Morphological Changes Accompanying Actinomycin Production in *Streptomyces antibioticus*

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The fine structure of the actinomycin producing bacterium *Streptomyces antibioticus* has been studied. The typical appearance of freshly inoculated cells is one of a generally electron dense cytoplasm. As cellular protein synthesis ceases and secondary metabolism (actinomycin biosynthesis) is initiated, a gradual loss of cytoplasmic density occurs, revealing in many cells membrane enclosed vesicles and an extensive cytoplasmic membrane system. Vesicles are seen to be compartmented by cell wall and may be released into the medium by a subsequent breakdown of the cell wall. The data suggest that the appearance of these internal structures is not due to a differentiation of *S. antibioticus* cells related to the initiation of secondary metabolism, but rather that these vesicles and membrane systems may be normal subcellular structures which become visible only after a loss of cytoplasmic material due to aging of the cells.

A large number of microorganisms have been examined for the production of useful antibiotic substances. Over a thousand antibiotics have been discovered to date, but only some fifteen of them have clinical utility. One group of such chemotherapeutic substances comprises the actinomycins, which are synthesized by certain species of the genus *Streptomyces*. The actinomycins constitute a group of chromopeptide antibiotics which differ only in the peptide portion of the molecule. (For a complete discussion of the chemistry of the actinomycins, see the reviews listed in references *2*, *3*, *6*, *12*, *27*.) Although much is known about the chemistry and production of actinomycin, the biosynthetic mechanisms involved in actinomycin formation have not been completely worked out. Precursor studies in whole *S. antibioticus* cells have shown that the D-valine and N-methyl-L-valine found in the pentapeptides of actinomycin are derived from L-valine (*13*, *15*, *23*); however, the details of the conversions are not clear. Similar studies have shown that methionine furnishes all the methyl groups in the molecule and that tryptophan is a precursor

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of the chromopore actinocin (*16*, *27*). The enzymatic synthesis of actinocin has been accomplished with the soluble enzyme phenoxazinone synthetase (*14*). No other enzymes which can be definitely implicated in actinomycin production have been reported. Recently, however, Walker et al. (*28*) have reported the purification of an enzyme from *S. antibioticus* which catalyzes ATP-pyrophosphate exchange in the presence of L-valine but which does not catalyze L-valine transfer to tRNA. Although this evidence is suggestive, this enzyme has not been shown to be involved in the cell-free synthesis of actinomycin.

Studies on the relationship between actinomycin synthesis and protein and RNA synthesis have revealed several interesting phenomena. As actinomycin production proceeds, there is a dramatic decline in the rate of protein synthesis in *S. antibioticus* cells (*15*, *17*). Jones and Weissbach (*7*) have shown that RNA synthesis also decreases in *S. antibioticus* with increasing age of the cells, but even after 36 hours, when cells are actively producing actinomycin, RNA can still be synthesized at a measurable rate. These workers have also shown that the enzyme phenoxazinone synthetase can be actively synthesized under conditions in which net protein and RNA syntheses are severely inhibited.

It is clear, then, that the induction of actinomycin production in *S. antibioticus* is accompanied by a marked decrease in the capacity of the cells to synthesize nucleic acid and protein. These biochemical features suggested to us that the *S. antibioticus* system might be a good model system in which to study differentiation. To further examine this possibility it seemed necessary to determine whether any morphological changes accompany the biochemical differentiation of *S. antibioticus* from nonproduction to production of actinomycin. The results of this study are detailed in this report.

MATERIALS AND METHODS

Streptomyces antibioticus, strain 3720, was employed throughout this investigation and was grown as described previously (*11*). 48-Hr NZ-amine cells were collected by centrifugation, washed with physiological saline, and transferred to galactose–glutamic acid medium (*10*). After selected durations of growth in this medium, 5-ml aliquots were removed to 25-ml flasks and 1 μCi of ^{3}H-L-valine (Schwarz/Mann, 17.2 Ci/mmole) was added. After a 30-minute incubation period at 28°C with shaking, mycelia were collected by centrifugation. The incubation medium was removed and assayed for radioactivity incorporated into actinomycin as described previously (*16*). Briefly, 3 ml of the incubation medium were extracted with 3 ml of ethyl acetate. The mycelial pellet was treated with 5 ml of 10% trichloroacetic acid (TCA) at 90° for 15 minutes, washed three times with 5 ml of cold 10% TCA and three times with 5 ml of ether:absolute ethanol (1:1 v/v). The resulting precipitate was heated to 90° in 2 ml of 0.5 *N* NaOH for 30 minutes (*7*). Aliquots of the ethyl acetate and hot alkali extracts were removed, and the radioactivity in these fractions was considered a measure of the incorporation of precursors into actino-

mycin and protein, respectively. Radioactivity was determined in a Beckman Model LS250 liquid scintillation system. Protein determinations were performed on the hot alkali extracts by the method of Lowry et al. (*20*), and the results are expressed as cpm/mg protein.

Aliquots for electron microscopic study were removed from NZ-amine and galactose–glutamic acid cultures after various periods of incubation. The cells were collected by centrifugation (1 500 *g*) and washed once with 0.1 M Tris-HCl buffer, pH 7.2, in 0.25 M sucrose. The incubation medium was in some cases centrifuged at 120 000 *g*, and the pellets were prepared as described below for electron microscopy.

Fixation of samples proceeded for 1 hr at room temperature in formaldehyde–glutaraldehyde solution diluted 1:1 with 0.1 M cacodylate buffer, pH 7.4 (*8*). The final concentrations were 2% formaldehyde and 2.5% glutaraldehyde. Cells were then washed with three changes of cacodylate buffer. Postfixation was carried out at room temperature for 2 hr in 1.3% osmium tetroxide buffered to pH 7.4 with s-collidine (*1*). After OsO_4 fixation, the cells were washed in cold sodium hydrogen maleate–NaOH buffer, 0.05 M, pH 5.2 (*4*), and were then placed in cold 1.0% uranyl acetate dissolved in this buffer (*9*). This *en bloc* staining was carried out for 2 hours at 4°C in the dark. Cells were then washed with three changes of the maleate buffer and were dehydrated in graded acetone series. Polymerization followed a four-step infiltration procedure. The embedding material used was Epon 812-Araldite 502 (*5*). Thin sections cut with a Porter-Blum MT2 ultramicrotome were stained with 2% aqueous uranyl acetate for 45 minutes or doubly stained with uranyl acetate and 2% potassium phosphotungstate, pH 6.8 (5 minutes). The sections were observed in a Philips EM 300 electron microscope.

Sonicated materials were prepared as follows. 48-Hr NZ-amine (time zero cells) and 36-hr galactose–glutamic acid cells were collected by centrifugation, washed once with 0.1 M Tris·HCl buffer, pH 7.2, in 0.25 M sucrose, then resuspended to 1/6 the original volume in this buffer. The cells were then sonicated for 2 minutes in 30-second bursts. This sonicated material was centrifuged at 5 000 *g* for 10 minutes. The pellets were discarded. The supernatants were centrifuged at 20 000 *g* for 20 minutes. These pellets were then prepared for electron microscopic observation as described above.

It should be noted that an alternate method of specimen preparation for electron microscopy was performed. However, this second procedure showed little or no preservation of the cytoplasmic membrane systems. This method consisted of fixation for 1 hr in phosphate buffered 2% glutaraldehyde, pH 7.2, followed by a 1.5 hr postfixation in 1% OsO_4, also phosphate buffered. Dehydration and infiltration proceeded as usual.

RESULTS

As a first step in this study, the time course of incorporation of ^{3}H-valine into actinomycin and protein was examined after inoculation of NZ-amine cells (not producing actinomycin) into galactose–glutamic acid medium. The results of this analysis are shown in Fig. 1. It can be seen that cellular protein synthesis decreases to nearly zero by 24 hr after inoculation. In contrast, the release of actinomycin into the culture medium reaches a maximum between 24 and 36 hr. Actinomycin production falls off somewhat after 48 hr, reaching a plateau which represents

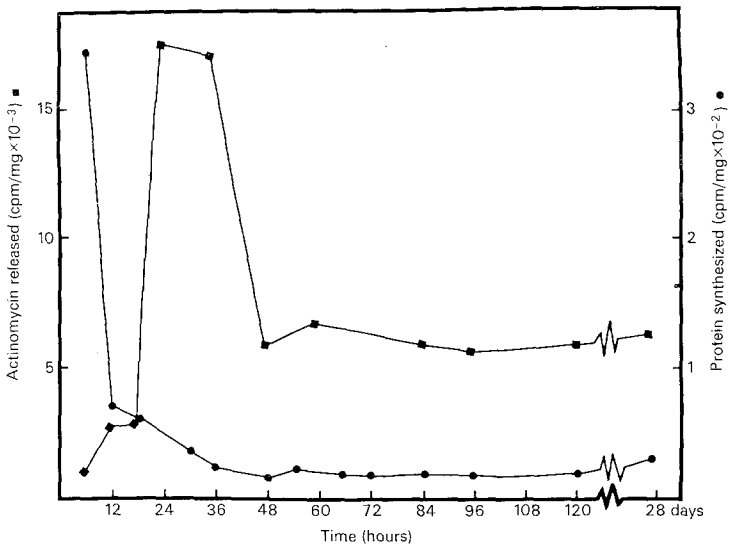


FIG. 1. Time course of incorporation of ^{3}H-valine into ethyl acetate-extractable and hot alkali-extractable products, representing actinomycin synthesis and release and protein synthesis, respectively. Results are expressed as cpm/mg protein.

about 30% of the maximum observed at 36 hr. This steady rate of actinomycin synthesis is maintained for as long as 28 days after inoculation into galactose–glutamic acid medium.

These findings confirm and extend those of Katz and Weissbach (*15*) and Katz et al. (*17*), and suggested the possibility that some morphological alterations might accompany these biochemical changes in *S. antibioticus* cells. To examine this possibility, cells obtained at varying times after inoculation into galactose–glutamic acid medium were examined by electron microscopy. 48-Hr NZ-amine cells were considered time zero cells with respect to actinomycin production, as this was the age of cells used for the transfer to the antibiotic producing (galactose–glutamic acid) medium. These cells have an electron dense, ribosome-filled cytoplasm (Figs. 2 and 3). The cell wall and membrane are also clearly visible, particularly in Figs. 2 and 3. In Fig. 2 are observed what appear to be membrane-bounded vesicles both inside and outside the cells. As actinomycin production is initiated and as protein synthesis ceases, the cytoplasmic electron density of many cells decreases and more of these vesicles and cytoplasmic membrane systems become evident. After 18 hr in glutamic acid medium, these changes are well underway (Figs. 4–6).

The clearing of the cytoplasm may be due to the loss of intracellular material, possibly involving the degradation of ribosomes. A progressive loss of cytoplasmic

material concomitant with an increase in visibility of the vesicular and membranous structures can be observed by comparing the three cell types in Fig. 5. Figs. 6 and 7 show adjacent cells which exhibit only small changes from the time zero appearance. However, in the 36 hr cell of Fig. 7, membranous structures are clearly visible.

Concomitant with the kinds of changes described above, certain cells appear to undergo a different type of cytoplasmic alteration (Fig. 8). By 72 hr, this class of cells has developed many vesicular electron transparent areas in their cytoplasm. It should be noted, however, that these cells also contain vesicles of the type observed in the previous figures (Fig. 8, arrow).

As the cells become still older (36 hr to 28 days) many different morphologies are observed. As early as 36 hr, a small percentage of the cells have completely electron transparent cytoplasm or cytoplasm in which vesicles and membrane systems can be quite clearly seen (Figs. 9 and 10). The variety of forms present at 7 days of incubation is displayed in Fig. 11. At this stage, when actinomycin production is continuing at the plateau level (Fig. 1), an increasing number of cells appear to have a light, rather amorphous cytoplasm often containing membrane-bounded structures (Figs. 11–13, 25). At the end of 28 days in glutamic acid medium, the majority of the cells have this appearance (Fig. 13), but in some cells this amorphous material has been completely lost, and only the cell wall remains (Fig. 13).

FIG. 2. 48 Hr NZ-amine-grown cell (time zero cells with respect to actinomycin production). Note vesicles in various regions of the cells and also vesicles of the same apparent size outside the cells (arrows). × 62 000.

FIG. 3. 48 Hr NZ-amine-grown cells. No vesicles are visible. × 62 000.

FIG. 4. 18 Hr galactose–glutamic acid-grown cells. Vesicles are clearly visible, surrounded by a dense cytoplasm. × 62 000.

FIG. 5. 18 Hr cells. Note the three different morphologies: (1) dense cytoplasm with several vesicles visible; (2) cytoplasmic clearing, revealing more vesicles and some internal membranes; (3) cells with extensive cytoplasmic membrane systems. × 62 000.

FIG. 6. 18 Hr cells. One can see all the stages noted in Fig. 5 in a single cell. Note also that these changes are not visible in an adjacent cell. × 48 000.

FIG. 7. 36 Hr cells. One cell is almost completely filled with small vesicles and also a larger vesicle (arrow). × 62 000.

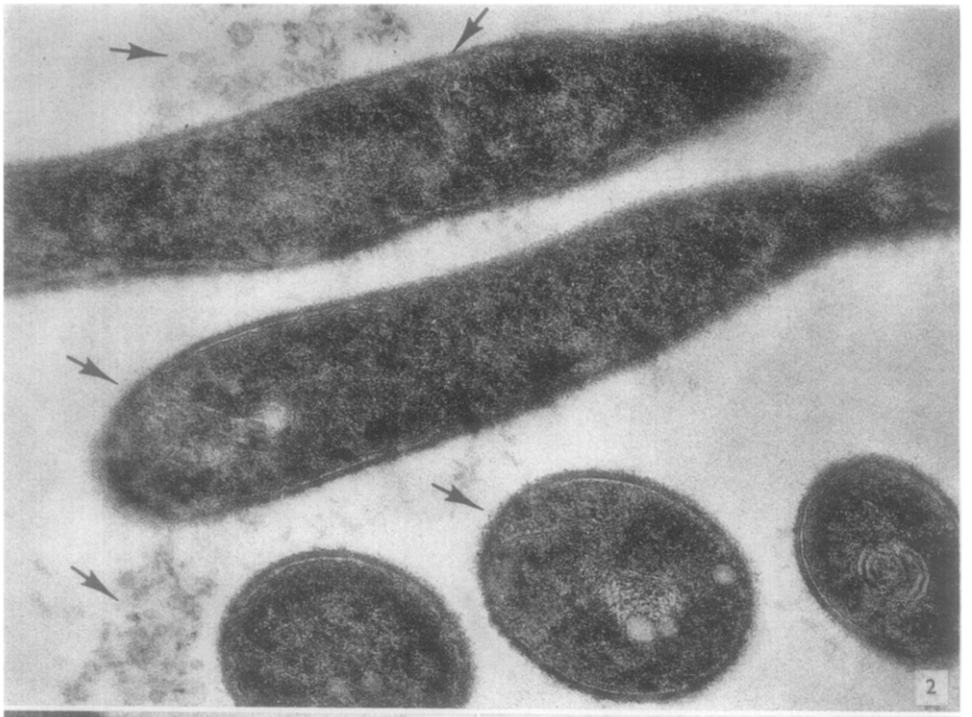
FIG. 8. 72 Hr cells. Loss of cytoplasm appears to be due to a localized "bleaching" of the cells, but in one area membrane-bounded vesicles are visible (arrow). × 48 000.

FIG. 9. 36 Hr cells. A few cells with this morphology are observed as early as 36 hr after inoculation into galactose–glutamic acid medium. It should be noted that the cells shown here are not of the general type found at 36 hr, but illustrate a cell type whose numbers increase as the cells become older. × 62 000.

FIG. 10. 7-Day cell. Membrane-enclosed vesicles and membrane systems are clearly seen. × 96 000.

FIG. 11. 7-Day cells. Some cells (a larger percentage than at 72 hr) have lost all cellular density except for a light, amorphous cytoplasmic substance (arrow). Other cells appear to represent earlier stages of this process. × 48 000.

FIG. 12. 7-Day cells. Notice the compartmentation of vesicles by what appears to be cell wall. There may then be a breakdown of the outer wall of the compartment and subsequent release of material into the surrounding medium (arrow). Vesicles can also be seen in the light, amorphous cytoplasm of the cell. × 62 000.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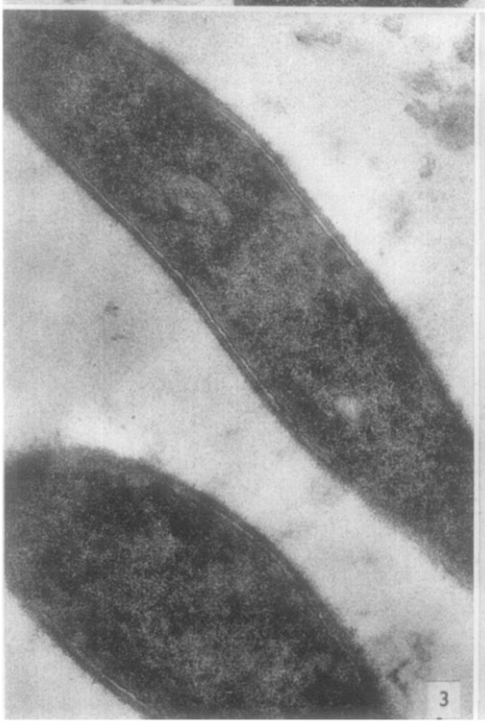
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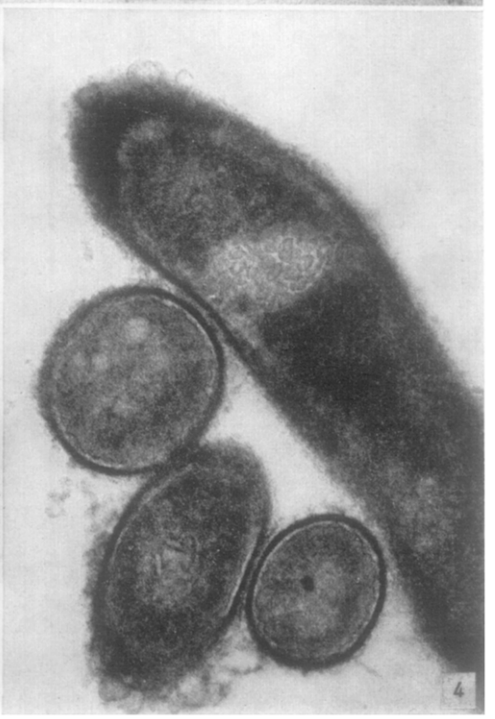
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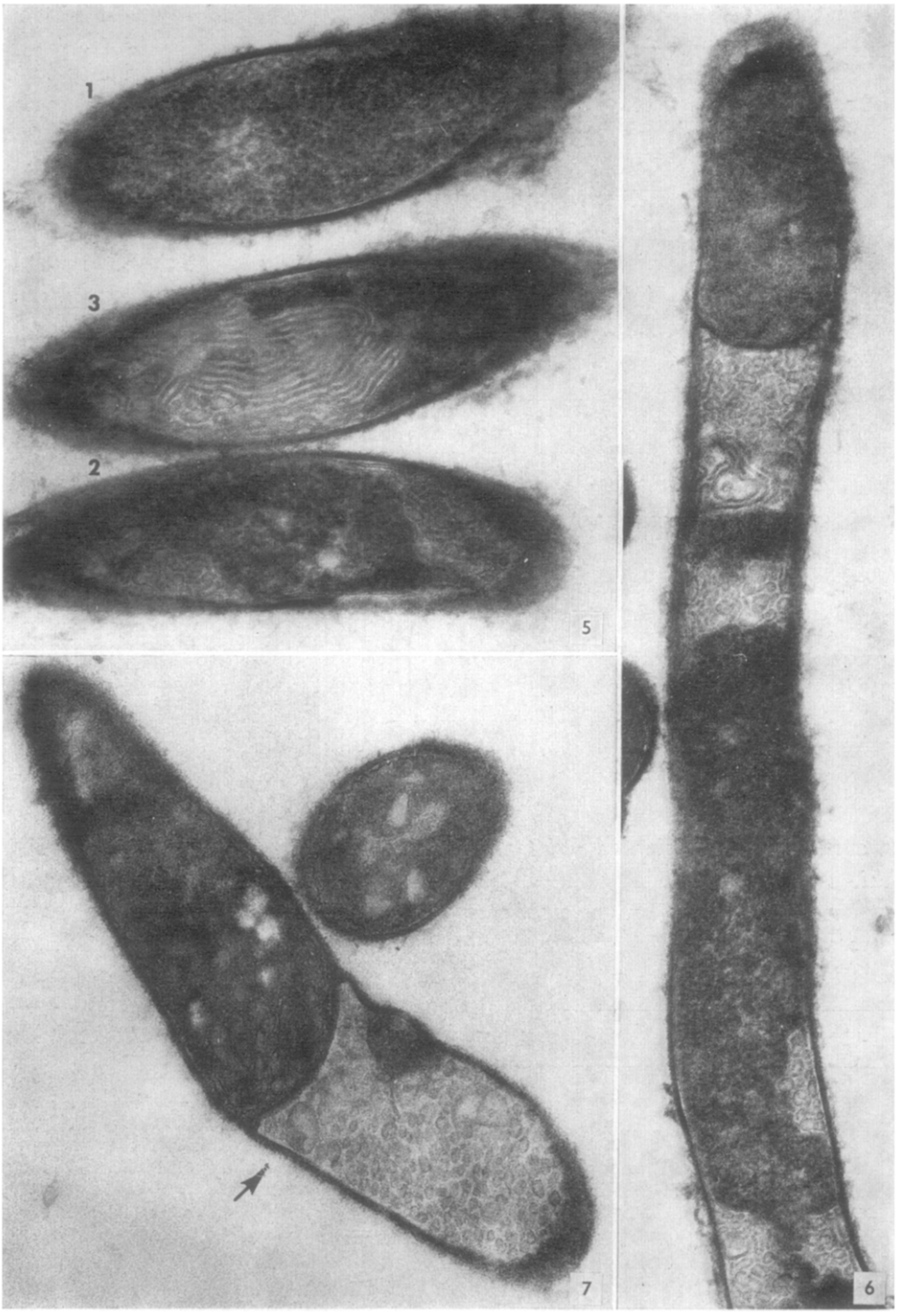
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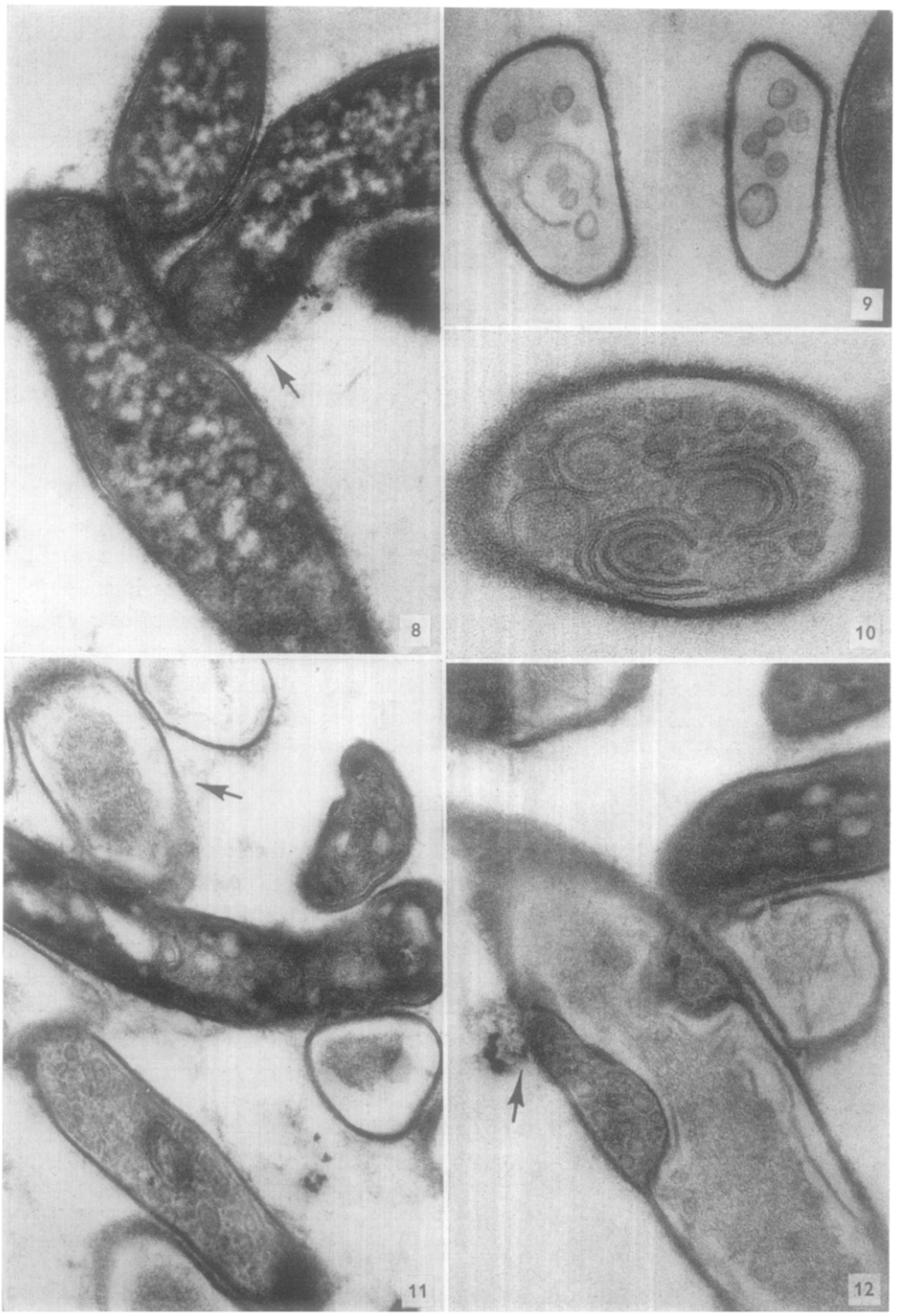
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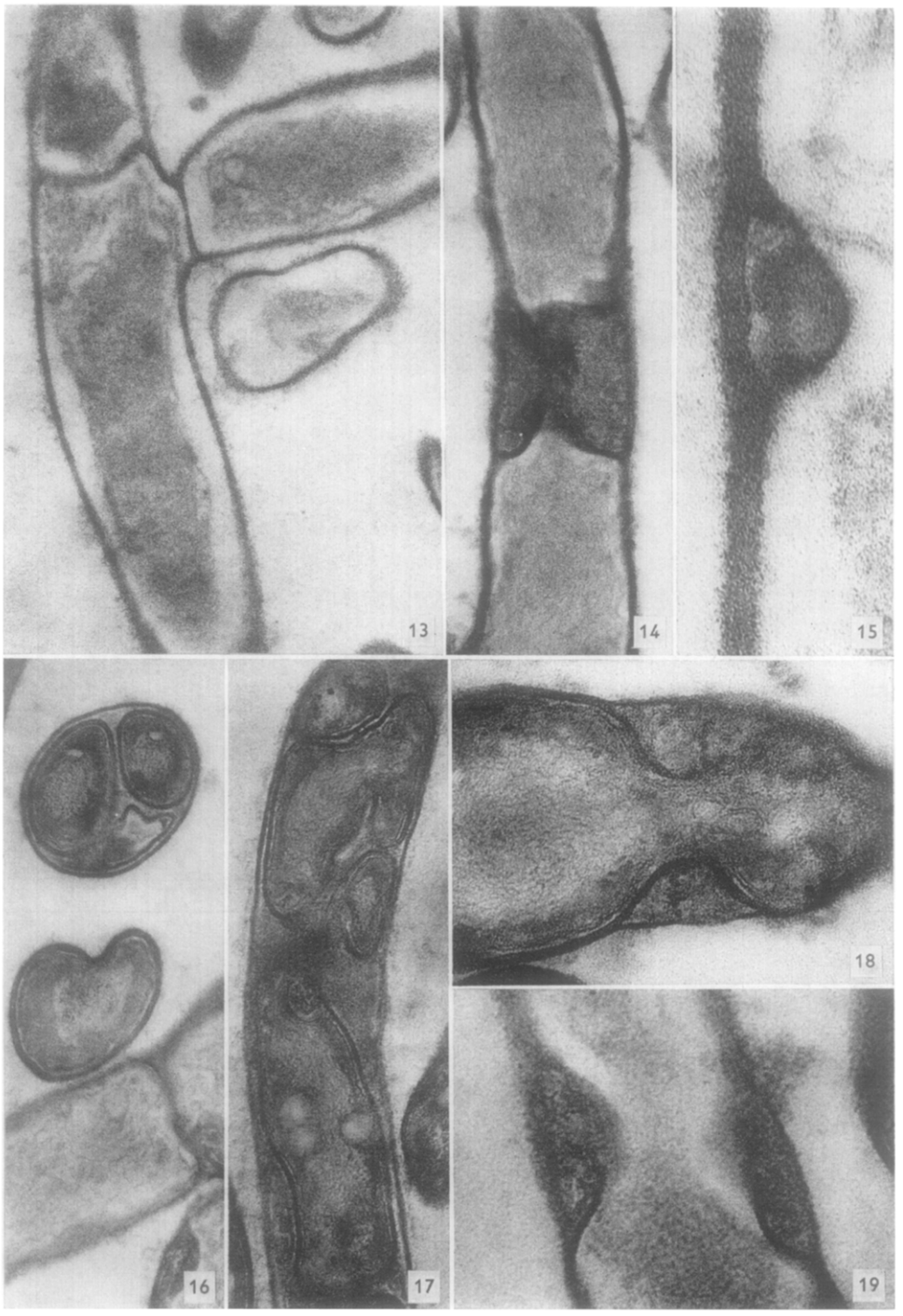
A most interesting phenomenon was observed in certain cells of age 7 days or older. In those cells, there appears to be an incorporation of the vesicular structures into compartments bounded by cell wall material (Figs. 12 and 14). These compartmented vesicles remain in close association with the cell surface (Fig. 15). In addition, in some cells, material having the same appearance as cell wall is found extensively, *within* the cell (Figs. 16–18), enclosing a relatively electron transparent, fibrous material. These structures will be discussed in greater detail below.

The rationale for the apparent compartmentation of the vesicular material by what appears to be the cell wall remains obscure, but a hypothesis can be formulated based on analogy with other bacterial systems. Similar vesicular formations enclosed in a "lock-chamber" formed by splitting of the cell wall have been previously described in *Bacillus licheniformis* 749/c (*25*). In that study, however, no cytoplasmic membrane systems were reported. The splitting of the cell wall of *B. licheniformis* 749/c was observed quite often in the area of the cell in which the vesicles were concentrated. Similarly, in *S. antibioticus*, the apparent breakdown of cell wall material can be observed in areas of compartmented cellular vesicles (Figs. 12, 19 and 20). These results suggest that the compartmented vesicles are released from the cells as a result of the degradation of the cell wall. Further evidence in support of this hypothesis was obtained by electron microscopic observation of extracellular vesicular material in a number of the micrographs (Fig. 2, for example) and by analysis of pellets obtained by centrifuging the incubation medium from 28-day cells at 120 000 *g*. Figs. 21 and 22 show that these pellets yielded exclusively vesicles and membrane fragments.

DISCUSSION

In summary, it has been confirmed that protein synthesis essentially ceases as actinomycin production is initiated in *S. antibioticus*. As the cells age in the actinomycin producing medium, they appear to lose cytoplasmic electron dense materials (including ribosomes), revealing numerous vesicles and extensive cytoplasmic membrane systems. This loss of density may occur by two different

FIG. 13. 28-Day galactose–glutamic acid grown cells. This is the predominant cell type found in 28-day cultures. Most of the remaining cells at this time appear to have lost the light, amorphous material. The cell wall, however, is still intact. × 62 000.
FIG. 14. 14-Day cell. Vesicles are visible within an enclosed compartment. × 62 000.
FIG. 15. 36-Hr cell. An early stage in the apparent splitting of the cell wall. Note compartmented material. × 257 000.
FIGS. 16–18. 7-Day cells. In these cells, cell wall-like structures appear in the interior of the cells, surrounding a less dense, fibrous material. Figs. 16 and 17, × 62 000; Fig. 18, × 96 000.
FIG. 19. 7-Day cells. The cell wall appears to have divided, forming an intracellular compartment. × 96 000.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mechanisms. With increasing age, the majority of the 7- to 28-day-old cells develop a light, amorphous cytoplasm, while the rest develop a cytoplasm which is completely devoid of electron-scattering material.

Several possibilities suggest themselves as explanations for these phenomena. First, it seems possible that the appearance of cytoplasmic vesicles and membranes is related to the ability of *S. antibioticus* cells to sporulate. It is known that the organism is a spore-former (*27*) and it has recently been suggested that antibiotic production is a necessary prelude to spore formation in sporulating bacteria (*24*). Indeed, it seems likely that the structures observed in Figs. 16–18 for 7-day-old cells represent the cellular nuclear region in the process of being encapsulated by a spore coat. However, only a small number of 7- to 28-day cells were observed with such an appearance. In addition, neither vesicles nor extensive membrane systems were observed in such cells. These findings suggest, then, that the appearance of these vesicles and membranes may be related to processes other than sporulation.

A second, more intriguing possibility, is that the appearance of the membranes and vesicles is directly related to the production of actinomycin in *S. antibioticus*. That such is the case is, at first glance, suggested by the fact that the appearance of these membrane systems coincides approximately with the time of appearance of actinomycin in the growth medium (about 36 hr after inoculation). Further, when the material from the 120 000 *g* centrifugation of 28-day medium is examined for its ability to incorporate ^{3}H-valine into ethyl acetate-extractable metabolites (actinomycin and its precursors), the vesicles and membrane fragments show considerable incorporating activity (unpublished results). That this incorporation is due to the presence of the membrane fragments is suggested by the finding that isolated vesicles do not seem to have incorporating ability.

Nevertheless, a third possibility, that the observed morphological changes are due to the normal aging of the cells, seems at this time to be the most tenable. When zero time (48-hr NZ-amine) cells are disrupted by sonication to release all

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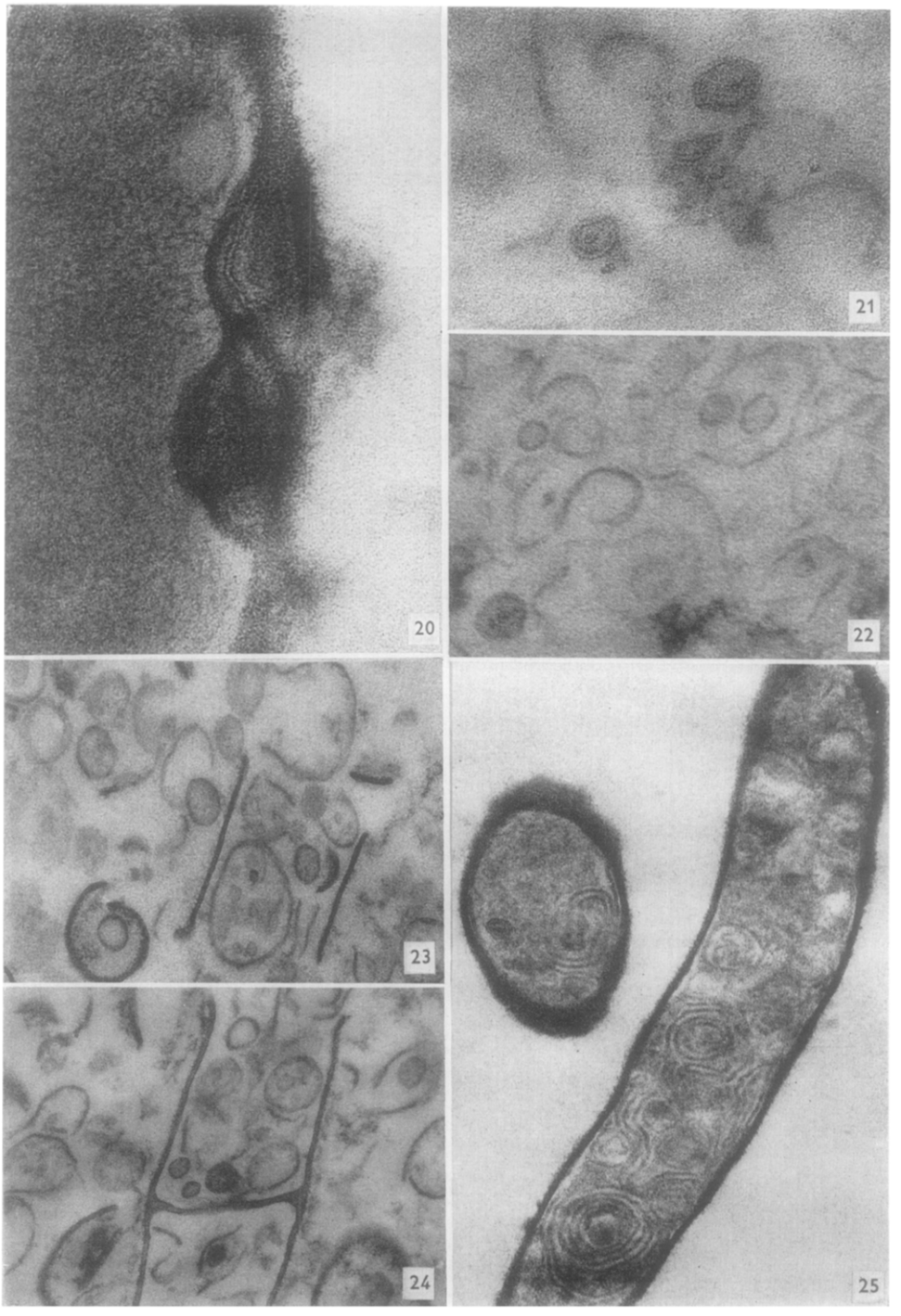
FIG. 20. 36-Hr cell. Cell wall material appears to be degraded exterior to the compartment. × 160 000.

FIGS. 21 and 22. 120 000 *g* pellet from 28-day galactose–glutamic acid medium. The cells were removed by centrifuging the culture at 1 500 *g*, and the incubation medium was filtered and recentrifuged at 120 000 *g*. Intact vesicles and membrane fragments can be seen. Fig. 21, × 154 000; Fig. 22, × 96 000.

FIG. 23. 20 000 *g* pellet of sonicated 36 hr galactose–glutamic acid-grown cells. A large number of vesicles of varying sizes and broken membranes can be seen. It is also quite easy to discern the cell wall and cell membranes. × 62 000.

FIG. 24. 20 000 *g* pellet of sonicated 48-hr NZ-amine (time zero) cells. The pattern is virtually identical to that seen in Fig. 15 for sonicated 36-hr galactose–glutamic acid-grown cells. × 59 000.

FIG. 25. 28-Day NZ-amine-grown cells. NZ-amine cells appear to undergo changes similar to those observed in galactose-glutamic acid-grown cells, although the changes may proceed somewhat more slowly in NZ-amine cultures. Extensive membrane systems are clearly visible. × 62 000.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soluble materials and the insoluble residue is compared with that obtained by subjecting 36 hr galactose–glutamic acid cells to the same procedure, no apparent differences are observed in the types of intracellular materials which are present (Figs. 23 and 24). Both preparations show vesicles of different sizes and various membrane and cell wall fragments. Indeed, vesicles and membranes can occasionally be seen in intact zero time cells (Fig. 2, for example). Further, aging NZ-amine cells show a loss of cytoplasmic electron density similar to that observed for galactose–glutamic acid grown cells, with a simultaneous appearance of membrane systems (Fig. 25). Most 28-day NZ-amine cells have the same light, amorphous cytoplasm observed in galactose–glutamic acid-grown cells. Thus it seems likely that these intracellular structures are always present in *S. antibioticus* to some extent, though in younger cells they may be obscured by other electron dense cytoplasmic material. This conclusion does not eliminate the possibility that there may be a specific proliferation of intracellular membranes in response to the signal(s) which turn on actinomycin production. However, this possibility remains to be thoroughly investigated.

Finally, as regards the apparent compartmentation and release of vesicles from older cells, there are known to be channels (or pores) in the cell walls of many bacteria and fungi (*18*, *19*, *21*, *22*, *26*), and such channels have been reported in *Bacillus licheniformis* (*25*). To date, we have not observed such channels in cells of *S. antibioticus*; rather, the release of vesicles into the cytoplasm appears to occur as a result of the breakdown of the outer wall of the compartment. Whether these vesicles contain substances related to actinomycin production is currently being investigated.

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