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# ON THE PHYSIOLOGICAL FUNCTIONS OF TEICHOIC ACIDS

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The choline-containing teichoic acids of pneumococci can be modified by biosynthetic replacement of the choline residues with certain structural analogues, such as ethanolamine (EA) or the N-monomethyl- (MEA) and N-dimethyl- (DEA) amino derivatives of ethanolamine. Cells containing such analogues in their teichoic acids develop pleiomorphic alterations in several physiological properties, which include resistance to detergent-induced lysis and inhibition of cell separation (chain formation). We report here the results of physiological studies on the mechanism of these two phenomena. Our results are summarized in the following: (a) Pneumococci grown on various amino alcohols produce cell walls of identical amino sugar and amino acid composition. (b) Both choline- and EA-containing teichoic acids seem to follow the same conservative pattern of segregation during growth and cell division. (c) Lysis sensitivity of pneumococci requires the juxtaposition of lysis-sensitive (choline-containing) cell walls and endogenous autolysin at the cell wall growth zone. (d) Upon readdition of choline to ethanolamine-containing cells, lysis sensitivity and catalytically active (C-type) autolysin reappear in the bacteria with the same kinetics. (e) The chains of EA-grown pneumococci contain fully compartmentalized cells and normal cross walls.

## INTRODUCTION

The unique localization of choline residues in the teichoic acid of pneumococci (1) and the nutritional requirement of these bacteria for an amino alcohol (2) have opened up the possibility of examining the physiological role of teichoic acids in this bacterium. An additional experimental handle for such studies was provided by the observations that the choline residues of teichoic acid can be biosynthetically replaced by certain analogues [e.g., ethanolamine (EA)] and that pneumococci growing on ethanolamine develop striking physiological changes in a whole series of surface-related properties, such as inability to undergo genetic transformation, defect in cell division, resistance to a large number of chemically unrelated bacteriolytic antibiotics, resistance to detergent-induced lysis, and so on (3). On the biochemical level, EA-grown bacteria are known to have at least two defects: EA-containing cell walls are completely resistant to the pneumococcal autolytic amidase (4), and EA-grown cells contain an abnormal, catalytically inactive autolytic enzyme (5).

The purpose of the experiments described in this communication was to try to establish the connection between these two (and possibly still other) biochemical defects and the physiological abnormalities of EA-grown pneumococci.

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## MATERIALS AND METHODS

Diplococcus pneumoniae strain R36A was used in all of the experiments. Several methods used in these experiments have been described in detail in previous communications; these methods include following bacterial growth by nephelometry (6), the composition of the chemically defined (Cd) growth medium (7), preparation of cell walls (4) and autolysin (4, 5), and determination of autolysin activity (4, 5). Ethanolamine- or choline-containing bacteria were grown for at least 10 generations in the medium with the appropriate amino alcohol before being used in an experiment.

Macromolecular (i.e., teichoic-acid linked) radioactivity from choline or ethanolamine label was determined by the following procedure. The isotope-labeled cells or lysates were chilled on ice; cold trichloroacetic acid (TCA) was added to a final concentration of 5–10%; after 10–15 min standing on ice, the precipitates were collected onto GFA filters and washed with 3 X 5 ml cold 5% TCA. The dried filters (100°C, 10 min) were counted in a toluene-base liquid scintillator using a Mark II spectrometer (Nuclear Chicago). In the preparation of specimens for electron microscopic examination a published procedure was used (8), with the exception that the mixture of methyl- and butyl-methacrylate was replaced by Epon. A Hitachi HU 11-C-1 electron microscope was used to 75 kV.

## RESULTS

As a first approach to understanding the mechanism of the physiological effects of amino alcohol replacement, we examined the possibility that pneumococci growing on choline analogs may produce cell walls of abnormal chemical composition or may have an anomalous mechanism for cell wall extension and segregation.

### The Chemical Composition of Cell Walls Produced by Pneumococci Grown on Different Amino Alcohols

Pneumococci growing in media supplemented with different amino alcohols (EA, MEA, or DEA) incorporate these molecules into the teichoic acids as structural choline analogues – that is, without a metabolic conversion to choline (9). Table I shows that such bacteria nevertheless produce cell walls of indistinguishable amino acid and amino sugar composition.

### The Mode of Segregation of Choline- and EA-Containing Teichoic Acids During Growth

It has been reported earlier that pneumococci labeled with $^3$H-choline and subsequently transferred to EA-containing medium exhibit a conservation of the choline label (10). Figures 1 and 2 show that this conservative mode of teichoic-acid segregation does not depend on the shift from one amino alcohol (choline) to the other (EA), but also holds true in experiments in which cells prelabeled with $^3$H-choline or $^3$H-EA are subsequently grown in the same (nonradioactive) amino alcohol. These experiments indicate that the replacement of choline residues with EA residues does not change the mode of enlargement and segregation of the pneumococcal cell wall.

## Detergent-Induced Lysis and the Nature of the Amino Alcohol Component of Teichoic Acid

In a series of experiments we examined the rate at which cellular lysis sensitivity or lysis resistance of pneumococci changed in response to shifts in the amino-alcohol composition of the cellular teichoic acids.

**TABLE I. Amino-Acid and Amino-Sugar Composition of Cell Walls Isolated from Pneumococci Grown on Different Amino Alcohols**

| | EA | MEA | DEA | Choline |
|---|---|---|---|---|
| Lysine | 1.0 | 1.0 | 1.0 | 1.0 |
| Glutamic acid | 1.08 | 1.08 | 1.13 | 1.09 |
| Alanine | 2.09 | 1.92 | 1.87 | 1.87 |
| Glucosamine | 0.60 | 0.60 | 0.64 | 0.65 |
| Muramic acid | 0.47 | 0.47 | 0.36 | 0.44 |
| Galactosamine | 0.80 | 0.93 | 1.10 | 1.08 |
| Glycine | 0.03 | 0.09 | 0.12 | 0.04 |
| Serine | 0.4 | 0.33 | 0.41 | 0.23 |
| Aspartic acid | 0.02 | 0.07 | 0.13 | 0.02 |
| Threonine | – | 0.03 | 0.04 | 0.01 |

Cell wall preparations were weighed into glass hydrolysis tubes and hydrolyzed with 6 N HCl, in vacuo, at 110°C for 16 hr. Analysis was done with a Durrum D 500 amino acid analyzer. Hexosamine values were corrected for decomposition (4). The table shows the molar ratios of components, normalized to the value of lysine, which was set at 1.0.

**Shift from ethanolamine to choline.** The rapid reappearance of detergent sensitivity upon readdition of choline to the growth medium of pneumococci grown on EA-containing medium has been reported earlier (3). Exposure of bacteria in EA-containing medium (40 μg/ml EA) to as little as 0.1 μg/ml choline for 2–3 min (about 8% of the culture's generation time) is sufficient to convert all the cells to lysis sensitivity, as evidenced by the complete clearing of such cultures upon the addition of deoxycholate (DOC) (250 μg/ml). Figure 3 shows that such an addition of choline results in the practically instantaneous cessation of EA-utilization by the bacteria, and Table II shows that the lysis sensitivity elicited by choline addition requires cellular metabolism (presumably for the biosynthesis and incorporation of choline-containing teichoic acids). Table II also shows the results of an experiment in which pneumococci growing on radioactive ethanolamine ($^{14}$C-EA) received a pulse of $^{3}$H-choline and were subsequently lysed by DOC. There was practically no release of the macromolecular EA label into soluble form, whereas over 90% of the teichoic-acid choline was released (i.e., became nonsedimentable at 10,000 × g in 20 min). These results indicate that under these conditions DOC-induced disintegration ("lysis") of the bacterial cells was not accompanied by the solubilization of the bulk of cell-wall material in the culture (represented by the EA-labeled wall fraction). An electron microscopic examination of such lysates confirmed this conclusion; the lysates contained mostly empty cell-wall fragments the size of which approached that of undisrupted coccal hemispheres (Fig. 4).

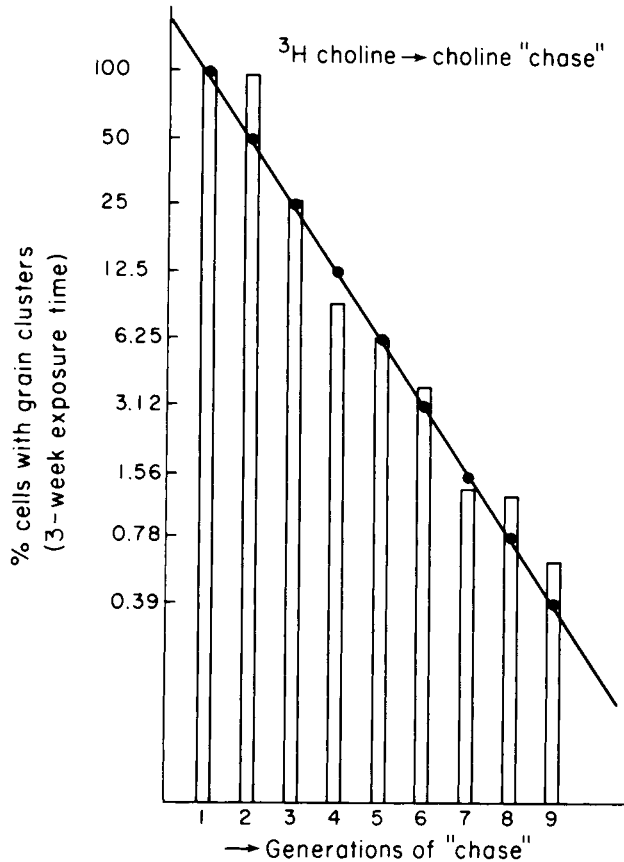


Fig. 1. The inheritance of $^3$H-choline-labeled teichoic acid. Pneumococci were grown overnight from a small inoculum ($2.5 \times 10^5$ cells/ml) in 10 ml growth medium containing radioactive choline (methyl-$^3$H-chloride; 7 μg/ml; specific activity, 0.7 mC/mg). At the cell concentration of $2.5 \times 10^7$ bacteria/ml, the culture was filtered (Millipore, 0.45 μ) and the cells were washed with 3–5 volumes of growth medium prewarmed to 37°C and containing nonradioactive choline. The washed bacteria were resuspended in prewarmed, choline-containing (nonradioactive) medium at a cell concentration of $2.5 \times 10^7$ cells/ml. Growth was followed with a nephelometer calibrated to allow estimation of the viable titer of the culture. The cell concentration of the exponentially growing culture was kept between 2.5 and $7.5 \times 10^7$ bacteria/ml by periodic dilutions with fresh, prewarmed growth medium. Samples were removed for autoradiography immediately after resuspension in the nonradioactive ("chase") medium and at each subsequent mass doubling time (generations of "chase"; see abscissa of figure). The samples were spread on glass microscope slides, air-dried at room temperature, and then exposed to a heating lamp 0.5 m away for 30 min to increase adhesion to the glass surface. After extensive washing in distilled water, the slides were dried, coated with nuclear emulsion (Ilford L-4), and kept in a $CO_2$-enriched atmosphere over Drierite at 4°C for exposure times of 2–3 weeks. Developed slides (Kodak D 19) were examined by a Zeiss microscope (planachromat 100/1.25 phase contrast oil immersion objective lens). The fraction of pneumococci containing grain clusters at the various sampling times is plotted as a function of generations of growth in the "chase" medium (vertical bars). The figure also shows the situation expected for the case of a single, centrally located zone of teichoic-acid incorporation followed by conservation of the radioactive label (solid circles connected by a straight line.

In addition to the autolysin resistance of their cell walls, EA-grown pneumococci are known to exhibit another biochemical defect. They contain an abnormal, enzymatically inactive autolysin that can be converted to the normal, catalytically active enzyme by exposure to choline-containing cell walls in vitro (5).

The rapid reappearance of sensitivity to DOC-induced lysis upon short exposure of EA-grown cells to choline prompted us to examine autolysin in such bacteria. Figure 5 shows that within 10 min after the addition of choline to EA-grown pneumococci, the low-molecular-weight (and inactive) E-form of autolysin (typical of EA-grown bacteria) is replaced by the high-molecular-weight, enzymatically active C-autolysin (typical of choline-grown pneumococci).

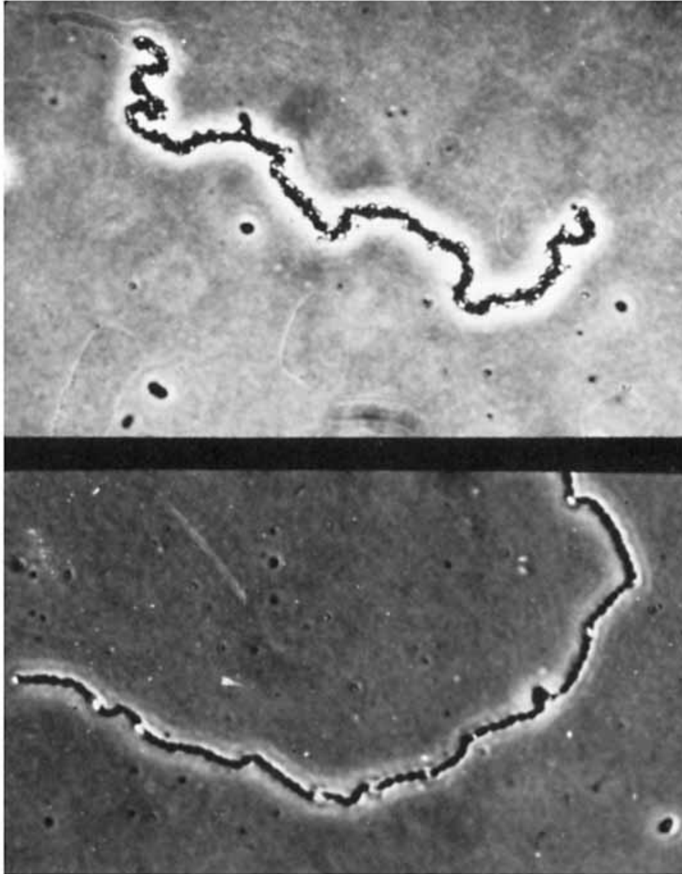

Fig. 2. The inheritance of $^3$H-ethanolamine-labeled teichoic acid. The design of this experiment was identical to that of the experiment illustrated in Fig. 1 (see legend to Fig. 1), except that the bacteria were prelabeled with radioactive ethanolamine (ethan-1-ol-2-amine-2$^3$H, Amersham-Searle Corp.; 40 $\mu$g and 1 $\mu$C per ml) and the "chase" medium contained nonradioactive ethanolamine (40 $\mu$g/ml) in place of choline. (Top) labeled pneumococci at the beginning (0 min) of growth in the chase medium. (Bottom) bacteria after several generations of growth in the chase medium.

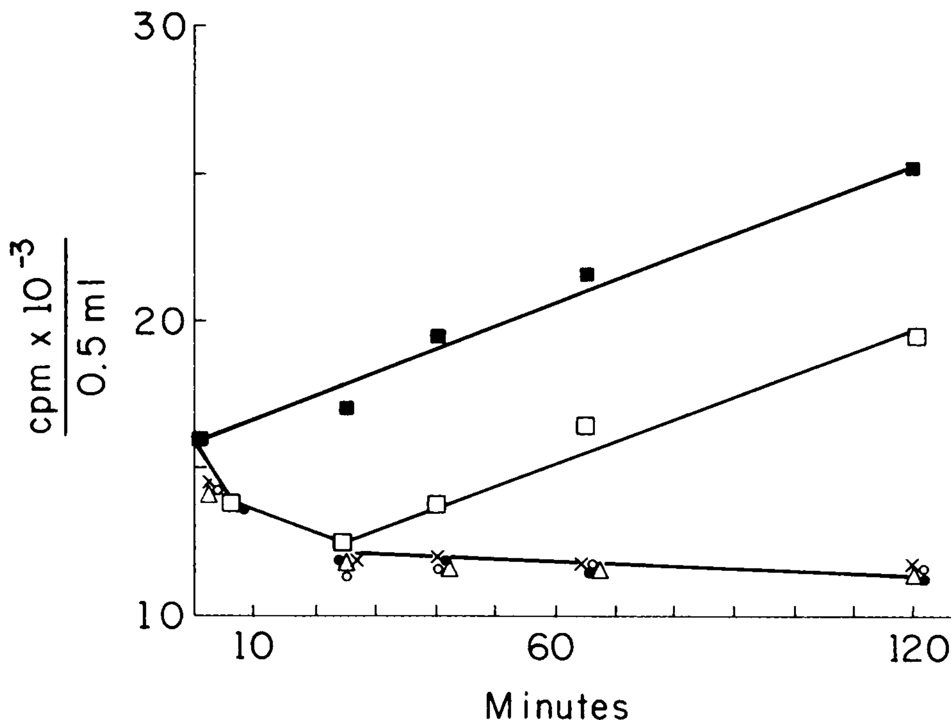


Fig. 3. Suppression of the cellular incorporation of $^{14}$C-EA by choline. Test tube cultures (10 ml) of pneumococci were grown in growth medium containing ethanolamine-1, 2-$^{14}$C (40 $\mu$g and 0.1 $\mu$C per ml). At time 0 (cell concentration; 5 × $10^7$ viable cells per ml) choline was added to some of the cultures at the following $\mu$g/ml concentrations: none (control, ■——■); 0.1 (□——□); 0.5 (△——△); 1.0(●——●);2.0 (○——○); 5.0 (x——x). Samples (0.5 ml) were removed at the times indicated on the abscissa; the bacteria were collected onto Millipore filters (0.45 $\mu$), and washed with 5 × 5 ml saline, and the cell-associated radioactivity was determined.

**Shift from choline to EA.** Figure 6 depicts the rate of development of resistance to DOC-induced lysis in a culture of pneumococci upon transferring the bacteria from choline- to EA-containing medium. Lysis resistance of the culture as a whole seems to develop in discrete steps and as a function of the number of cell generations in the EA medium. After one doubling of cell number in the EA medium the culture showed 100% lysis. Upon the second generation in EA, the culture showed about 50% lysis, upon the third generation the value has dropped to 25%.

**Double shift experiments: ethanolamine to choline to ethanolamine.** Addition of even a very small (0.08–0.1 $\mu$g/ml) complement of choline to the medium of EA-grown bacteria will result in the usual rapid appearance of lysis sensitivity. However, upon continued incubation the bacteria apparently deplete the choline and return to the utilization of EA in the growth medium (Fig. 3). Under such conditions lysis sensitivity of the culture is only temporary, and the culture as a whole reacquires lysis resistance within the time of a single cellular generation (Fig. 7).

**The conservation of lysis sensitivity and lysis resistance during cell division.** EA-grown pneumococci were labeled with radioactive EA and were then used to inoculate a

**TABLE II. Effect of Choline Incorporation on the Lysis Sensitivity of EA-Grown Pneumococci**

| Experimental condition | Detergent induced lysis (%) | Radioactivity released by DOC (%) | |
|---|---|---|---|
| | | $^{14}C$ | $^{3}H$ |
| EA-grown cells + choline (20 min) | 100 | – | – |
| EA-grown cells + choline | | | |
| + Actinomycin D (0.1 μg/ml) | 0 | – | – |
| + Rifamycin (0.1 μg/ml) | 0 | – | – |
| + Chloramphenicol (50 μg/ml) | 0 | – | – |
| – glucose and sucrose | 0 | – | – |
| EA-$^{14}C$-grown cells labeled with $^{3}H$-choline for 5 min | 100 | 2–5 | 85–95 |

A culture of pneumococci growing in EA-containing medium was distributed into a series of test tubes containing different metabolic inhibitors. An additional portion of the culture was filtered (Millipore) and resuspended in medium lacking amino alcohol and energy source (glucose and sucrose). After 2 min of incubation each test tube culture received choline (5 μg/ml). Sensitivity of the cultures to DOC-induced lysis was tested (for method see legend to Fig. 6) after 20 min of incubation with choline at 37°C.

In a separate experiment, also illustrated in this table, pneumococci growing in ethanolamine-1,2-$^{14}C$-containing medium (40 μg 0.1 μC per ml) were exposed to choline-methyl-$^{3}H$ (5 μg and 0.1 μC per ml) for 5 min. The bacteria were washed with medium on a Millipore filter, resuspended in saline containing 0.05 M $K_2HPO_4$, and lysed by DOC (250 μg/ml); pancreatic DNase (10 μg/ml) was added to decrease the viscosity. The lysates were centrifuged (10,000 × g, 20 min) and the distribution of $^{14}C$ and $^{3}H$ radioactivity between supernatant and pellet was determined (see Materials and Methods).

choline-containing medium in which they were subsequently grown for four generations. Upon DOC lysis of such cultures it was found that the EA-$^{14}C$ label of the inoculum cells (representing less than 6% of the total population) was quantitatively retained in a lysis-resistant form (i.e., remained sedimentable at low centrifugal fields) (Table III). Identical findings were observed in the symmetrical reverse experiments, in which $^{3}H$-choline-labeled pneumococci were inoculated into EA medium and were allowed to grow for five generations. Such cells were found to release quantitatively their isotope content into the supernatant when challenged with DOC.)

**The nature of the EA-induced chain formation.** Pneumococci growing on EA-containing media cannot separate at the end of cell division and form chains (3). Nevertheless, neighboring cells within the chains seem to be normally compartmentalized, since exposure of the EA-grown chains to mechanical shear results in a dramatic increase in the viable titre. For instance, 30 sec stirring in a Virtis homogenizer (at maximum speed setting) increased the viable counts of an EA-grown culture from $6 \times 10^6$ cells/ml to $5 \times 10^8$ to $1 \times 10^9$ cells/ml. Furthermore, electron microscopy of EA cells showed that neighboring cells in a chain were separated by cross walls of normal dimensions and normal depth of penetration. Frequently, cells within the chain seem to be held together by thin filamentous material that seems to be an extension of the polar cell walls (Fig. 8).

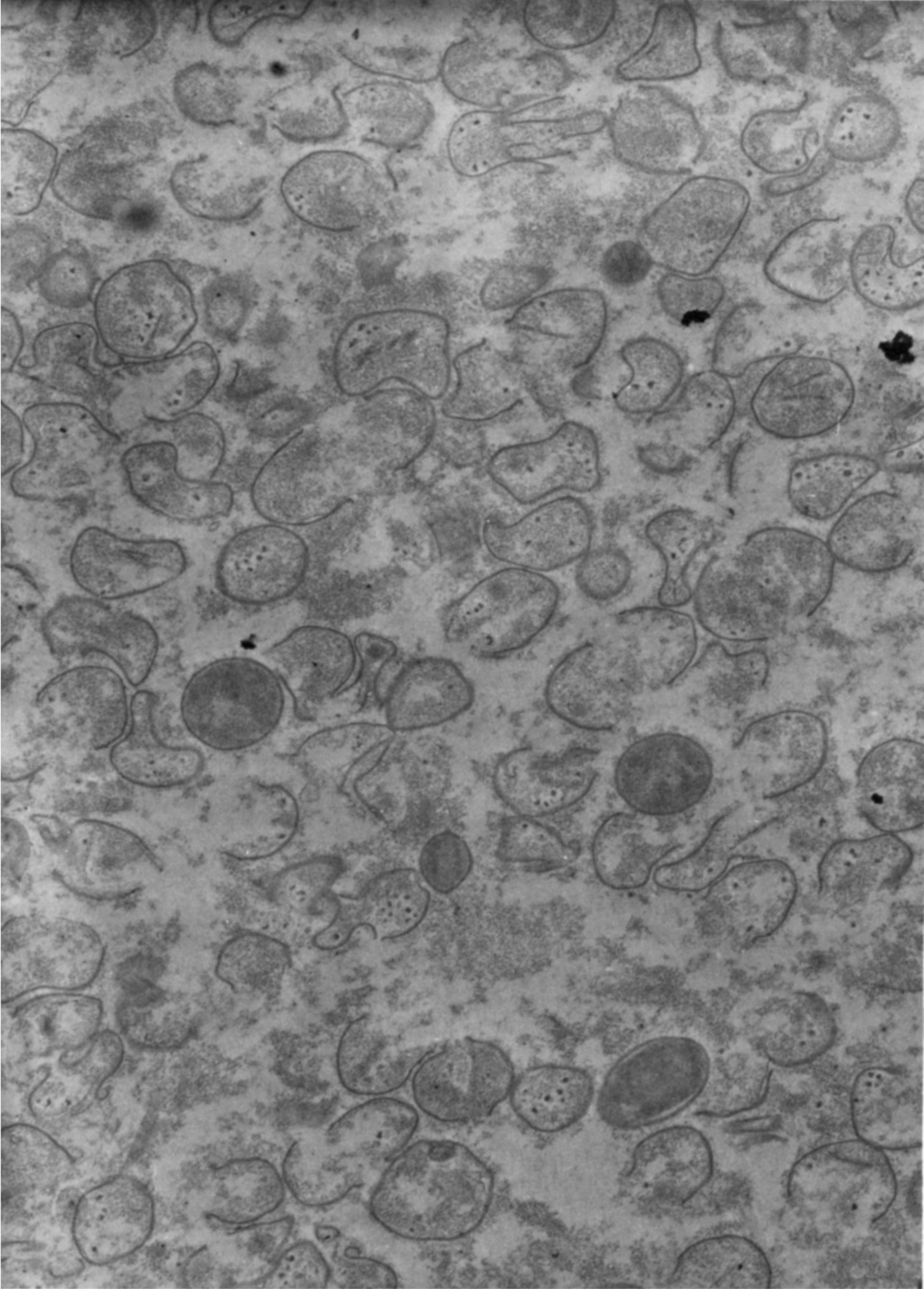

Fig. 4. Hemispherical cell wall fragments in the DOC lysate of choline-pulsed EA-grown bacteria. A pneumococcal culture (100 ml) was grown in EA-containing growth medium at 37°C. At the cell concentration of $3 \times 10^7$ bacteria/ml, choline (5 μg/ml) was added, and after 5 min of further incubation the culture was lysed by the addition of deoxycholate (DOC, 250 μg/ml). The lysate was briefly (15 min) treated with pancreatic DNase (10 μg/ml) and was filtered through a Millipore membrane (0.45 μ, 45 mm diameter). The particulate matter was eluted from the filter with 2 ml of potassium phosphate buffer (0.1 M; pH 6.6), fixed with 2.5% glutaraldehyde at room temperature for 30 min, and processed for thin sectioning and electron microscopy. (Magnification × 12,000).

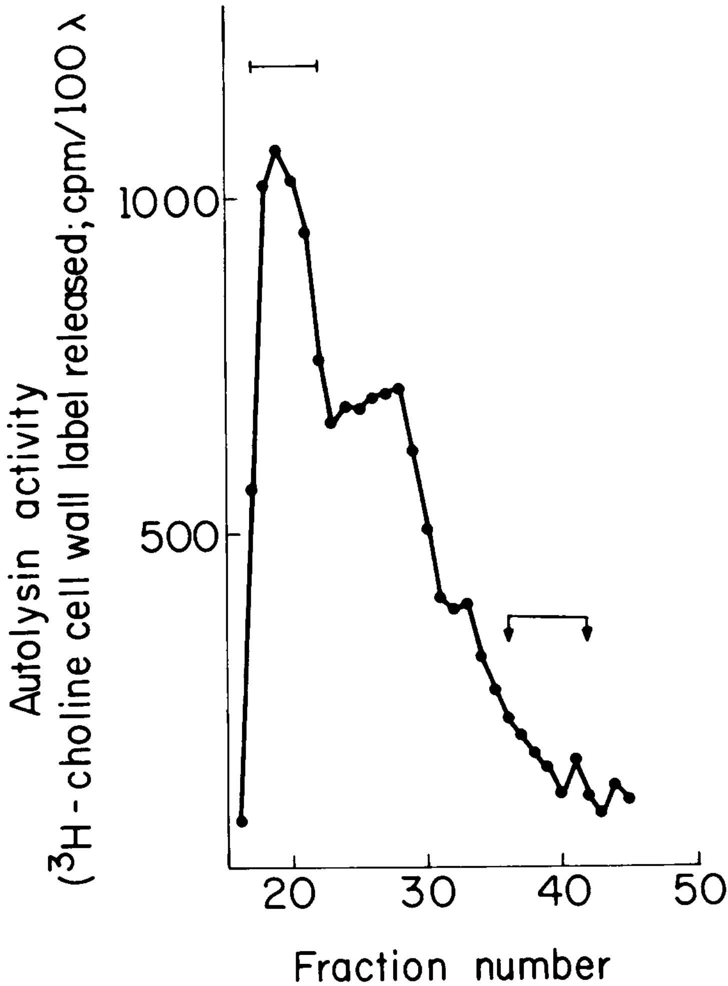


Fig. 5. The appearance of C-type autolysin in extracts of choline-pulsed EA-grown bacteria. A pneumococcal culture (100 ml) growing in EA-containing medium at 37°C received choline (5 μg/ml) at a cell concentration of $3 \times 10^7$ bacteria/ml. After 10 min of incubation at 37°C, the culture was chilled and centrifuged (7 min at 5,000 × g) and the cells resuspended in 2 ml of saline containing 0.05 M $K_2HPO_4$ (SP) and 5 μg/ml DNase. The suspension of pneumoccci was lysed by 10 min of incubation with DOC (250 μg/ml at 37°C. The lysate was centrifuged at 10,000 × g for 20 min, and the supernatant was passed through an agarose 5A column (equilibrated with SP) using SP as eluent; 0.5 ml fractions were collected. a 100 μl portion of each fraction was assayed for autolysin activity (4, 5). Horizontal bar shows the elution of blue dextran (MW $4 \times 10^6$); arrows indicate the position of E-type autolysin (5) in the elution profile.

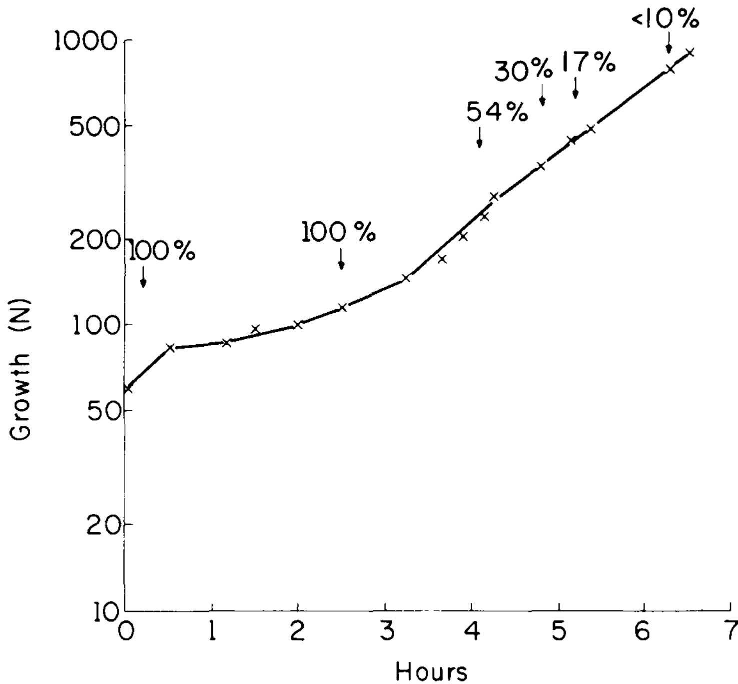


Fig. 6. Stepwise development of lysis resistance in pneumococci transferred from choline- to EA-containing medium. A culture of pneumococci (10 ml) exponentially growing in choline-containing medium at 37°C was filtered on a Millipore filter (0.45 $\mu$); the bacteria were resuspended in EA-containing growth medium (prewarmed to 37°C) at a cell concentration of $1.5 \times 10^7$ cells/ml, and the culture was allowed to grow at 37°C. Growth was monitored by a Coleman nephelometer. Sensitivity of the bacteria to DOC-induced lysis was tested periodically by pipetting 0.5 ml portions of the culture into test tubes containing 0.5 ml potassium phosphate buffer (0.1 M; pH 8); after recording the initial light scattering value of the suspension (in the Coleman nephelometer), 50 $\mu l$ of a 5% DOC solution was added, the suspension incubated at 37°C for 30 min (in order to induce lysis of the sensitive portion of the population), and the light scattering value of the suspension recorded again. Lysis sensitivity was determined at the times indicated by the arrows. The percentage values represent the degree of lysis sensitivity expressed as the fraction of initial light scattering value lost upon incubation with DOC. The curve in the figure depicts the growth of the culture after transfer (at 0 min) to the EA-containing medium.

## DISCUSSION

Attempts described in this paper to find major differences between the chemical compositions of the cell walls of choline-grown and choline-analogue-grown pneumococci yielded negative results. Similarly, replacement of choline residues by EA did not change the mode of cell-wall growth and segregation in the bacteria. Thus, the dramatic physiological changes observable in EA-grown bacteria (particularly the resistance of such bacteria to lysis) must be brought about by a more subtle biochemical mechanism.

The results of the amino-alcohol shift experiments described in this paper allow one to reconstruct the steps by which pneumococci recover their sensitivity to DOC-induced

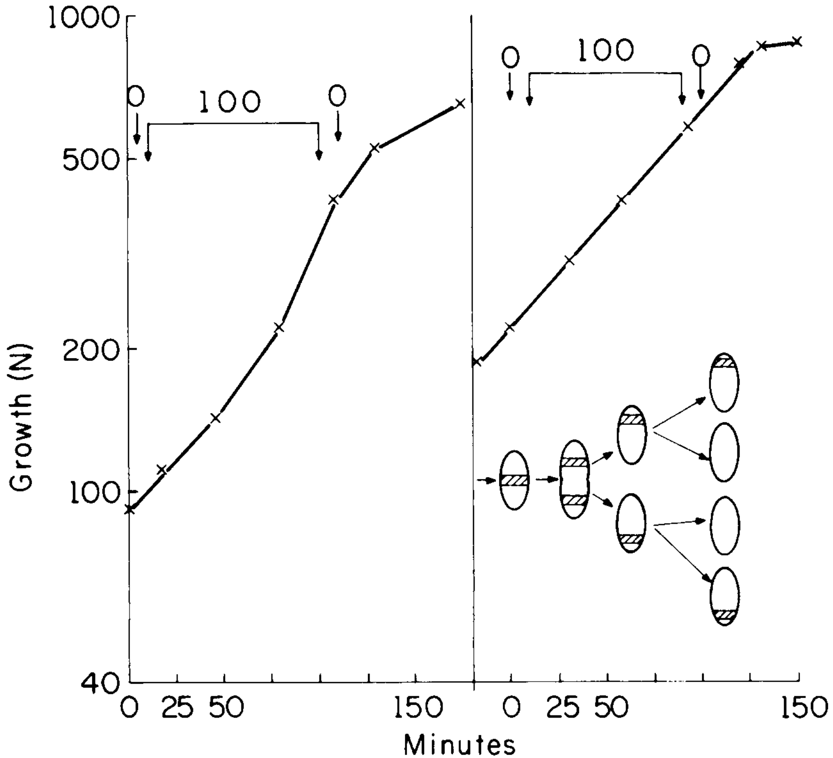


Fig. 7. Transient lysis sensitivity in EA-grown cultures in response to a very small pulse of choline. The figure illustrates two separate (and identically designed) experiments (see left and right halves of the figure). In both experiments, a 10 ml culture of pneumococci growing at 37°C in EA-containing medium (40 $\mu$g EA per ml) received a "minipulse" (0.08 $\mu$g/ml) of choline at the 0 min time of the experiments. The growth of the cultures was followed by nephelometry (solid lines). Sensitivity to DOC-induced lysis was determined at the times indicated by the downward pointing arrows (see method described in the legend to Fig. 6). The numbers above the arrows indicate the degree of lysis sensitivity (0 = complete resistance; 100 = complete lysis). The sketch in the right half of the figure illustrates in a schematic way the expected movement of the choline-containing zones of the cell envelope during the experiment.

lysis upon the addition of choline to EA-grown cells. After the addition of choline to the growth medium, pneumococci immediately shift to choline utilization and start the biosynthesis of phosphorylated choline compounds that appear to be metabolic precursors of the macromolecular (teichoic-acid linked) choline (9). The mechanism of the shift to choline utilization is not known at the present time. Next, the nascent, choline-containing teichoic acid units become attached to units of peptidoglycan synthesized at the same time (11) and these complexes incorporate into the cell wall at the equatorially located cell-wall growth zone (12). Metabolic inhibitors (Table I) possibly interfere with this step of incorporation into the cell surface.

The appearance of choline-containing cell-wall units has two important biochemical consequences: (1) the equatorial area of the pneumococcal cell wall becomes autolysin sensitive, and (2) catalytically active, C-type autolysin reappears in the cells (either through

**TABLE III. Retention of Ethanolamine-$^{14}$C-Labeled Cell-Wall Teichoic Acids in a Lysis-Resistant Form During Growth in Choline-Containing Medium**

| | Cell-associated, TCA-insoluble radioactivity (cpm/ml) | |
|---|---|---|
| | Before DOC treatment | After DOC treatment |
| Bacterial culture at end of labeling period (EA-$^{14}$C) | 35,060 | – |
| EA-$^{14}$C-prelabeled bacteria, after 5 generations of growth in choline medium | 35,200* | 38,200* |

*Counting rates were corrected for the 20-fold dilution of the EA-$^{14}$C-labeled inoculum into the choline medium.

A 10 ml culture of pneumococci was labeled by growing in Cd medium containing EA-1,2-$^{14}$C (0.6 $\mu$C and 38.9 $\mu$g per ml) for 5.5 generations. At the end of the labeling period (cell concentration, $1.47 \times 10^8$ cells/ml), the cells were removed from the radioactive medium by filtration on a Millipore filter (0.45 $\mu$) and washed with $5 \times 5$ ml of medium containing 100 $\mu$g/ml of nonradioactive EA. After resuspension (at the same cell concentration) in fresh medium free of amino alcohol, 100 $\mu l$ aliquots of the suspension were used to determine total and macromolecular (i.e., cold-TCA precipitable) radioactivity in the culture. Another portion of the washed bacteria (0.5 ml) was diluted into 9.5 ml of prewarmed (37°C) medium containing choline. After growth in this medium at 37°C for 5 generations, samples were prepared for the determination of total and lysis-resistant EA-$^{14}$C label retained by the cells. 0.9 ml portions of the culture were pipetted into two centrifuge tubes, each containing 0.1 ml of 1 M potassium phosphate buffer (pH 8); to one of the centrifuge tubes 50 $\mu$l of DOC (5% solution) was added. The other tube served as control. Both tubes were incubated at 37°C for 30 min and centrifuged at 15,000 $\times$ g for 15 min. The supernatants (containing less than 10% of the total radioactivity) were discarded; the residues were resuspended in 1 ml 0.15 M NaCl solution, 100 $\mu l$ portions were precipitated with cold 10% TCA, and precipitates were collected on GFA filters, dried, and counted in a scintillation spectrometer.

de novo synthesis or by in vivo "conversion" of the inactive E-type autolysin of EA cells to the active C-form). Upon addition of detergent (DOC), the C-type autolysin molecules gain "access" to the cell wall (possibly due to the partial dissolution of the plasma membrane) and digest the lysis-sensitive (choline-containing) and equatorially located ribbon of cell wall. The result is the "cracking" of the cell wall into two, mainly EA-containing hemispheres, followed by osmotic disruption of the emerging protoplast and escape of the cells' contents into the medium ("clearing").

Assuming that all the cells in the culture are growing (i.e., are incorporating teichoic acids) and that the degree of "clearing" in the culture is directly related to the number of disintegrating bacteria, the stepwise acquisition of lysis resistance during the shift from choline to EA medium is fully compatible with the mode of cell wall growth and segregation in this microorganism (10, 11). The rate of increase of DOC resistance simply reflects the rate of appearance of progeny bacteria that did not inherit the "old" (conserved), choline-containing surface segments of the parental bacteria.

In an apparent contradiction to this explanation, it was found that EA cells "pulsed" with a very small complement of choline develop a maximal DOC sensitivity for a brief

period only and return to mass DOC resistance within 1–1.5 generation times. If the presence of even a minimum complement of cholinated teichoic acid would make pneumococci DOC sensitive, one would expect a stepwise return of lysis resistance – that is, 50% resistance appearing about two generations after the short-lived lysis-sensitive phase. Instead, mass lysis resistance returns earlier, at a time when most of the cells in the culture must still be carrying choline-containing (lysis-sensitive) "bands" of cell-wall material (10). These results suggest that the active (C-type) autolysin molecules are localized at the cellular growth zone and that in the presence of detergent the breakdown of plasma membrane allows most of these enzymes to escape to the medium before they can encounter the thin, autolysin-sensitive bands of the cell wall, which are no longer in the direct vicinity of the growth zone. It seems, therefore, that cellular lysis requires spacial juxtaposition of endogenous autolysin and sensitive substrate at the wall growth zone.

The lysis-sensitive and lysis-resistant phenotypes remain fixed, apparently for indefinite periods, in all cells inheriting the cell-wall hemispheres constructed with the appropriate (choline- or EA-containing) teichoic acid. Thus, bacteria carrying cholinated cell-wall hemispheres will lyse upon exposure to detergent even after many generations of growth in EA-containing medium – that is, even in a situation in which the overwhelming majority of the population is made up of lysis-resistant bacteria. This type of pseudogenetic stability in a phenotypic trait is of course a direct consequence of the unique, conservative mode of inheritance of cell-wall structure in this bacterium.

The chain formation of EA-grown pneumococci suggests that a late or terminal step in the separation of daughter cells during cell division may involve the action of autolysin on the endogenous cell-wall substrate. In contrast to the quick return of lysis sensitivity the breaking up of chains of EA-grown bacteria seems to occur more slowly (within 1.5–2 generation times after choline addition) and gradually (3). The reason for this delayed recovery from the cell-divisional defect is not clear. Possibly, physical separation of daughter cells might occur only at septa which were initiated after the choline addition – that is, which contained exclusively choline-teichoic acids. Since the bacterial cultures used in these experiments were not synchronous in cell division, the incorporation of the majority of short choline pulses must have occurred at growth zones that had been initiated prior to the addition of choline and thus contained EA-teichoic acids also.

The results described in this paper strongly suggest that the defective autolytic system of EA-grown pneumococci forms the biochemical basis of several of the unique physiological features of these bacteria – namely, resistance to lytic conditions (e.g., stationary phase, detergents, antibiotics), chain formation, and possibly even the resistance shown by these bacteria against the newly isolated pneumococcal bacteriophage Dp-1 (13). The absolute requirement of the pneumococcal autolysin (an N-acetylmuramyl-L-alanine amidase) for choline residues in the cell-wall teichoic acid indicates that one of the main physiological functions of teichoic acid in this bacterium is in the control of the catalytic activity of the autolytic enzyme.

## ACKNOWLEDGMENTS

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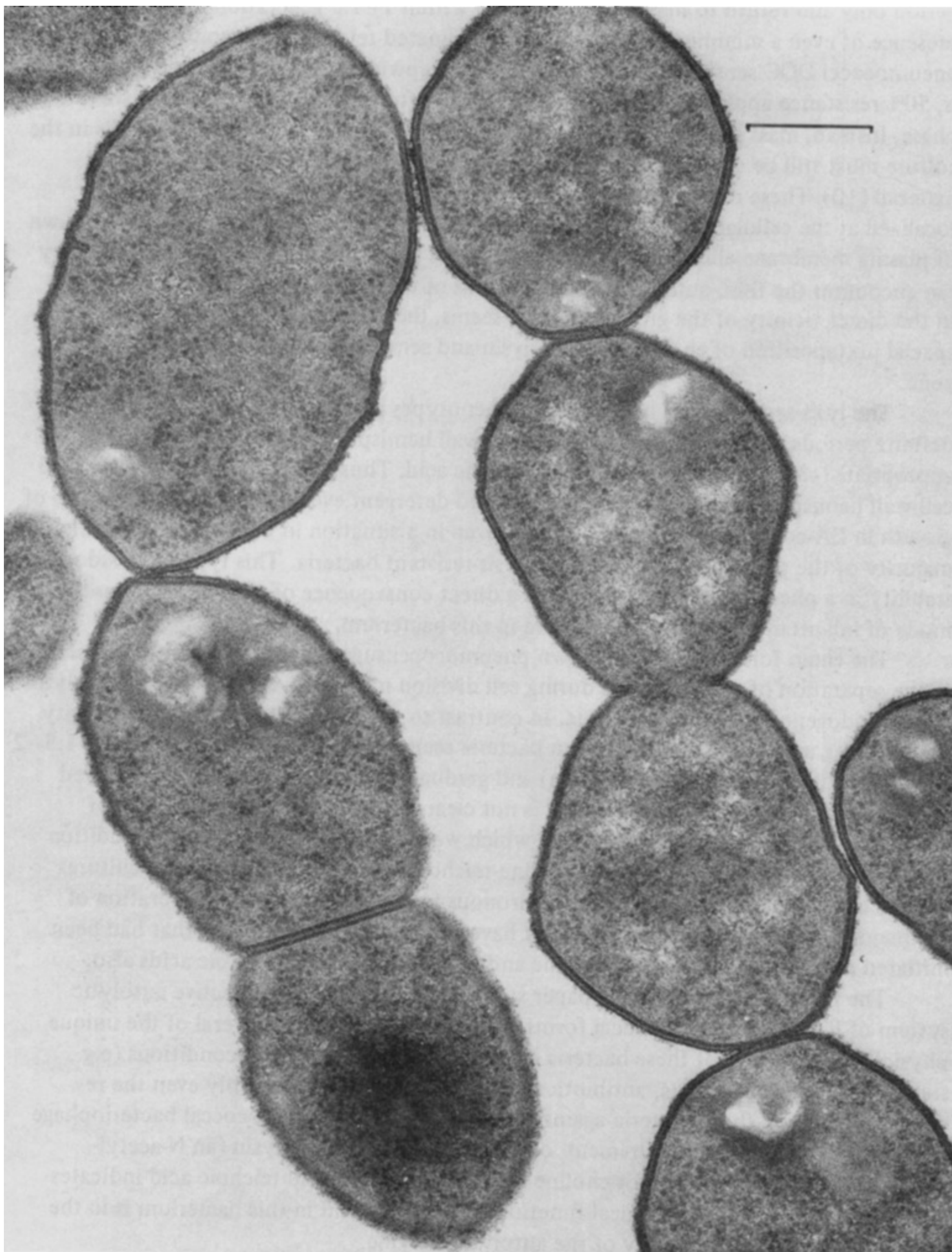
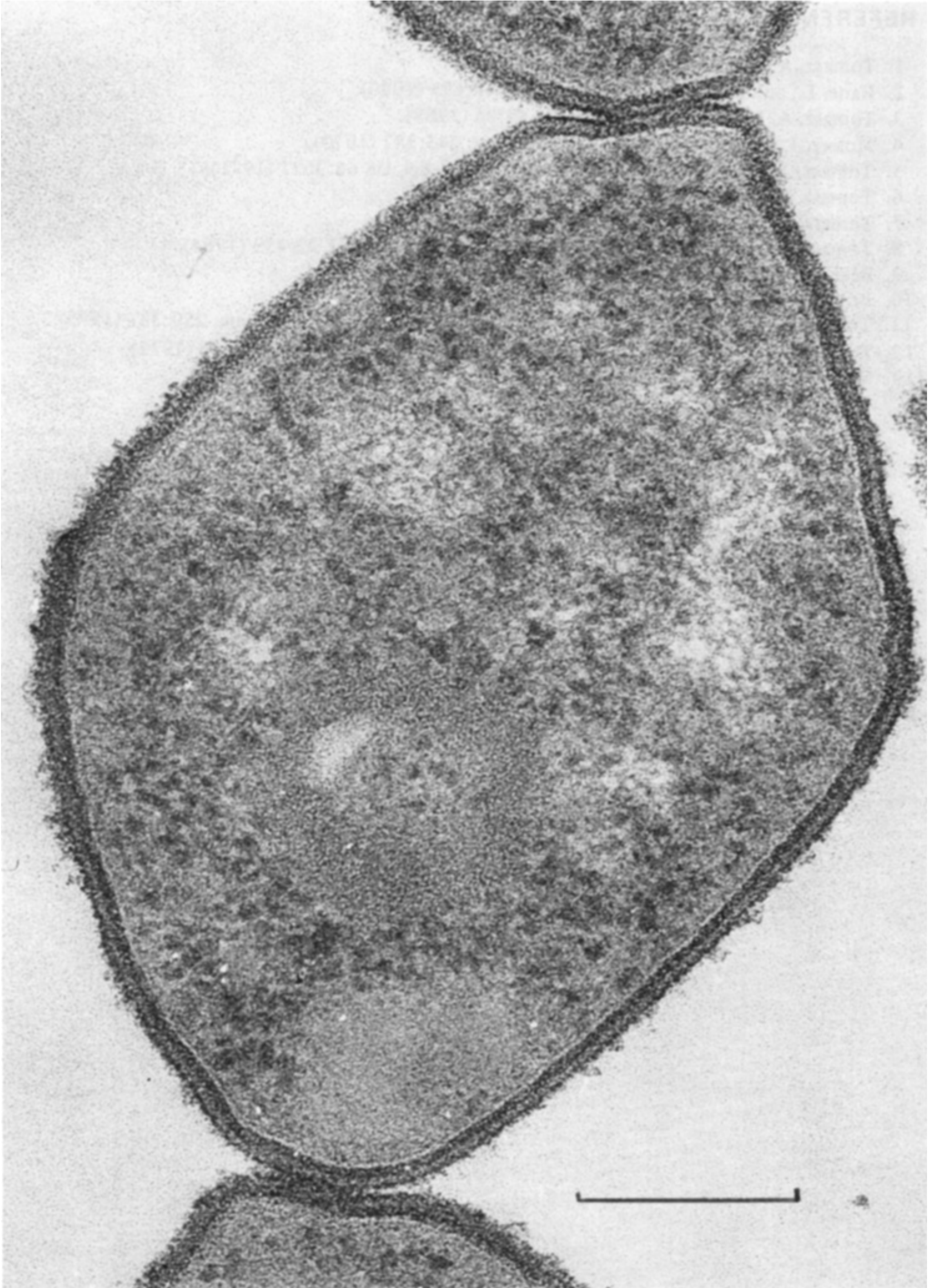


Fig. 8. Cross walls of chain-forming EA-grown pneumococci. Bacteria grown in the EA-containing medium were centrifuged; the pellet was resuspended in 0.1 M potassium phosphate buffer (pH 6.6) and fixed with 2.5% glutaraldehyde (at 0°C for 30 min). After postfixation with $O_sO_4$ (2%, room temperature, 60 min) the sample was worked up for thin sectioning and electron microscopic examination. Cross bars represent 0.2 $\mu$ (on A) and 0.1 $\mu$ (on B). (Magnification: A, × 90,000; B, × 300,000).



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