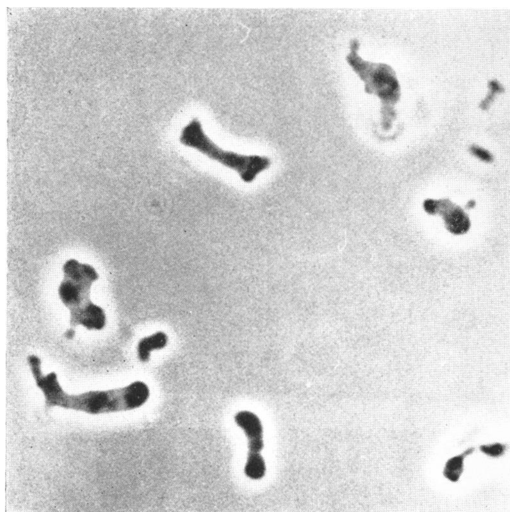

FIG. 10. *Spheroplasts reverting to rod form 1 hr after inactivation of penicillin.* × *2,320.*

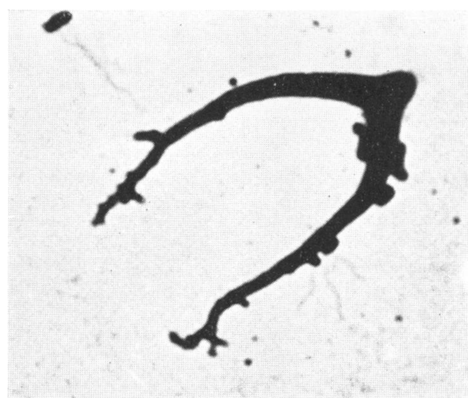

FIG. 11. *Flagella stain of a spheroplast reverting to rod form 2 hr after inactivation of penicillin. Leifson (1951) flagella stain.* × *1,820.*

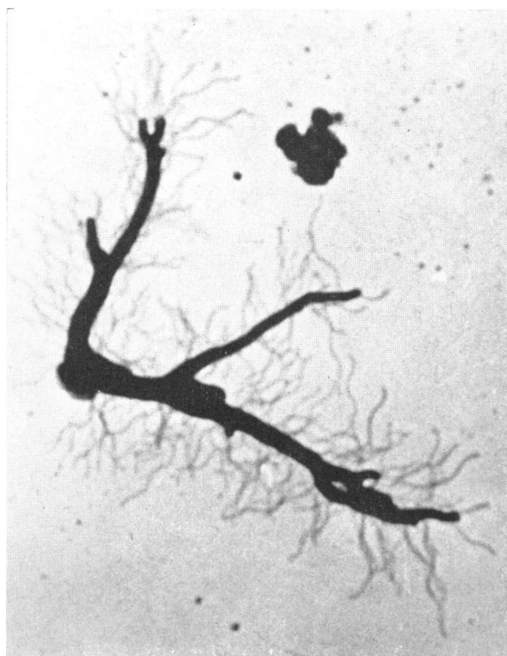

FIG. 12. *Flagella stain of a nonflagellated (44 C) spheroplast reverting to rod form 3 hr after inactivation of penicillin. Note the presence of flagella. Leifson (1951) flagella stain.* × *2,320.*

Table 3 summarizes the observations made during the reversion of nonflagellated spheroplasts to normal, flagellated, rod-shaped cells. Comparing these results with those for normal cells (Table 2), one sees that the appearance of flagella and the resumption of motility in reverting spheroplasts occurs approximately 2 hr later. This lag may represent the time required for the synthesis of a

normal rod-form organism bounded by a rigid cell wall beginning with a 3-hr-old spheroplast. The process, as described above, with its dependence on new cell-wall formation, is illustrated schematically in Fig. 15.

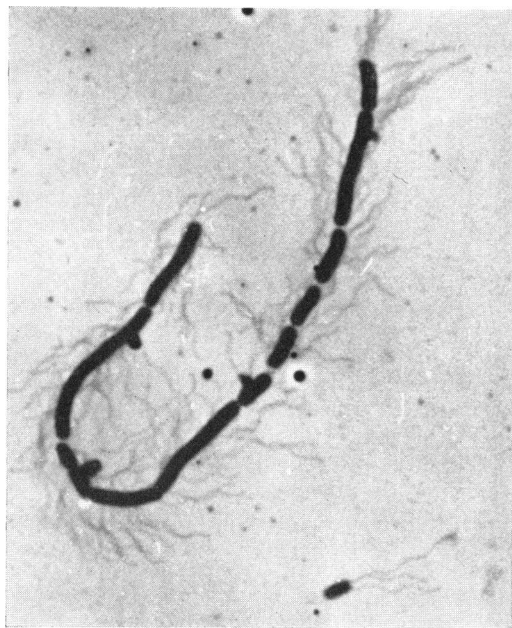

FIG. 13. *Flagella stain of a nonflagellated (44 C) spheroplast 4 hr after inactivation of penicillin. Flagellated branches are fragmenting to organisms of normal dimensions. Leifson (1951) flagella stain.* × *2,320.*

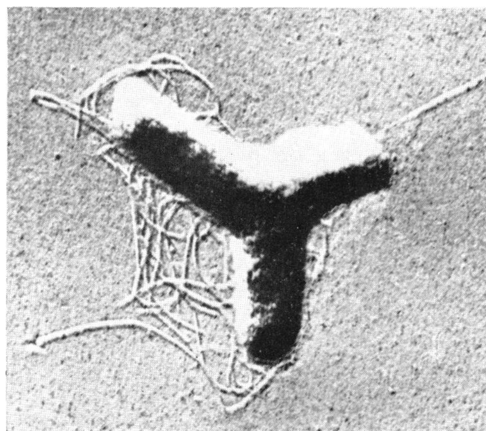

FIG. 14. *Flagellated "Y"-shaped fragment (originally part of spheroplast described in Fig. 13) of normal Salmonella typhimurium dimensions. Chromium-shadowed electron micrograph.* × *10,800.*

TABLE 3. *Time of regeneration of flagella and motility by Salmonella typhimurium spheroplasts transferred from 44 to 37 C, with penicillin inactivated 5 hr after addition**

| Time at 37 C | Motility | Flagellated cells | Avg length of flagella | Per cent reverting |
|---|---|---|---|---|
| *hr* | % | % | μ | |
| 0.5 | 0 | 0 | — | 50 |
| 1 | 0 | 0 | — | 90 |
| 2 | 0 | 1 | 1.5 | 90 |
| 2.5 | 0 | 5 | 2 | 90 |
| 3 | 1 | 40 | 3 | 90 |
| 4 | 10 | 60 | 5 | 90 |
| 5 | 30 | 95 | 6 | 90 |

* Average flagella per cell were not counted, since these are not normal rod-form cells.

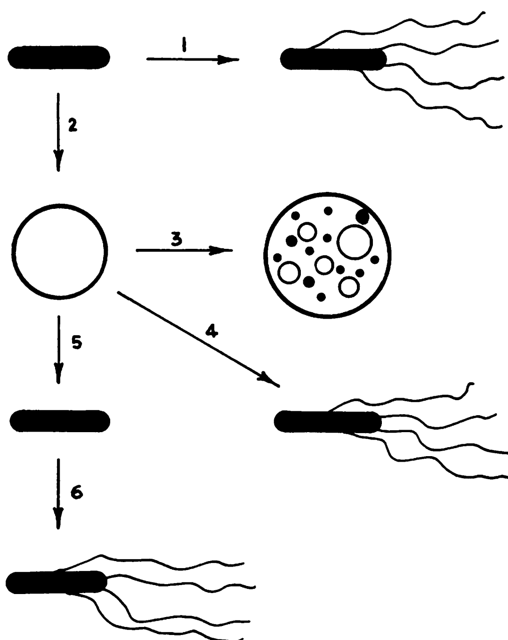


FIG. 15. *Summary of the process of flagella production by Salmonella typhimurium cells and spheroplasts in the presence and absence of penicillin when the incubation temperature is changed from 44 to 37 C. (1) Normal nonflagellated organisms at 44 C become motile and flagellated after 2 hr of incubation at 37 C. (2) Normal nonflagellated organisms at 44 C become spheroplasts 4 hr after addition of penicillin. (3) Spheroplasts [formed in (2)] incubated at 37 C for 24 hr with penicillin. Flagella are not produced. Spheroplasts enlarge and become vacuolated. (4) Spheroplasts become normal, motile and flagellated rod forms when incubated at 37 C for 4 hr after penicillin inactivation. (5) Penicillin-inactivated spheroplasts at 44 C produce normal rod forms in 4 hr. (6) Rod forms, upon transfer to 37 C, produce flagella and become motile in 2 hr.*

*Per cent spheroplasts reverting to normal rod-shaped organisms.* The per cent spheroplasts reverting to rod forms was estimated by phase microscopic counts. The reversion process was followed for a period of 5 hr (Table 4). The per cent of spheroplasts reverting decreases with time, and the process itself is initiated later and takes longer in older spheroplasts. The spheroplasts continued to enlarge, and, concurrently, there was an increase in the number of vacuoles and granules within the spheroplasts. These results support McQuillen's (1956) suggestion that older spheroplasts lose their pre-existing mucopolymer primer for the synthesis of a rigid cell wall. Old spheroplasts are, then, somewhat similar to L-forms, in that they no longer revert to rod shapes, even after penicillin is inactivated.

## Discussion

Lederberg (1956) described two processes of spheroplast formation when a young culture of *Escherichia coli* was grown in the presence of penicillin. The spheroplasts were formed by emerging as a bulging mass either from the middle of the bacterial body or from one of the ends. Thus, one organism gave rise to one spheroplast. A third type of formation, yielding multiple spheroplasts from one cell, was observed in this study, and it may be that this results when rapidly growing organisms divide in several places simultaneously.

As outlined in Fig. 8, the spheroplasts emerging from the center of an organism could possess two groups of flagella. Terminally formed spheroplasts would have one group of flagella. The newly formed nonflagellated spheroplasts could be those that are pinched off and float free from those cells forming two or more spheroplasts. A small per cent might lose their flagella in the preparatory steps preceding microscopic examination, but the number is assumed to be fairly constant, since all preparations were treated identically.

Lederberg (1956) believed that cell-wall fragments adhering to the spheroplasts withered away with time or were sloughed off as the cell grew larger. We found flagella in the areas where old parent cell walls remained, disappearing as the spheroplasts aged. The possibility exists, then, that, as the cell walls are sloughed off, the flagella are sloughed off with them. We propose that the definition of a spheroplast be expanded to include a reference to time elapsed after the addition of the spheroplast-inducing agent, since it is increasingly apparent that the age of the spheroplast is important in governing its synthetic capabilities, as shown here by the loss of flagella and in attempted reversion experiments (Table 4).

TABLE 4. *Relationship between spheroplast age and per cent reverting to rod forms upon inactivation of penicillin*

| Age | Spheroplasts reverting | Time required for reversion |
|---|---|---|
| *hr* | % | *hr* |
| 1 | 99 | 0.5 |
| 2 | 98 | 1 |
| 5 | 90 | 2 |
| 7 | 57 | 5 |
| 10 | 38 | 5 |
| 15 | 20 | 5 |
| 20 | 2 | 5 |

When penicillin was inactivated in young spheroplast preparations, the process of spheroplast reversion to rod forms was found to differ somewhat from earlier observations (Lederberg, 1956) in two respects. We observed that spheroplasts were able to produce new branched forms (Fig. 10), rather than lysing and disintegrating after their newly organized elements detached themselves. Secondly, the length of the branches was much greater than noted in previous reports. This difference may be accounted for by the use of fluid, rather than semisolid media.

The principal difference between a spheroplast and a normal bacterium is considered to be primarily the lack of the rigid cell wall or the mucopolymer layer. Specifically, it resides in the inability of the spheroplast to polymerize mucopolymer units of the cell wall (McQuillen, 1960; Martin, 1963). Our results support the suggestion of Stocker (1956) that a rigid or semirigid structure may be necessary for the flagella to exert their thrust upon to move the bacterium. We have consistently observed that motility was lost soon after the disappearance of cell-wall remnants on the spheroplast (Fig. 4). Chatterjee and Williams (1963), however, reported that glycine-induced spheroplasts of *Vibrio comma* were motile. These, in contrast to *S. typhimurium* penicillin-induced spheroplasts, are osmotically stable bodies. Thus, they must possess a protective layer of some sort, and the mucopolymer may still be present in some form.

Any special functional sites in the cell wall for the aggregation of flagellin molecules into flagella have yet to be discovered. From the results obtained here, one might speculate that the ability of *S. typhimurium* to synthesize flagella was lost with the removal of cell wall, and regained when mucopolymer layer was resynthesized. This suggests that the flagellin-aggregating mechanisms might be associated with intact cell wall. Abram and Koffler (1964) reported the aggregation of flagellin to flagella-like filaments in vitro by the manipulation of the *p*H and the salt concentrations of flagellin molecules in solution (*see also*

Asakura, Eguchi, and Iino, 1964). It is unlikely, therefore, that this polymerization is enzyme-dependent. If this be the case, then the intact cell-wall polymer might be required for a mechanical aggregation of flagellin molecules, dependent on a specific spatial arrangement of molecules between the plasma membrane and the complex outer layers of gram-negative bacteria (Martin, 1963). The presence of flagellin molecules in non-flagellated spheroplasts transferred from 44 to 37 C, or their medium, would lend support to the existence of this hypothetical mechanism and its dependence on the presence of the normal cell wall. This work, as well as work on other possible causes for the failure of *S. typhimurium* spheroplasts to form flagella, is in progress.

ACKNOWLEDGMENTS

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