SHRINKAGE OF *BACILLUS SUBTILIS*

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CHAPTER I

INTRODUCTION

Many approaches have been used to investigate the mechanisms by which antimicrobial agents affect bacteria. Among these approaches preliminary microscopy is usually done to assess possible morphological effects. All too often, however, such studies have been limited to visual memory comparisons of heat-fixed, simple stained cells observed through a bright field microscope. This method reliably detects only the most pronounced of morphological variations and is subject to artifacts arising from fixing and staining as well as to unintentional bias introduced by the operator in his selection of representative fields and in his imperfect recall of size and form.

Superior methods for morphological surveys are available. Phase contrast microscopy reveals almost as much information about living cells in wet mounts as does differential staining of fixed smears yet does not cause the physical and chemical distortion attendant to staining procedures. Photomicrography permanently records the appearance of specimens so that detailed studies of prints can be done later, including convenient measurements. The tendancy toward bias in field selection can be counteracted by using dense cell suspensions, thereby including a large sample size in each field. Although such observations of representative samples withdrawn at intervals have yielded much valuable information about morphological trends in a culture, it is obviously impossible to

follow the fate of individual cells by this discontinuous procedure. Many bacteriological phenomena, for example cell growth and division, are thought to be progressive, continuous events, and it thus seems appropriate to adopt microscopic methods which continuously record the morphologic aspects of such phenomena in the presence and absence of antimicrobial agents.

Suitable microculture techniques must immobilize the cells in an environment supportive of growth and satisfying optical requirements for critical microscopy. Quesnel (1969) has recently reviewed such methods and their associated technical difficulties. Motility and Brownian movement can be prevented by entrapping the cells between a cover slip and a block of soft agar growth medium. Since evaporation must be prevented by the use of a peripheral sealant such as petrolatum, oxygen becomes growth-limiting when strict aerobes are cultivated. This difficulty may be alleviated by the inclusion of air bubbles along the edges of the block within the sealed perimeter (Durham et al., 1967), or by the substitution of an oxygen-permeable Teflon membrane for the cover glass (Noller and Durham, 1968).

Time-lapse cinephotomicrography is a very useful method for recording dynamic morphological phenomena observed in slide cultures. Equipment is commercially available for automatically exposing single frames at regular intervals, thus preserving a consecutive record of the specimen appearance throughout the course of an experiment spanning several hours. To minimize focus instability and field drift, the temperature ambient to the apparatus must remain constant and under very precise control. A heated stage may be used to surround the slide culture with an elevated local incubation temperature optimum to the system

under investigation; this permits room temperatures more comfortable to the operator who must be present to maintain focus. When the resultant film has been processed and is viewed through a projector, the apparent motion is accelerated by a factor equal to the ratio of the projection rate to the camera exposure rate. Acceleration of gradual or subtle motion is a definite aid in discerning more exactly the mechanism of known processes and, in at least one instance, has disclosed previously unsuspected events (Durham et al., 1967). Analysis projectors are available which offer a broad selection of rates, convenient reversal and single frame projection, as well as frame number indication. Such instruments greatly facilitate time-motion studies of research films. When the enlargement factor is known, cell dimensions can be computed from direct measurement of the image projected onto a matt surface. For detailed comparisons and measurements prints can be prepared from selected single frames, relating the frame number to the time scale of the experiment. Thus time-lapse cinephotomicrography is potentially a powerful research tool in studies on dynamic morphological phenomena.

Relatively few investigations on the action of antimicrobial compounds have utilized time-lapse cinephotomicrography, perhaps due to the specialized apparatus required. A pioneering effort by Pulvertaft (1952) distinguished two types of bacterial death: explosive lysis, during which a cell enlarges suddenly and disappears, often moving adjacent particles, versus a less dramatic process in which cells cease to enlarge, become pale and transparent under phase contrast microscopy, and show no further changes. Critical concentrations of five antibiotics (aureomycin, terramycin, streptomycin, chloromycetin, and penicillin) each caused both types of death of Escherichia coli when the test agent

was incorporated in the slide culture agar block. Penicillin promoted explosive lysis in all concentrations tested, whereas high concentrations of the other antibiotics caused death by both modes. Lower, sublethal concentrations of the antibiotics retarded growth rate and caused pleomorphism of non-lysing cells. Because lysis of some cells always occurred in uninhibited controls, the author speculated that autolysis might have an important role in culture survival.

Using time-lapse cinephotomicrography Durham et al. (1967) discovered that Bacillus subtilis W 23 and Bacillus cereus undergo an abrupt shrinkage prior to forceful lysis when grown on nutrient agar containing 15 μg/ml vancomycin. The shrinkage occurred about 1 hour after exposure to the antibiotic, involved 10-15% reduction of both length and diameter within about 4 seconds, and preceded lysis by 40-50 minutes. Shrinkage of the cell wall as well as the protoplast seemed implicated since intercellular gaps within chains did not become apparent afterward. The cells remained dark after shrinkage and retained a normal phase halo. Indeed, since the change in size was the only alteration discernible, the shrinkage would probably not have been noticed without the accelerated action due to the time-lapse technique. On the other hand, lysis of the cells was characterized by loss of the phase halo as the cells became lighter and somewhat transparent. Local point rupture and ejection of protoplasm into the surrounding medium with a force sufficient to move the cell and adjacent particles was often seen. Less frequently, a gradual diffuse lysis was observed. With either type of lysis, continued disintegration was the eventual result.

The discovery of the shrinkage phenomenon prompted much curiosity

about its mechanism, its specificity as a vancomycin-induced effect, and its relation to the fate of the cell. This dissertation reports the results of further investigation directed toward characterizing the nature of the shrinkage phenomenon in B. subtilis W 23.

The dramatic change in size during shrinkage suggested that the bacterial surface was involved, perhaps causally. Electron micrographs of thin sections have revealed that the envelope of the gram-positive bacterial cell contains a thick cell wall surrounding a thin trilaminar cell membrane, these structures apparently connected by thin fibrils (Rogers, 1970). Despite their homogeneous appearance in electron micrographs, such cell walls usually contain at least two complex polymers both of which tend to exhibit taxonomic diversity: a peptidoglycan and a teichoic acid.

Although the cell wall peptidogylcan structure of strain W 23 of B. subtilis has not been definitely established, it is presumed to be of the A1γ type in the classification of Schleifer and Kandler (1972). This arrangement is typical of the vast majority of Bacillus (Schleifer and Kandler, 1972), has been established in the related strains B. subtilis Porton (Warth and Strominger, 1971) and B. subtilis 168 (Hughes, 1970; Schleifer and Kandler, 1972), and is consistent with the published cell wall analysis of B. subtilis W 23 (Hughes et al., 1968; Mauck, Chan, and Glaser, 1971). The A1γ peptidoglycan type contains the common glycan component consisting of alternating β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid residues. Amide-linked to the carboxyl of N-acetylmuramic acid is the peptide moiety, L-alanyl-γ-D-glutamyl-meso-diaminopimelyl-D-alanine. Direct interpeptide cross-linkage occurs between the ϵ-amino of meso-diaminopimelic acid of one peptide and the

carboxyl group of D-alanine on a proximal peptide. In B. subtilis 168 the average glycan length is 10 hexosamine units, roughly two-thirds of the peptide moieties are cross-linked, and the carboxyl group of diaminopimelic acid not involved in a peptide linkage is usually amide substituted (Hughes, 1970).

Under normal nutritional conditions the wall teichoic acid of B. subtilis W 23 is a glucosylated polyribitol phosphate (Chin, Burger, and Glaser, 1966). The rather rigorous conditions required to extract teichoic acid from cell walls as well as the finding that enzymic hydrolysis of cell walls produced fragments which contained both teichoic acid and peptidoglycan components suggests that these two polymers are covalently linked in the native cell wall (Young, 1966; Reaveley and Burge, 1972). A close association between peptidoglycan and teichoic acid is further implied by the identical turnover rates of these components observed in log phase cultures of B. subtilis W 23 by Mauch, Chan, and Glaser (1971). Ellwood and Tempest (1972) have reported that within the cell walls from phosphate limited chemostat cultures of B. subtilis W 23, teichoic acid was totally replaced by another anionic polymer, a teichuronic acid composed of alternating glucuronic acid and N-acetylgalactosamine. This study not only demonstrates that the cell wall composition is nutritionally dependent, but implies by the substitution of another complex anionic polymer (rather than deletion) that these compounds have an important physiological role. Several investigators have suggested that teichoic acids may bind divalent cations, availing them in the vicinity of the cell surface for stabilization of membrane structure and activation of certain synthetic and respiratory enzymes (Reaveley and Burge, 1972).

The discovery of autolytic enzymes associated with bacterial cell walls has stimulated much interest in their biological function, especially with regard to growth. One popular concept is that the autolytic enzymes catalyze the hydrolysis of specific bonds within the peptidoglycan fabric, exposing receptor sites to which activated precursor molecules could become attached (Ghuysen, 1968). Thus coordinated autolytic and synthetic activities would seem necessary for normal growth. If synthetic processes become inhibited--for example via nutritional deficiencies or antibiotic interference with precursor availability--the resulting unbalanced autolytic enzyme activity would weaken the peptidoglycan net until lysis would ensue when turgor could no longer be restrained. Support for this hypothesis was provided by Rogers and Forsberg (1971) who showed that lysis due to D-cycloserine and vancomycin was greatly attenuated in B. subtilis cells previously made autolysin-deficient by chloramphenicol inhibition of protein synthesis.

At least two autolytic activities have been reported in B. subtilis (Brown and Young, 1970; Rogers and Forsberg, 1971; Hughes, Tanner, and Stokes, 1970). The dominant activity was an N-acyl muramyl L-alanine amidase (or simply amidase) which cleaved the peptide subunits from the glycan chain. Also found was an endo-β-N-acetylglucosaminidase (glycosidase) which cleaves the glycan chain at the N-acetylglucosaminyl-β-1, 4-N-acetylmuramic acid linkage. Lysozyme, the useful glycosidase of animal origin, is an endo-β-N-acetylmuramidase, cleaving the N-acetylmuramyl-N-acetylglucosamine linkage (Strominger and Ghuysen, 1967). Although an autolysin with the lysozyme specificity has not been identified in B. subtilis, it is interesting that lysozyme separates these cells in temperature-induced abnormally long filaments, suggesting

that a lysozyme-like autolysin causes dechaining under normal growth conditions (Fan, 1970).

Several of the antibiotics used to investigate the shrinkage phenomenon are known to interfere with peptidoglycan biosynthesis (Ghuysen, Strominger, and Tipper, 1968). D-Cycloserine prevents the addition of D-alanyl-D-alanine to the uridine diphospho-N-acetylmuramic acid tripeptide (Strominger, 1962) by competitively inhibiting the enzymes alanine racemase and D-alanyl-D-alanine synthetase (Strominger, Ito, and Threnn, 1960; Neuhaus and Lynch, 1964). Vancomycin and the ristocetins interfere with the transfer of the N-acetylglucosamine-N-acetylmuramic acid-pentapeptide complex from a membrane lipid carrier to normal acceptors in the peptidoglycan net (Anderson et al., 1965, 1966, and 1967). Penicillin, a structural analogue of the D-alanyl-D-alanine terminus of the pentapeptide, prevents peptide cross linking by specifically inhibiting a transpeptidase and a carboxypeptidase (Izaki, Matsuhashi, and Strominger, 1966). The ultimate result of growth in the presence of lethal concentrations of peptiglycan biosynthesis inhibitors is an autolysin-weakened peptidoglycan network which eventually ruptures, thus failing to protect the protoplast from osmotic forces (Rogers, 1970).

Some antimicrobial agents act primarily upon the cell membrane. Because this organelle is so intimately involved in various physiological functions in procaryotes, membrane active compounds may ultimately influence not only permeability, but also DNA replication, cell wall biosynthesis and septation, as well as energy-yielding processes (Harold, 1970). The typical trilaminar appearance of the bacterial membrane in thin section reveals little about its organization and function, the subject of considerable research (Grula et al., 1967; Reaveley and

Burge, 1972). Singer and Nicolson (1972) have proposed a structural model consistent with much of the existing physicochemical and biological data. In their fluid mosaic model, heterogenous amphipathic globular protein and conjugated protein molecules are discontinuously distributed upon and within a viscous phospholipid bilayer matrix. Hydrophobic and hydrophilic interactions are considered the most important thermodynamic forces which maintain the general structure of this model, and these would readily permit dynamic interactions at the local level such as those presumed to occur during transport. The proteins would bear the major role in binding and catalytic functions. Proteins and phospholipids alike would be exposed on either inner or outer membrane surfaces, providing accessibility to either environment. Such accessibility--advantageous under ordinary circumstances--would become detrimental in the presence of antimicrobial agents; indeed it may partially account for the fact that such a variety of antimicrobial agents are membrane active.

The polymyxins and circulins comprise a group of cyclic peptide antibiotics whose primary action in bacteria is disruption of the permeability barrier (Harold, 1970). Newton (1956) reviewed the evidence which initially led him to this conclusion. He demonstrated that polymyxin B caused leakage of 260 nm-absorbing compounds from washed suspensions of cells as a function of concentration. Also the antibiotic readily increased the uptake of a normally excluded fluorescigenic dye, N-tolyl-α-naphthylamine-8-sulfonic acid. Newton then coupled a fluorescent compound (1-dimethyl-amino-naphthalene-sulfonyl chloride) to polymyxin and used fluorescence microscopy to display binding of this complex to isolated cell membrane fragments of Bacillus megaterium.

Other sites of action by polymyxin apparently exist in gram-negative organisms. Koike, Iida, and Matsuo (1969) reported that--in addition to breaks in the cytoplasmic membrane--polymyxin induced numerous projections originating from the outermost layer of the cell envelope in E. coli and Pseudomonas aeruginosa. Similar bleb-like projections have been reported in polymyxin-treated Chlamydia psittaci (Matsumoto, Highashi, and Tamura, 1973). Koike and Iida (1971) studied the alteration by polymyxin of specific phage receptors in E. coli and concluded that lipopolysaccharide, but not lipoprotein, is altered both functionally and morphologically. Their study augments an earlier report that polymyxin treatment caused discernible changes in ribbons of lipopolysaccharide isolated from E. coli (Lopes and Inniss, 1969). Therefore in addition to the well documented membrane perturbation which satisfactorily explains many of the effects of polymyxin, evidence is mounting for other primary sites of action.

In contrast to the relatively specific lesions caused by antibiotics, some of the chemical agents used in this study are known to cause extensive denaturation of many biomolecules, especially proteins and lipids. For example, concentrated strong acids or alkalies overwhelm natural buffers and disrupt ionic associations and hydrogen bonding which, in part, maintain the biologically active conformation of enzymic and structural proteins. Trichloroacetic acid is a traditional protein precipitant which breaches the permeability barrier of cells, releasing from them small molecular weight compounds. High concentrations of organic solvents such as ethanol and acetone also cause macromolecular denaturation. Protoplasts are lysed by ethanol, and such damage probably results from the disorganization of the hydrophobic

regions of membrane lipids (Harold, 1970). Detergents should also affect the membrane since these amphipathic molecules would tend to concentrate at such surfaces (Hamilton, 1971). Suspensions of erythrocytes, cultured animal cells, or bacterial protoplasts rapidly become clear when treated with detergents, the apparent result of membrane damage (Gilby and Few, 1960; Salton, 1968). Detergent treatment of intact bacteria causes delayed, protracted clearing of suspensions, suggesting that autolytic enzymes must attack the cell wall before the detergent effects become apparent by turbidimetric methods (Salton, 1968). Studies which show leakage of a variety of metabolites from whole bacteria in response to several detergents have led Salton (1968) to conclude that cell membrane disorganization is the primary effect of these compounds, and that various autolytic events follow permeability disruption.

Formaldehyde, on the other hand, has been shown to strengthen protoplast membranes, increasing their resistance to osmotic lysis (Corner and Marquis, 1969). A widely used disinfectant and fixative, formaldehyde, is known to produce intra- and intermolecularly cross-linked proteins via methylene bridges between amino groups (French and Edsall, 1945). Thus the microbicidal effect of formaldehyde certainly involves membrane perturbation as well as widespread denaturation of intracellular proteins (Hamilton, 1971). Membrane action therefore seems to be a common denominator among most chemical agents used as general microbicides. Although the specific membrane lesion may vary, alteration in permeability properties is the ultimate result shared by this group of compounds and is considered sufficient for lethality (Hamilton, 1971).

Due to the high osmolarity of the cytoplasm relative to that of the

usual external menstruum, bacterial cells normally possess osmotic turgor. This internal osmotic pressure has been estimated at 20-25 atmospheres for gram-positive and 2-3 atmospheres for gram-negative bacteria (Mitchell and Moyle, 1956). Although the cell wall may restrict transport of large molecules, the principal osmotic barrier is the cytoplasmic membrane (Salton, 1967). Osmotic turgor normally distends this structure tightly against the cell wall and causes lysis if the wall is defective (Salton, 1968). When gram-negative bacteria are suspended in hypertonic, non-toxic solutions of impermeable solutes (commonly 1 M sucrose) they are readily plasmolyzed: the protoplast shrinks within the relatively rigid cell wall. Plasmolysis of gram-positive bacteria is more difficult, perhaps due to the higher osmotic pressure and possible adhesions between the membrane and the cell wall (Mitchell and Moyle, 1956). Knaysi (1930) reported plasmolysis of B. subtilis with 25% w/v NaCl. Recently Marquis (1968) has demonstrated salt-induced contraction of the Bacillus cell wall. Since cell wall contraction would tend to obscure protoplast shrinkage during plasmolysis in strong salt solutions, this finding may partially explain why some attempts to plasmolyze Bacillus have yielded inconsistent results (Mitchell and Moyle, 1956).

CHAPTER II

MATERIALS AND METHODS

Test Organisms

The principal culture used throughout this investigation was *B. subtilis* W 23, a prototrophic strain originally isolated by Anagnostopoulos and Spizizen (1961) for transformation studies. *Pseudomonas fluorescens* NND and *E. coli* B were cultures from the collections of Dr. Norman N. Durham and Dr. Edward A. Grula, respectively. Stock cultures were maintained on glucose salts agar slants at 4 C.

To obtain morphological uniformity, exponential phase cells were routinely used to inoculate the slide cultures. Such cells were grown in 5 ml of glucose salts medium in 18 x 150 mm cotton-plugged test tubes on a reciprocal shaker (100 excursions per minute) at 37 C. Growth was followed by measuring the increase in absorbancy at 540 nm with a Coleman Junior II spectrophotometer, using the culture tubes as cuvettes. Slide culture inoculation was done when the A_{540} was within the range of 0.5 to 0.9. To secure a homogeneous inoculum, at least four serial 0.1 ml transfers to fresh medium were made at about 10 hour intervals.

Chemicals

A list of the sources for special chemical agents used in this study follows: vancomycin, Eli Lilly and Co.; ristocetins A and B, Abbott Laboratories; penicillin G, Mann Research Laboratories, Inc.; polymyxin

sulfate, Charles Pfizer and Co., Inc.; chloramphenicol, lysozyme, and Triton X-100, Sigma Chemical Co.; sodium lauryl sulfate (SLS) and trichloroacetic acid (TCA), Fisher Scientific Co.; cetyl pyridinium chloride (CPC) and cetyl trimethyl ammonium bromide (CTAB), Eastman Kodak Co.; Tween 80 and sodium desoxycholate (DOC), Nutritional Biochemicals Corp.; formaldehyde, Merck and Co.

The water used had been glass distilled. All solutions are aqueous unless otherwise specified. The pH of solutions was not adjusted unless indicated in the text.

Media

The principal growth medium employed in these studies will be termed glucose salts and had the following composition: 1.4% (w/v) K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.5% glucose. Other essential elements were supplied by adding 0.1% (v/v) of a saturated trace mineral solution consisting of 5 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $MnSO_4$, 1 g $FeCl_3$, and 0.5 g $CaCl_2$ in 100 ml of water. The trace mineral solution, the basal salts solution, and a 10% solution of the carbon source were prepared separately, sterilized in the autoclave at 121 C for 15 minutes, cooled, and then combined to yield the desired final concentrations. The final pH was 7.0. Five ml aliquots of this complete glucose salts medium were aseptically dispensed into sterile culture tubes for use in cultivating the inoculum for the slide cultures.

A similar medium containing agar was used in the slide cultures. Because sterility was not essential, and because it permitted the convenient incorporation of a variety of agents into the medium, the procedure for preparing this glucose salts agar differed from the above.

A quadruple strength glucose salts medium was prepared by dissolving 1.4 g K_2HPO_4, 0.6 g KH_2PO_4, or 0.2 g $(NH_4)_2SO_4$, and 0.5 g glucose in water to 24.9 ml of solution, then adding 0.1 ml of the trace mineral solution. This non-sterile medium concentrate could be stored at 4 C for several days or until contamination or precipitation was evident. A double strength aqueous agar gel was prepared by dissolving 0.8 g of Difco Special Agar (Noble) in 50 ml of water within a 125 ml Erlenmeyer flask heated in a boiling water bath. The flask was covered by a glass sphere to minimize evaporative water loss. To ensure homogeneity of the gel, heating was continued about 10 minutes after clearing; then the flask was cooled to 50 C in another water bath. Any unused fraction could be stored at 4 C and later remelted. Test agents to be included in the slide culture medium were prepared as quadruple strength aqueous solutions. With each solution preheated to 50 C, the complete medium was prepared by combining in a test tube 1 ml of the quadruple strength glucose salts medium, 1 ml of the test agent solution or water, and 2 ml of the melted aqueous agar. After briefly vortex-mixing, each 4 ml aliquot of medium was poured into a plastic petri dish. The dish was tilted until the medium had covered the entire bottom, then placed on a level surface until the agar had solidified, forming a layer about 0.6 mm thick. The plates were then sealed with stretched Parafilm to retard evaporation and could be stored satisfactorily for a few days at 4 C.

Slide Cultures

Two types of slide cultures were used in this study: a sealed agar block type wherein the test agent was a constant component of the medium, and a wick slide culture which allowed perfusion of the agar block with

several test agents in sequence during the course of an experiment.

To prepare the sealed slide culture, a stainless steel spatula with a 1.1 cm squared tip was used to slice and remove a block of the desired agar medium from a petri dish. An auxiliary spatula was then used to slip the agar block onto a clean 1 x 3 inch microscope slide, exercising care to neither tear the thin layer of soft agar nor entrap agar fragments beneath it. The often distorted edges of the agar block were then trimmed. The final dimensions of the block were approximately 1 cm square by 0.6 mm. If inspection under reflected light did not reveal a smooth surface, the block was discarded and its preparation was repeated.

Inoculation with an exponential phase culture was done by sliding a loopful across the surface and off the edge of the agar block. The loop could be further used to gently spread the inoculum on the surface, but great care was required to avoid scratching or tearing the medium. Once again the surface was ascertained to be smooth, without liquid pools or agar fragments. Inoculation was done quickly to prevent drying or excessive aeration. The nichrome wire inoculating loop (approximately 4 mm) was not permitted to become encrusted with oxide.

An oxygen-permeable Teflon coverslip (Yellow Springs Instruments Co. No. 5352 1 mil FEP Teflon membrane, Noller and Durham, 1968), pre-cut to approximately 1.4 cm square, was applied to the inoculated agar block. Flat forceps were used to grasp this thin membrane, first touching an edge to the medium, then rolling it onto the block. It was important not to slide the membrane or to lift and reposition it or the result would often be entrapped air bubbles, and usually a mottled and inhomogeneous background would be seen in the microscope. Melted

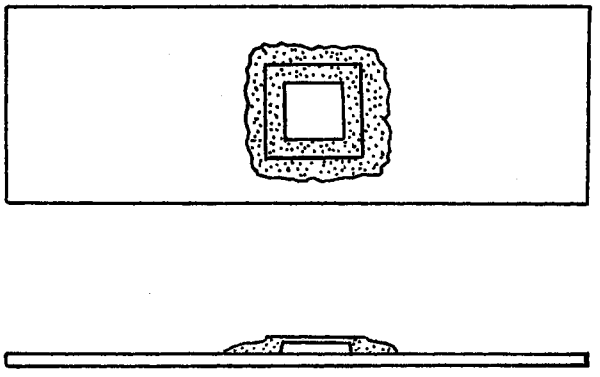
petrolatum from a warmed syringe was applied to the perimeter of the agar block, sealing the space between the cover slip and the glass slide. This operation was done quickly, completing the seal before any of the petrolatum solidified. Evaporation of the liquid through a seal failure would result in field drift and focus instability. The sealed slide culture is diagrammed in Fig. 1.

Because in this type of slide culture the cells were exposed to the test agent in the medium from the moment of inoculation, a premium had to be placed upon haste during the final stages of slide preparation, microscope adjustment, and selection of an appropriate field in order to begin filming early events as soon as possible. To obviate disadvantages arising from haste and to secure increased experimental flexibility, the wick slide culture was devised. It is diagrammed in Fig. 2.

To prepare the wick slide culture, 1.5 x 6 cm strips of Whatman No. 1 filter paper were cut, and a 1/4 inch hole was punched 2.5 cm from one end. The opposite end was folded twice at approximately 1 cm intervals to provide an upward flute as shown. Then the paper wick was placed on a glass microscope slide and saturated with water. As described previously a block of glucose salts agar was removed from the petri dish and deposited over the hole in the wick, taking care not to include air bubbles within the hole. The agar block was inoculated as before. An 18 x 18 mm No. 2 glass cover slip was then eased down onto the agar block, touching one edge first and avoiding air bubbles. Next, warm petrolatum was used to seal the perimeter of the cover slip and to form a surface tension barrier around the fluted end of the wick (the exit reservoir) and around the opposite end (the entrance reservoir). Although the melted petrolatum thus sealed the cover slip to the top of

Figure 1. Sealed Slide Culture. Above the glass slide is an inoculated glucose salts agar block fitted with a Teflon cover slip and sealed with petrolatum.

Figure 2. Wick Slide Culture. Above the glass slide is a filter paper wick containing a 1/4 inch hole, an inoculated glucose salts agar block, a glass cover slip, and a petrolatum seal. The entrance reservoir is at the right; the exit wick is folded upward at the left.



the wick, it did not penetrate the wet paper and thus did not prevent capillary conduction between the agar block and the reservoirs. The entrance reservoir was filled immediately with water, and the entrance wick was not permitted to dry throughout an experiment. Test solutions were added dropwise to the entrance reservoir as required, commonly at intervals of 3-5 minutes. This manual replenishment compensated for the evaporation from the exit wick. The resulting capillary flow through the wick under the agar block thus provided the means by which the aqueous environment of the cells could be changed while the cells were under constant observation.

Time-Lapse Cinephotomicrography

The apparatus for conducting time-lapse cinephotomicrography was marketed by Wild-Heerbrugg (Heerbrugg, Switzerland and Farmingdale, New York). The basic M 20 research microscope was illuminated by a tungsten-halogen lamp through heat absorbing and green filters. A long focal length phase contrast condenser was necessitated by the thickness of the slide cultures. A 100 X oil immersion phase contrast objective lens was ordinarily used. The body tube was equipped with a 1.25 X magnifier and beam splitter which partitioned the image between a binocular eyepiece and a vertical monocular tube containing a 10 X photoeyepiece. Above this was attached a cine focusing viewfinder which directed most of the light toward the motion picture camera, yet provided a constant image to the operator for monitoring. Preliminary scanning to find an appropriate field was most conveniently accomplished while sighting through the binocular eyepiece, then critical focus was maintained throughout the experiment by using the image through the cine focusing

tube. The dim and granular image on the ground glass of the camera reflex viewfinder was of little help, therefore this light path was kept turned off to ensure against stray light leakage through it. For the same purpose, the binocular eyepiece was hooded when not in use, and the room lights were dimmed.

The Bolex H-16 movie camera was equipped with two lenses: a 50 mm f/1.8 and a 75 mm f/2.8. The low available light required their use at full aperture for cinephotomicrography; they were set at infinity focus. Although the use of either lens was possible, the 50 mm lens was favored for cinephotomicrography while the 75 mm lens was preferred for sequence slating. Optical linkage to the microscope was accomplished by inserting the camera lens front into a light trap funnel without direct mechanical linkage in order to avoid vibration of the microscope.

Attached to the camera was an auxiliary motor to power the single frame exposures and film advance. This motor was actuated by a pulse transmitter unit which offered a wide variety of available rates. Most of the long growth experiments were filmed at 2 frames per minute, while the shorter studies with the wick slide cultures were usually done at 10 frames per minute. For experiments using sealed slide cultures the microscope was fitted with an electromagnetic shutter which shielded the slide culture from illumination except during the exposure or focus checks. This shutter, which was triggered by the pulse transmitter, was the effective control of exposure duration in this type of experiment; it was routinely set to open for 0.8 second. During experiments using the wick slide cultures, focus was so unstable that a constantly illuminated field was more convenient to the operator. Consequently, the electromagnetic shutter was not used on such occasions, and the

camera shutter provided 1 second exposures.

Eastman Kodak Tri-X Reversal black and white 16 mm movie film (No. 7278) was used throughout the investigation. The exposure index of this film with tungsten lamps is ASA 160. To obtain proper exposure, the light output of the microscope lamp was adjusted via a potentiometer on its power supply. A photocell in the cine focusing tube wired to an external microammeter indicated the intensity of the illumination. Just prior to beginning the filming, the luminescence was set to indicate 0.5 μA when using the 50 mm camera lens or 1.0 μA for the 75 mm lens. Further lamp regulation during the experiment was not required.

The best microscopic techniques were practiced in order to photograph the specimens with the greatest possible detail. Kohler illumination conditions had to be re-established for each photographed field since slide culture thickness affected this adjustment. Alignment of the phase rings was required only when the objective was changed. Lamp centration was necessary only when the bulb was replaced. Dust was blown from accessible optical elements of the microscope and camera at weekly intervals. Such particles, particularly on the lamp condenser lens, the camera viewfinder prism, and within the camera lens, would be rendered as distracting Airy disks on the processed film. Although the microscope was supported by a massive table of stone slabs designed to dampen vibration, the operator had to avoid bumping the stage and thus moving the field.

By far the most irksome difficulty was focus instability caused mainly, in the case of the sealed slide cultures, by temperature fluctuation. The slide culture was locally warmed to 37 C by an electrically heated stage which provided constant heat output. Thermoregulation of

the room to 28 C with minimum bandwidth fluctuation was accomplished by a sensitive thermostat which controlled a 2 kw resistive heater and by a fan which circulated the air. Despite this rather good control, focus on sealed slide cultures usually required adjustment each 10-15 minutes. Because evaporation contributed to focus difficulties, the wick slide cultures required constant attention during these experiments.

The exposed rolls of film were reversal processed by a commercial laboratory (United Films, Inc., Tulsa, Oklahoma).

Maintenance of Records

To avoid potential mistakes with external labels, particularly after editing, a typed note of standard format listing a code number and experimental conditions was filmed immediately preceding each experiment. The code number consisted of LA, the roll number, and the experimental sequence number on that roll. Complete experimental conditions were described on a standard data sheet in loose leaf form for convenient sorting after editing of the film. The slating was accomplished by illuminating a 4 x 6 cm label with a 40 watt bulb at about 40 cm, then focusing it through the 75 mm camera lens fitted with a +2 diopter close-up attachment, stopping the lens down to f/11, and running the camera at 2 frames per second for 32 seconds. Since the label was directly linked to the experimental film sequence, considerable re-sorting of sequences could be done by editing without necessitating additional records. Obviously this slating also facilitated the study of the films during their projection by eliminating frequent reference to the data sheets in the darkened room.

Analysis of the Films

Study of the time-lapse films was facilitated by the use of a Lafayette Analyzer projector AAP-300 (Lafayette Instrument Co., Lafayette, Indiana). Subtle motion could be rendered more apparent by the judicious selection of projection rates. Details were typically scrutinized by running through brief segments in forward and reverse. The frame counter was valuable for kinetic studies of projected events. Frame by frame and single frame projection at full brightness permitted unhurried observations.

When an experimental sequence was analyzed, a sketch of the initial field appearance was made on the data sheet. The fate of each cell was then noted on this sketch as the film segment was repeatedly projected. Thus the data sheet became a brief summary of the events captured on the motion picture film and was thus a convenient reference.

The observed acceleration of motion by the time-lapse technique is equal to the ratio of the projection rate to the filming rate. For example, a growth study filmed at 2 frames per minute and projected at 16 frames per second is seen at a 480 X acceleration.

Magnification

The Wild-Heerbrugg instructional literature gives the formula for computing the magnification on film using their apparatus as the product of the objective lens magnification, the phototube magnification factor, the photoeyepiece magnification, and the ratio of the camera lens focal length in mm to 250. During this investigation the usual choice of variables was a 100 X objective lens and 50 mm camera lens which resulted in a 250 X magnification on the 16 mm film. The accuracy of

this computation was verified by photographing a stage micrometer.

At 250 X magnification, the horizontal distance across a projected 16 mm frame was thusly found to be the equivalent of 40 μm of specimen. Consequently, to obtain a convenient enlargement factor, the projector was routinely positioned to give an image exactly 40 cm wide so that 1 image cm equaled 1 specimen μm. The projected images of cells on single frames were therefore directly measured in cm, and these dimensions were recorded on the data sheets as μm, the specimen equivalents.

Prints

When positive prints of single frames were desired, a 35 mm negative was first prepared. The selected frame was projected onto a matt white surface in a darkened room and photographed on Kodak Plus-X black and white film with a Nikkormat FTN 35 mm single lens reflex camera through a 135 mm lens. The camera was mounted on a foam rubber cushioned board as close as possible (25 cm) to the projector lens to minimize distortion. Although the light meter in the camera dictated the exact exposure settings, 1/8 second at f/5.6 was typical.

To simplify labeling, a light box was mounted beside the screen through which an adding machine paper roll was passed. On the paper strip the experiment code number and the frame number were conspicuously written. This label was photographed with the screen image. Thus each negative and print possessed the necessary numerical reference such that sorting errors during negative filing and print preparation were avoided.

Conventional darkroom procedures using Eastman Kodak products were followed. The Plus-X film was processed with Microdol-X developer

diluted 1:3. A Beseler 45 H enlarger (no filter) was employed to expose Polycontrast paper, which was then developed in Dektol.

All prints included in this dissertation are enlargements of complete 16 mm movie frames to 72 x 52 mm. These prints are collected in the Appendix as Figs. 3-50. Figs. 3-28 and 33-50 are 1800 X enlargements of the specimen (1.8 mm on the print is the equivalent of 1 μm on the specimen). Figs. 29-32 are 2700 X enlargements (2.7 mm from 1 μm). The prints were included to aid the identification of particular cells and to depict certain characteristic appearances. Measurements were better made directly from the projected image, however, and all of the data were therefore collected via analysis projection. The original motion picture films generated from this study were deposited with the Department of Microbiology, Oklahoma State University.

Calculation of Cell Volume

B. subtilis W 23 is a cylindrical bacterium with hemispherical ends. For the purpose of calculations, the cell volume was considered to be composed to two equal hemispheres of diameter D (= 2R) and a cylinder of the same diameter and with height H. The total measured length of the cell (L) = H + D. Adding the volume of the sphere, $4\pi R^3/3$, to the volume of the cylinder, $\pi R^2 H$, and converting the dimensions to L and D (which were conveniently measured from the photographs), the formula for computing the volume of the cell V was obtained:

$$V = \frac{\pi D^2(3L-D)}{12}$$

When D and L were expressed as μm, the unit of volume V was μm^3.

CHAPTER III

RESULTS AND DISCUSSION

Choice of the Primary Test Organism

The morphological and nutritional aspects of several species were considered before B. subtilis W 23 was selected as the primary test organism in this study. The use of a synthetic minimal medium was desired, both to obtain better-defined conditions and to suppress the growth of contaminants in the slide cultures. Several possible cultures, including B. cereus, were not used due to their inability to grow on the glucose salts minimal medium.

The specimen size, shape, and characteristic arrangement were also important considerations in the choice of the test organism. An optimum size range existed. Relatively large bacterial species with cell diameters in excess of approximately 1 μm produced microscopic images surrounded by such prominent phase halos that peripheral and intercellular details were obscured. B. megaterium KM and an unidentified Sarcina species were photographed and judged to be too large. Another difficulty was that the thickness of these cells apparently exceeded the depth of focus of the optical system, because critical focus on the entire organism could not be achieved. Depth of focus problems were the basis for rejecting all species, such as Micrococcus lysodeikticus, which form three-dimensional aggregates.

Toward the other dimensional extreme, cells with diameters of less

than 0.5 μm such as P. fluorescens NND lacked the phase halo problem but also did not appear as dark against the background. Not only were such cells difficult to measure precisely, but it was almost impossible to distinguish intact cells from lysed cells on the basis of contrast.

Because the measurement of longer distances was less subject to interference from the phase halo, rod-shaped organisms were favored over cocci. Although both length and width shrank by approximately the same percent, the length could be measured more precisely and therefore was selected as the dimension for routine comparison. Unless otherwise noted, the percent change cited throughout this work is the percent change in length during the described event.

B. subtilis W 23 did not exhibit most of the difficulties inherent to the other cultures surveyed. Its size (0.8 x 3-6 μm) was large enough to present a dark contrast against the background yet not so large as to cause significant depth of focus or phase halo problems. Intact cells appeared darker than lysed cells and could thus be easily distinguished. This strain grew well on the glucose salts minimal medium. In addition, B. subtilis W 23 has, in recent years, become a popular research organism. It also was one of the strains used when the shrinkage phenomenon was discovered (Durham et al., 1967). For these reasons the choice of B. subtilis W 23 as the primary research culture seemed appropriate.

Refinement of Experimental Techniques

During the course of this investigation many technique modifications were attempted before the procedure was standardized. For example, to minimize the change in composition of the agar block due to dilution

during inoculation, growth from 5-18 hour glucose salts agar slant cultures was at first employed to inoculate the slide cultures. However it proved difficult to obtain an even distribution of such cells on the agar block surface. Typical fields contained long, tangled chains with many pleomorphic and lysed members. With patience it was usually possible to locate a field with short chains and individual cells of uniform morphology. Frequently, however, such cells either failed to grow or showed an unpredictable lag of 1-2 hours. These inconsistent results probably were caused by the varied microenvironmental circumstances which exist within any agar surface growth. As the inoculating loop moves through the mass, it undoubtedly collects cells in a variety of physiological states, thus leading to heterogeneous morphology and growth responses.

To secure a more consistent inoculum, the use of late log phase glucose salts liquid cultures was adopted. As noted earlier, log phase growth was maintained by periodic transfers to fresh medium. After a few subculture cycles, the cells were remarkably consistent, were slightly larger relative to agar grown cells, had rounded ends, and occurred predominantly in singles and pairs rather than in chains. A conjectural disadvantage of the liquid inoculum was its dilution of the slide culture medium in the region local to the cells. In our experience, this did not appear to cause difficulty, but it should be recognized that the concentrations of ingredients in the agar block medium were subject to alteration due to this dilution as well as due to evaporation during the operations which expose the medium directly to air.

Adequate aeration of liquid and slide cultures of B. subtilis W 23 was essential for growth and avoidance of autolysis. Removal of

exponential phase cultures from the incubator shaker for longer than two minutes prior to the inoculation of the slide cultures resulted in a noticeably increased proportion of lysed cells. To reach the cells under observation in the sealed slide cultures, oxygen must diffuse through the Teflon membrane and through the agar medium. To increase the area of this special cover slip in contact with the atmosphere, the immersion oil droplet was made as small as practicable to seal the optical path. No conclusive evidence existed, but a number of early growth failures may have resulted from excessively large oil seals. Glass cover slips were used with the wick slide cultures after it had been experimentally established that sufficient aeration for growth was achieved via diffusion and convection from the reservoirs to the cells.

An effort was made to prepare slide cultures as quickly as possible. Dehydration due to prolonged exposure of the agar block to air resulted in an uneven surface which made the eventual selection of a satisfactory field of immobilized, coplanar cells almost impossible. Exposure of the inoculum film to air for more than a few seconds seemed to be inhibitory or lethal to the cells, therefore the cover slip was positioned without delay.

The nichrome wire loops used for inoculation were quite susceptible to corrosion when heated to incandescence after exposure to the glucose salts medium. Not only did a corrosion-encrusted loop tend to scratch the surface of the agar block, but its use as an inoculation vehicle seemed to affect the growth of the cells in the slide cultures. This correlation was first noticed upon scanning mature slide cultures in which cells surrounding the dislodged chips of oxide exhibited excessive pleomorphism and lysis. Subsequently it was noted that growth failure

often resulted following use of a corroded loop even if oxide chips could not be found on the slide culture. Presumably some toxic salt or oxide had contaminated the system. The difficulty was satisfactorily circumvented by rinsing the medium-contaminated loop with water prior to flaming it, and by replacing the wire at the first sign of pitting or encrustation. The apparent inhibition was not further investigated.

Short chains of 2-8 cells were not unusual in B. subtilis W 23 cultures. After each cell in a chain had shrunk, the percent decrease in the length of the entire chain was found to approximate that of single individual cells. Consequently, when all cell units in a chain were observed to shrink it was more convenient to measure and compare the length of the entire chain, especially since the intercellular septa were often indistinct.

Characteristics of Normal Growth

Experimental sequence (LA) 23-2 was filmed at 2 frames per minute (fpm) and recorded the normal growth of B. subtilis W 23 on glucose salts agar in a sealed slide culture. The size of individual cell units was 0.8 x 3-6 μm. Growth was obvious at the beginning of the sequence and continued to the end. Cells usually did not separate as septa appeared but instead developed chains which continued to elongate until a tip collided with other cells or seemed to encounter increased friction. At this time the chain would tend to buckle, often shearing at one or more of the septa.

Although the films resolved little detail of septum formation, the first detectable sign was a slight decrease in contrast of an area equidistant from the poles of an elongated cell unit. This area gradually

became lighter, and a definite constriction was eventually seen. Meanwhile, cell elongation appeared to continue at a constant rate. The first indication of septation was regularly observed when a cell unit became approximately 6 μm long. Following the lowest tip unit of the chain derived from the centermost cell (a) on LA 23-2 through three cell divisions (Figs. 3-6), a 6 μm length of serial daughter cells was noted at frames 27, 141, 247, and 380 which corresponded to length-doubing times of 57, 53, and 66 minutes respectively. The average time required for a daughter unit from a 6 μm parental cell to itself become 6 μm was thus used to estimate generation time. In LA 23-2 the average generation time based on the growth of 10 cells was 59.6 minutes or approximately 1 hour.

Growth rate was not considered to be a very reliable measure of the action of antimicrobial agents in these slide culture studies because it was subject to so many possible influences. Physiological stress prior to or during inoculation could cause growth lag. Moreover, growth rate would depend upon various local microenvironmental conditions such as redox potential, pH, and availability of nutrients--factors influenced in part by the density and physiological activity of regional cells. Because these multiple influences could not be adequately controlled, the growth rate was used in this study only in a very general manner.

Effect of Several Antibiotics on Growth in Sealed Slide Cultures

Vancomycin

LA 9-5 (Figs. 7-10) was a typical experiment which showed the effect of including vancomycin at 15 μg/ml in the growth medium. The filming

rate was 2 fpm and 6.5 hours. Although the filming was started just 8 minutes after inoculation, no growth was seen. Cell a shrank at frames 70-71. The diameter decreased from 0.8 to 0.7 μm (13%); the length decreased from 3.0 to 2.6 μm (13%). This represents a change in volume from 16.5 μm^3 to 9.1 μm^3 or a 45% decrease. The entire shrinkage event occurred between two frames and therefore required no more than 30 seconds. Nothing could be detected leaving the cell during shrinkage.

Cell a lysed between frames 584 and 587. Protoplasm was extruded from a region on the pole adjacent to the sister cell. The loss of protoplasm preceded the complete loss of cell density by 2-3 frames, suggesting a gradual release over a minute or longer. Cell a moved slightly at the beginning and at the end of the lysis sequence. Most of the cells had lysed by the end of the experiment. Although several cells lysed without overtly shrinking on the film, shrinkage may have occurred before the filming was begun.

The shrinkage and lysis due to vancomycin in this glucose salts agar system was similar to that reported earlier in a nutrient agar medium by Durham et al. (1967). Studies were next conducted to determine if shrinkage was a specific effect due to vancomycin, or if it would occur in the presence of other stress-causing agents.

Ristocetins

Shrinkage was also observed in the presence of ristocetin A or B. LA 5-5 showed the effect of ristocetin B at 120 μg/ml, filmed at 2 fpm for almost 4 hours. Of 7 cells, 4 were observed to shrink, each requiring only one frame or less than 30 seconds. A good example was the centermost cell which shrank at frame 206 from 4.3 to 3.6 μm (16%).

The lighter appearance of the cells some 20 hours later indicated that lysis eventually ensued. In other sequences shrinkage was noted at much lower concentrations, for example 10 μg/ml for ristocetin A and 2 μg/ml for ristocetin B.

Penicillin

LA 6-3 (Figs. 11-18) showed cells subjected to penicillin G at 100 μg/ml in the standard growth medium. Filming was started 10 minutes after inoculation and continued uninterrupted for 6.5 hours at 2 fpm. During this period several cells were seen to grow slightly, shrinking at irregular intervals, and later lysing. For example, the length of cell a increased from 4.2 to 4.4 μm until frame 26 where it abruptly shrank to 4.1 μm (6.8%) and then lysed at frame 111. Cell b grew to 6.2 μm, shrank to 5.8 μm (6.5%) at frame 99, and then lysed at frame 167 through a bleb-like rupture in the lateral wall. Cell c appeared to shrink slightly at frame 152, changing from 2.9 to 2.7 μm (10%). Then cell c seemed to undergo lysis at frame 175. It abruptly lost density and was simultaneously surrounded by a diffuse, spreading halo, presumably of protoplasmic origin. However, some image density persisted and at frame 400 a protoplast was seen to extrude from the upper pole. This protoplast became oval and underwent no remarkable change until the filming was interrupted for 5 hours. When filming was resumed, the protoplast derived from cell c (Fig. 18) had lysed. The experiment was continued after the delay to show that some surviving cells in the region d had begun growing rapidly, albeit very pleomorphically. It could not be determined with certainty which cells eventually grew or if they had previously shrunk.

A lower concentration of penicillin, 5 µg/ml, was used in LA 40-5. Initial growth was evident. Then as growth subsided, the chains of cells appeared to shudder as their component members shrank and later lysed. The shuddering appearance left the intriguing impression that some cell units may have undergone multiple shrinkage. Septa were not clearly defined so this conclusion could not be substantiated. Since intercellular linkage appeared to be rather rigid, movement at any point in the chain could have been readily transmitted along its length, causing some regions to appear to shrink more than once. Both shrinkage and lysis were seen during this sequence and either could have contributed to movement or redistribution of frictional forces. Therefore, although LA 40-5 suggested multiple shrinkage, other explanations for this appearance were available.

D-Cycloserine

LA 22-2 (Figs. 19-22) showed the effect typical of D-cycloserine at 25 µg/ml. Filming was begun 15 minutes after inoculation and continued for 5 hours at 2 fpm. All of the cells eventually lysed, most after shrinking. For example, cell a shrank from 3.5 to 3.0 µm (14%) at frame 15, then gradually faded during frames 50-70. Cell b shrank gradually from 2.5 µm at frame 117 to 2.1 µm at frame 151. After its companion cell lysed at frame 160, cell b appeared paler, yet did not seem to have lysed. Beginning with frame 193 (Fig. 21) a protoplast was seen to slip from the lower end of the cell, becoming rounded. This protoplast survived until frame 345 when it abruptly lysed.

Growth in the presence of D-cycloserine resulted in a gradual local increase in the diameter of cells, usually at one of the poles.

Eventually many banjo-shaped cells reminiscent of partially inflated tubular balloons could be seen, some of which underwent shrinkage prior to lysis. For example the swollen cell c shrank at frame 371 and lysed at frame 416. The diameters of the swollen regions became as large as 1.7 μm or about twice the normal value. The cylindrical portion of such cells was less refractive than the swollen spherical bulb probably because it was not as thick. There was no indication that the protoplast had migrated from the cylindrical region into the bulb: abrupt expansion of the bulb was not seen, nor was the cylindrical part observed to become suddenly less dense. Thus it seemed probable that the protoplast filled the entire cell wall. When banjo-shaped cell d lysed at frame 306, the protoplasm was expelled from the pole opposite the swollen bulb. Lysis of other swollen cells seemed diffuse but usually occurred within the duration of one frame interval or 30 seconds.

Polymyxin

LA 11-1 (Figs. 23-24) showed the effect of 200 μg/ml polymyxin B sulfate filmed at 3 fpm for 7.5 hours beginning 10 minutes after inoculation. Growth of most cells was apparent. At frame 300 cell a was converted to a protoplast through the upper pole; it had not been observed to shrink. At irregular intervals most of the cells were seen to shrink and eventually lyse. Cell b seemed to shrink twice, gradually during frames 60-80 from 3.5 to 3.3 μm then more abruptly during frames 190-194 from 3.5 to 3.0 μm. The average decrease in length of several randomly chosen cells was 11%. Shrinkage usually required 2 frames (approximately 40 seconds). Lysis was sometimes abrupt, requiring but a single

frame; but more often it was diffuse and gradual.

Circulin

The effect of circulin at 500 µg/ml was shown by LA 10-3. Filming was begun 5 minutes after inoculation and continued at 2 fpm for 12 hours. The initial growth slowed and ceased after about 1 hour. Several cells shrank at irregular intervals, averaging 12%. By the end of the sequence two cells had lysed, both rather abruptly.

Chloramphenicol

LA 9-2 recorded the effect of chloramphenicol at 50 µg/ml. This sequence was started 5 minutes after inoculation and continued for 7.5 hours at 2 fpm. After very slow initial growth subsided, about half of the cells shrank at irregular intervals. The length decrease averaged 15%. When the field was checked at 15 hours post-inoculation, several cells had lost density and appeared to have lysed.

Discussion

The inability to distinguish some septa within a chain caused difficulty in significantly expressing the degree of shrinkage. Cells longer than 6 µm probably consisted of two or more units although a septum often could not be discerned. Frequently during shrinkage only a portion of such "cells" seemed to change, further implying that the aggregate length involved more than one cell unit. When the septum could not be distinguished, the entire length was used as the basis for calculating the percent shrinkage. Therefore, if the entire length had not shrunk, the percent shrinkage was artificially low. Consequently

this datum is intended only as a rough guide and should not be regarded as a precise and reliable statistic for comparing the effects of various agents.

Due to the difficulty of these experiments, relatively few concentrations of the test agents were tried. No organized attempt was made to determine dose-response patterns or if a threshold effect was involved. In general, the minimum growth-inhibitory concentration for an agent was estimated from the literature, and this concentration was employed in initial trials. Although some adjustment may have been required in subsequent trials in an effort to obtain shrinkage, insufficient information was available for conclusions regarding optimum concentrations resulting in either shrinkage or lysis. Typically both shrinkage and lysis resulted from concentrations of the antibiotics which retarded growth.

Two general patterns of lysis seemed apparent; these could be termed point and diffuse. Point lysis was characterized by local extrusion of protoplasmic materials with apparent force from a single region on the cell periphery, most commonly a pole. That this event was forceful was concluded because small particles were seen streaming from the cell along rather straight trajectories to distances of several μm. In some instances, point lysis appeared to disturb nearby cells and particles as well as to propel the lysing cell. Theoretically, if the frictional forces immobilizing the cells were suddenly disturbed by the release of protoplasmic materials, Brownian motion might account for abrupt random movements. However, the films left a rather convincing impression that the event was more organized. The lysing cell and the released particles often moved away from the point of lysis in

opposite directions as though in accordance with Newton's third law of motion (to every action there is an equal and opposite reaction) before ceasing movement. Thus forceful seemed an appropriate adjective. Turgor pressure could be the origin of such a force. After point lysis, the residual ghost was typically quite transparent, retaining little internal definition. Such ghosts never showed further biological integrity and often faded away.

Diffuse lysis apparently was less catastrophic. Density usually declined gradually over many frames. In the less common instances in which diffuse lysis was abrupt, a phase-dark material spread as an expanding halo about the cell and disappeared within a very few frames. Particles were never seen leaving the cell, nor were neighboring cells and particles disturbed. Diffuse lysis usually left a persistent granular ghost. An unusual and interesting case was cell c in LA 6-3 which underwent diffuse lysis, then later liberated a protoplast which retained turgor. The cell membrane may have ruptured via a relatively minor lesion at a hidden locus beneath or about the cell, later becoming repaired. On the other hand, a sudden and reversible fluctuation in permeability might account for the efflux of liquid without the permanent loss of membrane integrity. This hypothesis was consistent with the absence of discernible particles exiting the cell and with the time available for diffuse lysis, since at 2 fpm an event may actually require up to 30 seconds, yet appear instantaneous when projected. Although the liberation of a protoplast by cells which have undergone diffuse lysis was rare, an intact protoplast may have commonly survived within the wall of such cells and account for their intermediate refractility.

Since both types of lysis were usually seen regardless of the lytic agent, the basic mechanisms may be similar. Whether a cell exhibits point lysis rather than diffuse lysis may depend upon such factors as its turgor and autolytic enzyme activity. Theoretically, local attack by autolysins in the presence of strong turgor pressure would favor point lysis, and the damage wrought by the explosive outrush of protoplasm would seem severe.

That a liberated protoplast can survive for any length of time in the absence of conventional osmotic or membrane stabilizers is also surprising. Perhaps in the rare instances in which survival occurred, the agar medium provided mechanical stabilization in lieu of an intact cell wall. Such protoplasts were rarely spherical, even after they had been seen slipping from a cell wall, and did not continue to expand. Thus, mechanical influence of the agar medium seemed probable. Persistence of a protoplast in the presence of polymyxin, a known membrane perturbant, seemed particularly curious, since this antibiotic has been reported to cause breaks in the cell membrane.

The swelling of cell diameters during growth in the presence of D-cycloserine was quite remarkable and was not seen with any other agent tested. The normal growth pattern seemed to be reversed in that as the diameter of such a cell began to rapidly increase, its length became static. The swelling was gradual and, from all appearances, the result of a modified pattern of cell wall biosynthesis. The bulged regions attained twice normal diameters without lysing, suggesting that the swollen wall either retained considerable strength or that turgor pressure was deficient. The latter seemed unlikely since point lysis often appeared quite explosive. Point lysis was usually seen in the cylindrical

region rather than the swollen bulb. This further supported the concept of a strong cell wall in the swollen region, since the total force exerted upon this zone would have been much greater than the force exerted upon the cylindrical part assuming that osmotic turgor was uniform throughout the cell. Diffuse lysis was also common in the presence of D-cycloserine and frequently was observed in only one or two frames. Due to its diffuse appearance and unknown nature it was not possible to determine if this type of lysis directly involved the swollen bulb.

Shrinkage and lysis thus were observed after growth in the presence of a variety of antibiotics notwithstanding their different mechanisms of action. It seemed possible that the physiological stress exerted by these drugs might have caused unbalanced growth, and that the observed effects followed excessive autolytic enzyme activity.

Autolysis Induced by Limited Aeration

To test the hypothetical association between autolysis and shrinkage, it was desirable to obtain autolysis without using antibiotics. Since B. subtilis W 23 has a strict requirement for oxygen and tends to autolyze in its absence, limiting the oxygen to a growing slide culture was considered a promising approach.

A modified sealed slide culture was prepared. The edges of the glucose salts agar block were heavily inoculated from a 10 hour slant culture, and the surface was inoculated as usual from an exponential phase liquid culture. A glass cover slip was used rather than one of Teflon, and the edges were sealed with petrolatum. The rationale was that the respiration of the concentrated growing cells would rapidly lower the dissolved oxygen tension until growth would be inhibited and

autolysis would be promoted.

LA 10-5 (Figs. 25-28) showed the results of such an experiment. Filming was begun 15 minutes after inoculation and was continued for 3.75 hours at 2 fpm. All of the cells grew for about 45 minutes, and then growth subsided and had ceased by about frame 100. Some 35 minutes later, shrinkage began at frame 169, and each cell unit had shrunk by frame 219--a period spanning 25 minutes. All cells then lysed in a gradual diffuse manner. Lysis was complete by frame 300.

To obtain simultaneous autolysis, the heavy inoculum around the edge of the agar block was essential, presumably because the respiration of a large number of cells was necessary to rapidly deplete the oxygen. It is conceivable that nutrient exhaustion and waste product accumulation due to the heavy peripheral inoculum also influenced the observed autolysis. Attempts to achieve autolysis on agar blocks of Difco Brewer Thioglycollate Agar were not successful; however, the reducing agents contained in this medium may have become oxidized in the air before the slide culture could be prepared and sealed.

Since shrinkage and lysis could be obtained without the use of any antibiotic or other added growth inhibitor, it was clear that shrinkage was not a direct effect of such compounds. Rather it seemed likely that shrinkage was a secondary consequence of environmental conditions which exerted physiological stress, especially those circumstances which abruptly inhibited growth and promoted autolysis.

Effect of Pre-Inhibition of Protein Synthesis

In view of the report by Rogers and Forsberg (1971) that chloramphenicol treatment of B. subtilis for two normal generation times

prevented lysis in the presence of vancomycin evidently because autolysin synthesis had been inhibited, it was of interest to determine if shrinkage could likewise be prevented. Preliminary experiments established that after adding 50 μg/ml chloramphenicol to an exponential phase glucose salts culture at an A_{540} of 0.3, the increase in turbidity slowed and the absorbancy stabilized at about 0.4 within 1-1.5 hours. The cells were then concentrated by centrifugation (10,000 x g for 5 minutes) or used directly to inoculate the slide cultures.

The slide culture medium in sequence LA 10-1 contained 50 μg/ml chloramphenicol and 15 μg/ml vancomycin. The inoculum cells had been incubated with 50 μg/ml chloramphenicol for 3.5 hours and concentrated by centrifugation. The filming of this sequence was begun at 5 minutes after inoculation and continued for 7 hours at 2 fpm. No growth was observed. Of 14 cells, 9 shrank at irregular intervals between frames 21 and 405. Shrinkage typically required 1 or 2 frames. One cell had lysed before the film began and another was seen to fade very gradually. No other cells lysed, even after 36 hours. Thus, this sequence indicated that exposure of the cells to chloramphenicol before exposure to vancomycin prevented growth and lysis but did not prevent shrinkage. A control experiment (LA 21-2) which employed both antibiotics in the slide culture medium but used fresh cells not pre-exposed to chloramphenicol also resulted in no detectable growth but did show increased lysis (7 of 19 cells), in some cases after shrinkage. LA 21-3 was similar to LA 10-1 except that only 2 hours of prior exposure to chloramphenicol were used, and the inoculum was not centrifuged; the result was almost identical. Increasing the chloramphenicol concentration to 75 μg/ml and decreasing the exposure time to 1 hour produced an equivalent result in LA 25-2.

Most of the cells shrank but did not grow or lyse.

Chloramphenicol inhibited the growth of the inoculum culture, and no cellular growth was seen in the slide cultures. The study by Rogers and Forsberg (1971) demonstrated that such cells were autolysin-deficient. The near-lack of lysis, even after a very long interval in the presence of vancomycin, could thus have resulted from the inability or reduced ability of the cells to synthesize autolytic enzymes in the presence of chloramphenicol. Possibly some autolytic activity was present, but it was apparently insufficient to cause lysis.

Shrinkage, however, was not prevented by inhibition of protein synthesis. This observation suggested that either shrinkage required a lower concentration of autolysins than lysis or perhaps that the mechanism of shrinkage was not dependent on autolytic enzyme activity.

Lysozyme Action

Effect of Lysozyme in Sealed Slide Cultures

It seemed possible that the shrinkage observed during autolysis was initiated by the action of one or more of several autolytic enzymes. Lysozyme was used to explore this possibility since it also attacks the cell wall. Although hydrolysis of a different bond is involved, the general effect of lysozyme was expected to resemble that of the autolytic glycosidase because the cell wall glycan moiety is cleaved by either enzyme.

To preserve consistent activity of lysozyme, technical aspects of these experiments were a bit more critical. The melted agar medium was cooled quickly after adding lysozyme to avoid unnecessary heating of this enzyme. Although there was no evidence of loss of activity

resulting from storage at 4 C, the medium was prepared fresh daily for these experiments. Preliminary trials established that a useful concentration range for lysozyme existed. Above 100 µg/ml lysis occurred too quickly and was often noticeably in progress before a suitable microscopic field could be selected and filming could be initiated. On the other hand, concentrations below 50 µg/ml gave inconsistent results, with cells sometimes growing without lysing. Perhaps the failures at lower concentrations resulted from the destruction of the enzyme by bacterial proteases or from adsorption of most of the lysozyme molecules to agar.

Besides the usual glucose salts agar basal medium, 0.8% aqueous agar or 0.8% agar prepared in 5 mM potassium phosphate buffer, pH 7.0, were also employed as slide culture media in these studies. Control experiments without lysozyme showed that no detectable change (such as shrinkage or autolysis) occurred except on glucose salts agar, which supported growth. The effects of lysozyme were similar with each of the three agar compositions.

The action of lysozyme at 100 µg/ml in aqueous agar was noted in LA 13-3 (Figs. 29-32). Filming of this sequence was begun 5 minutes after inoculation and continued for 21 minutes at 6 fpm. The magnification on the motion picture film for this sequence was 375 X, consequantly the cells appeared 1.5 X larger than on the usual 250 X. Some events may have transpired during the 5 minutes required to begin the sequences. In the initial frames the cells were uniformly dark. Then they progressively lost phase density and increased in size, especially in diameter, until they could barely be discerned against the background. For example cell a measured 0.8 x 2.5 µm in the first frame,

0.9 x 2.8 μm in frame 35, 1.1 x 3.1 μm in frame 70, and 1.3 x 3.3 μm by frame 105. During those 17 minutes, cell a had thus increased 32% in length and 63% in diameter. Lysis of all the cells was diffuse and gradual. Shrinkage was not seen in this sequence, but the possibility remained that it had been missed before the camera had been started. The interval between inoculation and the exposure of the first frame needed to be reduced to explore this possibility.

To expedite slide culture preparation, the petrolatum seal was omitted and a glass cover slip was used. Evaporation from the agar block caused focus instability which necessitated constant attention, but this arrangement was generally satisfactory for brief sequences. In LA 17-1 through 7 the camera was started 2 minutes after inoculation.

Some shrinkage was detected during the action of lysozyme, perhaps because the camera had been started sooner. For example in LA 17-1 (Figs. 33-34, 20 fpm) cell a shrank at frame 17 and cell b shrank at frame 41, each about 8%. Other cells were not observed to shrink, and all eventually expanded and became pale. The agar block contained 75 μg/ml lysozyme in 0.8% agar buffered to pH 7.0 with 5 mM potassium phosphate.

Influence of Mg^{++} on Lysozyme Action

Because Mg^{++} is known to increase the stability of bacterial protoplasts, it was desired to determine the effect of Mg^{++} on the action of lysozyme. The series LA 17-1 through 7 portrayed the dose-response to $MgCl_2$. The $MgCl_2$ concentration was varied at exponential intervals from 10^{-7} M in LA 17-2 to 10^{-2} M in LA 17-7. LA 17-1 and 2 were filmed at 20 fpm; the remaining sequences were filmed at 30 fpm. Other condi-

tions were as uniform as possible. $MgCl_2$ had no apparent effect at 10^{-4} M or less; only one or two cells shrank, often quite early. Approximately half (6 of 13) of the cell population shrank by frame 47 in the presence of 10^{-3} M $MgCl_2$ (LA 17-6). Most (10 of 12) of the cells shrank during the sequence using 10^{-2} M $MgCl_2$ (LA 17-7). The initiation and progress of lysis seemed delayed at this highest concentration, and one cell pair failed to shrink or lyse by the end of the sequence at frame 800 (27 minutes). Material was ejected from several cells during lysis. Shrinkage always preceded lysis, which usually followed immediately. The average shrinkage was 14%.

These results suggested that at the higher concentrations $MgCl_2$ inhibited lysozyme action and may have enhanced the detection of shrinkage. Although these trends were repeatable, the mechanism was obscure. The inhibition of lysozyme lysis could have resulted from effects upon the enzyme, upon the cell wall substrate, or upon intervening surface polymers. The fact that more shrinkage could be seen at higher $MgCl_2$ concentrations may have been derived from the inhibition of lysis so that early lytic events, perhaps including shrinkage, did not occur before filming began. It is also possible that the cell membrane has a major role in the shrinkage phenomenon, and that Mg^{++} stabilization of the membrane delayed the onset of shrinkage or enhanced its detection.

Effect of Lysozyme on Cells Treated with Heat or Formaldehyde

To further explore the possibility of cell membrane involvement in the shrinkage associated with lysozyme lysis, cells were subjected to lethal and supposedly membrane-destructive treatments (exposure to

formaldehyde or heat) before filming in the presence of lysozyme.

An exponential phase glucose salts culture was incubated with 10% (v/v) formalin for 15 minutes at room temperature, washed once with three volumes of water, and then suspended in water. LA 18-5 (Figs. 35-36) followed the fate of those cells inoculated onto the phosphate-buffered agar containing 75 µg/ml lysozyme. The filming was begun 6 minutes post-inoculation and continued 84 minutes at 5 fpm. No shrinkage was seen. The cells gradually expanded in a manner similar to the lysozyme effect previously seen except that the density of the cytoplasm became rather uneven. The phase halo gradually diminished. Cell a was typical and expanded from 0.7 x 1.8 µm to 1.4 x 2.5 um during the sequence. The smaller initial size of the cells suggested that shrinkage may have occurred prior to the sequence, very probably during the formalin treatment phase.

A similar exponential phase culture was heated to 121 C for 10 minutes in an autoclave and used to inoculate a phosphate buffered agar block containing 75 µg/ml lysozyme. Exposures in LA 18-6 (Figs. 37-38) were started 5 minutes after inoculation and continued for 109 minutes at 10 fpm. Again, no shrinkage could be seen. The phase-dark portion of the cells did not change size during the entire sequence. A typical cell measured 0.7 x 1.7 µm. Some activity during the first 100 frames was indicated by the gradual separation of cell units in the chains. Presumably this was due to lysozyme action upon transparent cell wall material in the intercellular gaps. Detail within these gaps was obscured by a rather prominent phase halo.

Heat thus appeared to have rendered the cell rigid. The darkness of the cytoplasm and the large and static phase halo suggested that such

cells retained their initial thickness throughout the sequence. Formaldehyde-treated cells were apparently more flexible since the cytoplasm seemed to expand and lose density as the phase halo diminished.

The smaller than normal size of the cells at the beginning of these sequences and the lack of shrinkage during them indicated that shrinkage may have occurred during the formalin and heat treatment steps. Both of these microbicidal agents were rigorous and should have caused extensive denaturation of macromolecules, especially proteins. The formalin-treated cells expanded, and it seemed clear that the autolytic enzymes had no responsibility for that action. Also the separation of adjacent autoclaved cell units indicated that heating had not destroyed the lysozyme substrate.

Action of Lysozyme in the Wick Slide Culture

The wick slide culture technique was devised to resolve the question of whether early events, such as shrinkage, had been missed before observations and filming could begin. Because this method permitted the introduction into the system of a test agent such as lysozyme after field selection and while filming was in progress, all shrinkage due to the agent could be detected.

In LA 37-5 the agar block medium was glucose salts agar less the glucose and mineral salts solution. Lysozyme at 1 mg/ml was added to the wick as the camera was started (frame 1). The filming rate was 6 fpm. Definite expansion of cells was noted by frame 70, indicating that lysozyme had reached the cells. Between frames 85 and 100 at least five cells shrank, each within a single frame interval. Such cells typically began to expand and lose density, abruptly shrank and became darker,

then resumed expansion until they soon could not be distinguished from cells which had not shrunk. The average shrinkage was 28% of width and 10% of length. Thus the wick culture method permitted detection of shrinkage even in the absence of Mg^{++} in the medium. By frame 117 all of the cells had expanded extensively and their phase halo had concomitantly diminished. The cytoplasm was lighter but homogeneous.

In an effort to determine if such cells possessed an osmotic barrier and retained turgor, polymyxin at 500 µg/ml was added at frame 117. Due to its smaller relative molecular size, polymyxin was expected to reach the cells more rapidly than had lysozyme. The cells continued to expand. Then at frame 137 the cytoplasm abruptly became quite granular. Progressive expansion continued without any sudden dimensional change resembling either shrinkage or lysis. The abrupt granulation suggested that polymyxin had reached the cells under observation and caused their disintegration via membrane action. The absence of an abrupt change in dimensions implied that the lysozyme treatment had resulted in loss of osmotic integrity and turgor.

Discussion of the Lysozyme Experiments

The observations that lysozyme caused the expansion of cells, and that the diameters increased by a greater factor than the lengths, were intriguing. During expansion the gradual decline of phase density and halo prominence was regularly seen.

Since lysozyme promotes the cleavage of cell wall glycan chains, the dramatic increase in diameter suggests that these chains may run circumferentially. As the wall peptidoglycan became weakened, it could be stretched by the turgid protoplast. Kelemen and Rogers (1971) have

used molecular models to propose possible arrangements of units within the peptidoglycan of gram-positive bacilli consistent with various biological and physico-chemical data. They proposed three possible orientations for the glycan chains: circumferential, longitudinal, and radial. The appearance of lysozyme action did not support the longitudinal arrangement, since the relative increase in length was not as great as that shown by the diameter. The radial arrangement was consistent with the observed action of lysozyme only if the cell wall were part of the phase-dark measured zone so that thickening of the cell wall during glycan cleavage would result in an apparent increase in cell diameter. However, the transparent appearance of lysed cells, isolated cell walls, and gaps between autoclaved cells suggested that the cell wall was poorly visible under these conditions of microscopy and perhaps was not included in the measured zone. Consequently the circumferential glycan arrangement seemed most consistent with the lysozyme data.

It was possible, however, to explain the data even without obligatory expansion of the cell wall. If turgor and protoplasm were lost so that the cylindrical shape collapsed into an essentially planar configuration, the cell diameter would appear to increase from 0.8 μm to half the circumference, or 1.25 μm. This mechanism seemed consistent with the observed dimensional change and the decline in refractility. It did seem improbable, however, that the pressure of the agar medium against the cover slip could satisfactorily account for the consistent degree of spreading that was observed. These two hypothetical mechanisms explaining expansion are not mutually exclusive, and may, in fact, both contribute to the observed effect. Conceivably, initial surface distention might alter membrane permeability, allowing subsequent leakage

and collapse.

Shrinkage could result from the sudden leakage of liquid through a damaged cell membrane, relieving turgor and permitting the relaxation of elastic surface structures such as the cell membrane and possibly the cell wall. Since nothing could be discerned exiting the cell during shrinkage, either in a diffuse or local manner, submicroscopic particles such as ribosomes were likely retained. The increased intracellular concentration of such particles might account for the increased phase density of cells seen just after shrinkage. The dependence of shrinkage upon turgor might explain why it was seen only during the early stage of lysozyme action, assuming that expansion was accompanied by loss of turgor. Membrane perturbation and osmotic manipulation of turgor therefore seemed promising experimental approaches to test the hypothetical shrinkage mechanism.

Effect of Membrane Active Agents in Wick Slide Cultures

To determine if destruction or perturbation of the cell membrane would induce shrinkage, various agents known to affect bacterial membranes were tested in wick slide cultures.

The standard wick culture system was used including glucose salts agar medium, an exponential phase inoculum, and a glass cover slip. Water was always the initial wick irrigant, and ordinarily a few minutes during this period were filmed to establish a control appearance before the introduction of the test agent. LA 33-5 was the control sequence to determine the effect of continued water irrigation. Normal growth continued during this sequence for as long as 110 minutes after

inoculation, thus demonstrating that the wick slide culture environment satisfied growth requirements for at least this duration. The absence of shrinkage and lysis from this control implied that aeration and nutrition remained adequate, and that physiological stress deriving from the method was not detectable.

As the test agent was introduced into the entrance reservoir, 2 or 3 blank frames were exposed to mark the addition. The time required for the agent to reach the vicinity of the observed cells was subject to many variables such as the concentration, molecular size and adsorption affinity of the test agent, the location of the specimen cells on the agar block, and the rate of evaporation from the exit wick. The time elapsing between the introduction of an agent and the appearance of its effect ranged approximately from 5 to 30 minutes.

The concentration of the agent reaching the cell was also subject to variables not under precise control in this method. Since the aim of this line of experimentation was to determine the morphological effect of sudden exposure to agents known to damage membranes, relatively high concentrations of such agents were added to the wick to ensure that the dosage eventually diffusing to the cells would be adequate to produce an insult to the cell. Under these circumstances it was expected that the concentration of the agent in the microenvironment of the observed cells would increase from zero to an effective level within a few seconds.

Table I summarizes the effect of various agents on cell size, using the average percent change of length for comparison. In most of these experiments every cell on the field, including the units comprising filaments, underwent a dimensional change within a short period of time.

TABLE I

EFFECT OF VARIOUS AGENTS ON CELLULAR LENGTH IN WICK SLIDE CULTURES

	Agent	Concentration	% Change*	Sequence
1.	Ethanol	95% v/v	-17 R	LA 29-4
2.	Acetone	100% v/v	-21 R	LA 42-5
3.	Formaldehyde	37% v/v	-25 S	LA 42-4
4.	$HgCl_2$	1% w/v	- 4 S	LA 41-5
5.	NaOH	1 N	-11 R	LA 42-1
6.	HCl	1 N	-23 R	LA 42-2
7.	TCA	1% w/v	-22 R	LA 34-2
8.	SLS	5% w/v	-13 R	LA 41-6
9.	DOC	5% w/v	-10 R	LA 41-7
10.	Triton X-100	1% v/v	- 8 S	LA 33-1
11.	Tween 80	5% v/v	- 5 S	LA 46-1
12.	CTAB	1% w/v	-16 R, +8 S	LA 42-7
13.	CPC	1% w/v	-18 R, +8 S	LA 42-8
14.	Polymyxin	1 mg/ml	-17 R	LA 32-3
15.	Circulin	1 mg/ml	-13 R	LA 33-4
16.	Vancomycin	120 µg/ml	-10 R	LA 33-2
17.	Penicillin	50 µg/ml	-14 R	LA 39-5
18.	Sucrose	2 M	- 7 S	LA 43-3
19.	NaCl	5 M	-20 S	LA 32-2

*Average percent change in length based on the initial length; R, rapid, requiring less than 1 minute per cell; S, slow, requiring more than 1 minute per cell.

Thus this concerted percent change in length would seem somewhat more significant than that measurable from sealed slide culture experiments wherein shrinkage was an unpredictable sporadic occurrence. Agents 1-15 were selected because they were all known to cause membrane damage at the concentrations employed. Other cellular lesions were also expected for many of these agents, but membrane action was a trait held in common. No attempt was made to standardize the pH or concentration, since that might have altered the activity attributed to these antimicrobial agents. All of the agents 1-15 caused dimensional changes; however, differences in their action could be seen, particularly with regard to the rate and extent of the observed shrinkage.

In the presence of the organic solvents ethanol and acetone all of the viewed cells shrank abruptly and nearly simultaneously. In addition to dissolving membrane lipids, both agents cause dehydration which should have precipitated proteins and perhaps led to loss of cytoplasmic volume due to water extraction. Concomitant with the shrinkage, a deterioration of the image optical quality was seen, and the focus became unstable. These difficulties perhaps resulted from the dehydration of the agar gel and an alteration of the refractive index of the medium.

The protein fixatives formaldehyde and $HgCl_2$ caused very gradual shrinkage. In the presence of formaldehyde the cells shrank 25%, but required 14 minutes for this slow activity. This bactericidal compound is known to fix protein structures by covalently linking amino groups. The shrinkage due to $HgCl_2$ was very slight (4%), gradual, and was only detected via comparative measurements. Heavy metal cations such as Hg^{++} link sulfhydryl groups, binding adjacent protein molecules. The

slowness of the shrinkage induced by these agents suggested that their action on membrane proteins may have retarded the loss of water and solutes. That formaldehyde caused the greatest degree of shrinkage (25%) implied that intermolecular bonding had caused contraction (not merely relaxation) of protein structures. $HgCl_2$, however, could be expected to produce a similar result, and an explanation for the observed difference was not obvious.

The 11% total shrinkage in the presence of the strong alkali NaOH seemed to consist of two phases: about half the shrinkage was lost very abruptly, and the remainder declined gradually during a 5 minute period. On the other hand, the strong acids HCl and TCA caused a characteristic optical "banding" of the cytoplasm followed by an abrupt shrinkage of approximately 22%. The banded appearance--characterized by alternate bright and dark zones--persisted after shrinkage, and the phase halo seemed accentuated. Such extremes of pH could be expected to inflict havoc upon the structure and function of many cellular organelles. Certainly the isoelectric precipitation of membrane proteins alone would severely alter permeability properties and could lead to leakage. Also, protonation of anionic groups of the cell wall could possibly lead to dimensional changes of this structure. For example, electrostatic repulsion between the normally electronegative groups of teichoic acid and peptidoglycan may contribute to an expanded wall structure. Protonation of such anionic groups would cancel this repulsive force, allowing increased wall compactness and dimensional contraction.

The surfactant properties of the six detergents tested would predict membrane action. The observed effects seemed correlated to the general type of detergent used: anionic, non-ionic, or cationic. The

anionic detergents SLS and DOC induced an abrupt shrinkage and no further change. The non-ionic compounds Triton X-100 and Tween 80 caused a very gradual, moderate shrinkage. The cationic detergents CTAB and CPC caused an abrupt shrinkage of approximately 17% followed by a gradual expansion of approximately 8%. This cellular expansion was quite interesting, since it implied the possible restoration of partial turgor after its apparent initial loss. Addition of water to the wick after CPC or CTAB caused no further change, suggesting that the expanded size was not dependent on a particular environmental concentration range of the detergents. Both of these cationic detergents are known to promote leakage of small molecules from cells. Restoration of turgor would seem to require replacement of leaked substances with osmotically active compounds and a retention or at least restoration of the osmotic function of the membrane. Restoration of all normal biological functions was unlikely since these two compounds are potent bactericides. The slower, moderate shrinkage in the presence of the less toxic non-ionic detergents also implied that a different mechanism prevailed. Although the basis for the qualitative differences in the observed shrinkage response to the various detergent types could not be elucidated, the relationship between shrinkage and the presence of a known membrane perturbant had been augmented.

The wick slide culture system was also used to compare the effects of polymyxin and circulin, which are known membrane perturbants, with those of vancomycin and penicillin, which interfere with cell wall biosynthesis. The two membrane active antibiotics caused an abrupt shrinkage similar to that seen with the anionic detergents and organic solvents. Each cell shrank within the span of 2 frames (12 seconds) and, in general,

all of the cells shrank within a few minutes. Such a concerted shrinkage of cells frequently exhibited a directional pattern across the field which implied the arrival and diffusion through the field of a critical concentration of the agent. In some such experiments an occasional cell shrank before the addition of the agent or before most of the cells shrank, probably as the result of autolytic changes triggered by unknown stresses. However, the general pattern exhibited by all of the agents 1-15, which were expected to cause membrane perturbation, was a concerted shrinkage (rapid or slow) of nearly all of the cells on the field. The immediacy of this characteristic action indicated a primary effect by the agent on cellular structures, particularly the cell membrane.

The pattern of the incidence of shrinkage resulting from the wick addition of vancomycin or penicillin was quite different: growth subsided, and shrinkage, then lysis, occurred at seemingly random intervals and locations on the field. This sporadic pattern implied that the shrinkage resulting from these two antibiotics was a secondary effect expressed only after cell wall lesions had resulted from unbalanced synthesis and autolytic activity. It is plausible that subtle variations in the physiological state of cells within the population viewed caused a differential susceptibility to the antibiotics which led to the varied development of the consequent cell wall lesions. The observation that such shrinkage occurred at random intervals over a relatively long time period was consistent with the hypothesis that shrinkage was the direct result of the sudden failure of the cell membrane. In the case of the antibiotic inhibitors of cell wall biosynthesis, this membrane failure appeared to await the development of lesions in the cell wall such that

the turgid protoplast could no longer be restrained.

Kinetics of the Shrinkage Induced by TCA

Strong acids such as TCA and HCl seemed particularly useful agents for studying the shrinkage phenomenon since the cytoplasmic banding effect could serve as a cue, signaling the contact of the agent with the cells. Typically this zebra-like appearance was noticeable a few frames prior to shrinkage, forewarning the operator that shrinkage was imminent. During shrinkage the cells became obviously smaller and somewhat more refractile. This increased refractility together with the cytoplasmic banding, both of which persisted afterward, identified cells that had shrunk.

By using these optical cues it was possible to film the shrinkage of cells in several fields of the same slide culture by merely skipping ahead of the advancing acid front as it diffused through the agar block. LA 34-2 showed six sequential fields from the same slide culture filmed at 10 fpm after adding 1% TCA. Four frames (24 seconds) were typically required for the shrinkage, which averaged 21%. The first field showed the paralyzing effect the acid had upon motility: concomitant with the banding, and some 20-30 seconds prior to their shrinkage, several loose, highly motile cells abruptly became quiescent. The TCA probably affected energy metabolism, the integrity of flagella and other cellular structures, and perhaps the tenacity of the compressed agar gel. Although it was not possible to determine what initially caused the abrupt immobilization, this striking effect emphasized the catastrophic nature of the events which accompanied TCA-induced shrinkage.

To reveal possible intermediate stages and to further characterize

the time-motion aspects of shrinkage, the effect of TCA was filmed at the highest rate available with the apparatus, 120 fpm. This rate was achieved by actuating the continuous run switch. Using this unorthodox procedure, the effective camera shutter speed was limited to approximately 0.2 second. To compensate for this abbreviated exposure, the lamp intensity was increased to give a light meter current of 2.5 μA, and the substage mechanical shutter was removed. To conserve film, the camera was started when banding was first noted. LA 35-3 included four sequential fields filmed from one slide culture. Figs. 39-41 respectively show a field before banding, after banding but prior to shrinkage, and after shrinkage. Figs. 42-43 show the shrinkage of another field after banding has occurred. Studied at this lower 8-fold acceleration, these sequences revealed that the shrinkage typically required 8 frames (4 seconds) per cell unit. Because not all of the units within a chain shrank simultaneously, long chains of cells shortened over a much longer period; nonetheless, the individual cell units appeared to exhibit similar kinetics, whether within a chain or not. Even after using this advanced filming rate, nothing could be seen leaving the cells during shrinkage. Because loss of material accompanied both diffuse and point lysis, shrinkage and lysis thus seemed fundamentally different.

The characteristic banding seen in the presence of acids was interesting, but its exact nature remained obscure. It probably was not an optical artifact due to the increase in refractive index of the medium since it was observed only with TCA and HCl and not with any of the other wick additives. Possibly these strong acids caused intracellular chemical changes which emphasized differences in the refractive index of cytoplasmic regions. For example, areas in which the cellular DNA is

concentrated are thought to be deficient in ribosomes and protein. A differential effect by the acid on protein-enriched as opposed to protein-deficient regions could lead to discontinuous zones of refractive index which would be rendered as light and dark bands under phase contrast microscopy. Direct effects on the nuclear material may also occur since aggregation of chromatin by environments of high ionic strength has been suggested (Whitfield and Murray, 1956).

Although some variation in banding patterns was seen, a correlation may exist between the dark bands and sites of septum formation. For example, individual cells exhibited a single central phase-dark zone. Cell pairs possessed three such dark areas which partitioned the pair into four equal lighter zones. Longer filaments displayed a similar pattern having equal spacing of the alternate light and dark zones. Thus their characteristic location and spacing suggested the possibility that the dark zones were associated with septa or pre-septal structures. Although the phenomenon was not further investigated in this study, the intriguing possibility that TCA had differentiated septum-related structures under phase contrast microscopy should be further tested, perhaps with division-inhibited filaments which electron microscopy has revealed to be without septa (Grula, Smith, and Grula, 1968).

Effect of Osmotic Effectors in the Wick Slide Culture

To investigate the possible relationship between osmotic turgor and shrinkage, plasmolysis of the cells was attempted by adding concentrated solutions of low toxicity to wick slide cultures.

Sucrose was considered a promising osmotic effector in view of its

popularity as a stabilizer of protoplasts. A previous experiment (LA 12-1) had incorporated 1 M sucrose in the glucose salts agar block of a sealed slide culture. The cells initially appeared pale and displayed a heterogeneous cytoplasm, suggesting that plasmolysis may have occurred before filming had been started. No shrinkage was obvious. Eventually some cells darkened and most began slow growth, evidence of the capacity to compensate for the high osmolarity of the medium.

LA 43-3 showed the effect of applying 2 M sucrose to the standard wick system. Filming was done at 10 fpm and sucrose was added at frame 1. Very gradual shrinkage ensued and reached approximately 7% by frame 350 (35 minutes). The syrup-like high viscosity of the 2 M sucrose solution was rather incompatible with the wick system. Evaporation caused the development of a thickened surface layer over the entrance reservoir which threatened to interfere with aeration. The exit wick eventually became dry, indicating that capillary flow through the system had practically ceased, perhaps exacerbated by sucrose crystallization. Therefore, although sucrose was a traditional osmotic effector, its manifest disadvantages in the wick system prompted the search for an alternate substance.

Ultimately 5 M NaCl was selected as a routine experimental osmotic effector. The high water solubility and low solution viscosity of this salt made it fairly compatible with the restrictions imposed by the wick slide culture technique. Also the high osmolarity of 5 M NaCl solutions offered better prospects for plasmolyzing the gram-positive test organism despite the great osmotic pressure reputed to such cells. The ionic nature of this salt did, however, raise the possibility of effects beyond simple plasmolysis.

LA 40-2 showed the effect of adding 5 M NaCl and water alternately to the wick system. The filming rate was 6 fpm, and water was the initial irrigant. The responses of filaments a and b seemed typical, and their average percent changes were reported relative to their size at frame 180 (30 minutes), when 5 M NaCl was first added. Figs. 44-50 and Table II summarize the dimensional changes observed. Filaments a and b appeared to behave as osmometers, gradually shrinking and swelling in response to the osmolarity of the external menstruum. By frame 319, at the end of the first exposure to 5 M NaCl, the protoplasts within several cells (such as those of a) appeared shrunken and retracted from the cell wall, especially at the ends, suggesting that plasmolysis had indeed occurred. After swelling in response to another water treatment and then shrinking again in response to the NaCl, the protoplasts once again appeared withdrawn at the ends.

Cell c abruptly shrank 10% at frame 149, before addition of NaCl. This fortuitous spontaneous shrinkage afforded the opportunity to determine what further effect NaCl and H_2O would have on a cell which had already shrunk in the conventional abrupt fashion. Table II shows that the salt-induced dimensional changes of cell c were much less than those of other cells. This implied that the spontaneous abrupt shrinkage had affected the osmotic barrier of this cell, and that turgor had been lost and could not be fully regained.

TCA (1%) was applied to the expanded cells in LA 40-2 to determine if they remained susceptible to this potent shrinkage agent. An abrupt shrinkage did occur in all cells although it was somewhat less pronounced than the 17-23% normally obtained with previously untreated specimens.

To determine if lower concentrations of NaCl were sufficient to

TABLE II

SUMMARY OF LA 40-2

Frames	Time in Minutes	Irrigant	Cells a, b Ave. % Change	Cell c % Change
1- 179	0- 30	H_2O	Growth	Spontaneous 10% Shrinkage
180- 319	30- 53	5 M NaCl	-17	-7
320- 519	53- 87	H_2O	+14	0
520- 619	87-103	5 M NaCl	-15	-1
620- 919	103-153	H_2O	+12	+4
920-1000	153-167	1% TCA	-16	-12

cause a similar effect resembling plasmolysis, a dose-response experimental series was done (LA 45-5 through 8). The average percent shrinkage obtained was related to the NaCl concentration as follows: 0.5 M, 7%; 1.0 M, 13%; 2.0 M, 16%; 5.0 M, 23%. Thus, the greatest degree of shrinkage was seen at the highest concentration used, 5 M. Moreover, the protoplast appeared most clearly shrunken at this value. Therefore 5 M NaCl was chosen as the agent for inducing plasmolysis.

Shrinkage of Heat-Treated Cells, Autolyzed Cells, and Isolated Cell Walls

In view of the results of LA 40-2 and the report that salt induces cell wall contraction (Marquis, 1968), control experiments were appropriate to determine to what extent the action of strongly ionic agents on the cell wall might contribute to the total shrinkage observed.

An exponential phase inoculum (5 ml) was heated to 121 C for 10 minutes in an autoclave to determine what effect heat denaturation of thermolabile molecules would have on the shrinkage caused by NaCl or TCA. LA 46-6 showed that 5 M NaCl caused a gradual 9% shrinkage or about half that seen with unheated cells (17-23%). The rather homogeneous cytoplasmic region showed no discernible change, and nothing resembling a contracted protoplast developed. Water was added to the wick after NaCl to determine if this would cause expansion. No size change was seen during 15 minutes. However, this period may have been inadequate to purge the salt from the system since about 30 minutes had been used to accomplish this in previous experiments (such as LA 40-2). LA 46-7 showed that 1% TCA caused a rather abrupt 12% shrinkage of heated cells which compared with 17-23% previously seen with unheated cells. During

shrinkage the cytoplasm became somewhat less homogeneous, but it did not display the obvious banding observed in unheated controls. A similar experiment, LA 46-8, showed that polymyxin at 1 mg/ml caused a very gradual 10% shrinkage of these heat-treated cells compared with an abrupt 17% shrinkage of unheated controls.

The effect of NaCl and TCA on cell walls isolated from the test organism was next determined. The cell wall preparation was generously provided by Donald F. Haslam. His isolation procedure involved mechanical rupture, heating to a boil in 1% SLS buffered to pH 9.0, trypsinization, washing, and lyophilization (Best and Durham, 1965; Haslam, 1973). A 1 mg/ml aqueous suspension of the lyophilized cell wall preparation was examined in the wick slide culture system. The image of the transparent cell walls was very faint and could barely be discerned. Although most of the cell walls appeared to have the general cylindrical form of intact cells, their transparency and tendency to cluster made precise measurements very difficult. A typical cell wall measured approximately 0.8 x 3.5 μm. LA 47-5 showed the effect of 5 M NaCl on these cell walls. Although no detectable change was noted during analysis projection, measurements indicated that a 4% average shrinkage occurred. The limited number of walls suitable for measuring and their poor visibility cast some doubt on the reliability of this figure, however, but some degree of shrinkage seemed evident. LA 47-6 showed the effect of 1% TCA on cell walls. An unfortunate slight drift in the microscopic field interfered with projection-analysis of the TCA effect. Comparative measurements indicated that TCA caused an abrupt 13% shrinkage. One central cell wall became separated as though it had been torn due to excess tension, perhaps as the result of shrinkage.

Although the image was poor, some shrinkage seemed definite albeit less than that observed using intact cells.

To investigate the effect of shrinkage agents on autolyzed cells an early stationary phase culture (A_{540}, 1.0) was vortex-mixed for 5 seconds to promote autolysis, then used immediately to inoculate a wick slide culture. Likely due to the combined physiological and mechanical stresses, a greater proportion of autolyzed cells was seen, and fields were sought which contained both intact and autolyzed specimens. LA 47-7 filmed the effect of 5 M NaCl, water, and then 1% TCA in sequence. The filament of intact cells showed a normal response to these agents in respective order: slow 23% shrinkage, slow 18% expansion, then rapid 19% shrinkage with banding. The response of the autolyzed filament was: slow 10% shrinkage, slow 7% expansion, then a rapid shrinkage and segmentation due to TCA that could not be measured because the filament ends could not be discerned. Analysis projection of this TCA action conveyed the impression that the cell wall shrank about 5%, while internal substances consolidated into much shorter phase-dark segmental units of an unknown nature. LA 47-8 showed the effect of subjecting intact and autolyzed cells to polymyxin at 1 mg/ml. Many of the dark cells on the field failed to shrink and were assumed to have shrunk spontaneously before filming began. However, roughly half of the dark cells did show a simultaneous and abrupt shrinkage of about 17% in response to the antibiotic. During this same interval, measurements indicated that the autolyzed cells shrank only 2%.

The effect of heat on the reactions to shrinkage agents was curious. The autoclaved cells appeared darker, more refractile, and slightly smaller than unheated controls, implying that they had shrunk during

the heat treatment. Extensive changes were expected to result from autoclaving, since this procedure is more than adequate to kill vegetative bacteria. The predicted effects of this moist heat treatment on the cellular proteins alone suggested that enzyme activity would be lost, that the structural and biological properties of the cell membrane would be radically altered, and that the cytoplasm would perhaps coagulate and therefore display increased rigidity. Indeed the static size of the phase-dark area of autoclaved cells treated with lysozyme (LA 18-6) had implied an increased structural rigidity, consequently the shrinkage noted in this experimental series was unexpected and not easily rationalized. Some degree of cytoplasmic rigidity was suggested by the absence of protoplast shriveling after NaCl and by the failure to display the usual banding after TCA. Any such implied rigidity was insufficient to prevent dimensional changes, however. The slow shrinkage due to polymyxin seemed anomalous, since heat had presumably destroyed the osmotic barrier, permitting leakage and loss of turgor. The polymyxin effect may reflect non-membrane sites of activity, at least in heat-treated cells. The shrinkage caused by the strongly ionic agents NaCl and TCA seemed attributable to electrostatic cell wall contraction (Marquis, 1968) rather than to plasmolytic relaxation, since heat was assumed to have destroyed the osmotic barrier. Although the direct effect of heat on the cell wall was presumed to be less drastic than on other structures, many indirect effects could be imagined. For example, the peptidoglycan molecular net could have become impacted with denatured protein molecules as the autoclave cooled, thus creating an entirely artificial barrier of unpredictable properties. The lack of detailed knowledge of the molecular effect of heat on cellular structures

therefore compromised the interpretations of the results with the autoclaved cells.

The isolated cell walls provided the most direct evidence of cell wall shrinkage, but their transparency made dimensional measurements very difficult. Autolyzed cells were better visible, likely due to the presence within them of refractile cytoplasmic remnants. With either type of cell wall specimen some alterations from the native state were possible due to the isolation procedure or to the action of autolytic enzymes. However, these hypothetical changes may have been minor, especially compared to those rendered by heat. These collected results implied that the cell wall had an active role in the shrinkage induced by NaCl, TCA, and possibly polymyxin, but that the amount of this wall-associated shrinkage could not account for the total effect observed in normal intact cells. The total shrinkage observed when an intact cell was subjected to an agent such as TCA could therefore represent the sum of two effects: one being a cell membrane perturbation resulting in the reduction of osmotic turgor and the consequential relaxation of the cell surface, and the other being contraction of the cell wall. The observation that the nature and the extent of the cell wall contraction depended on the shrinkage agent used may partially account for the quantitative and qualitative differences in shrinkage due to various agents (Table I).

Sequential Treatment with Shrinkage Agents

To determine if an additive effect due to different shrinkage agents could be demonstrated, cells were exposed to two or more agents in sequence.

LA 43-4 tested the effect of adding 1 mg/ml polymyxin, then 1% TCA. Polymyxin induced the typical abrupt shrinkage of cells, averaging 8%. TCA, added at frame 320 (32 minutes), after the polymyxin activity had ceased, caused an additional rapid shrinkage, averaging 10%. The shrinkage due to polymyxin may derive mainly from membrane action and therefore indicate the response to sudden relief of turgor. TCA caused additional shrinkage but less than half that when it was used alone. In this instance the 10% additional shrinkage due to TCA may indicate that portion of TCA action due to cell wall contraction. The fact that the sum of polymyxin and TCA shrinkage was less than that induced by TCA alone suggests that wall contraction simultaneous with turgor relief results in a more dramatic size change. In view of these observations, it seemed little wonder that 5% TCA is such an effective agent for extracting soluble "pool" compounds from bacteria.

In LA 46-5, 1 mg/ml polymyxin caused 12% abrupt shrinkage. Water was then added to determine if it would reverse the polymyxin effect. No change was seen during the 10 minutes of filming.

LA 43-2 recorded the effect of sequentially applying 1% TCA, 5 M NaCl, and then water. TCA caused a typical 22% abrupt shrinkage, leaving the cells banded. The addition of the NaCl caused a minor 2% shrinkage, detected only by measurement of the cells. No change was seen after water was added. Thus the changes rendered by TCA prevented significant additional shrinkage by NaCl, implying some related site of activity. It seemed likely that TCA not only destroyed the osmotic barrier, but also caused cell wall contraction, possibly by a mechanism related to that of NaCl.

Because the shrinkage in the presence of 5 M NaCl seemed partially

attributable to plasmolysis, it was of interest to determine if exposure to this agent could prevent, or at least attenuate, the shrinkage induced by other agents. LA 43-1 showed the effect of adding in sequence 5 M NaCl, 1% TCA, then water. NaCl caused a gradual shrinkage of 20%. The TCA effect was characterized first by cytoplasmic banding, then by a brief and slight expansion, followed by rapid shrinkage of approximately 5%. Water irrigation caused no further changes, and the banded appearance persisted. Thus the TCA shrinkage was considerably attenuated by prior exposure to NaCl. Plasmolysis and cell wall contraction may have both contributed to the attenuation of the TCA effect. The brief expansion before TCA shrinkage likely was due to plasmoptysis, since the aqueous TCA solution was not isotonic with the NaCl solution. It is interesting that the cytoplasmic banding appeared before expansion, indicating that TCA caused an internal effect prior to the putative osmotic swelling.

LA 40-1 was a similar experiment except that the NaCl treated cells were then treated with 1% TCA dissolved in 5 M NaCl in an attempt to better isolate the TCA effect from osmotic effects. A gradual 17% shrinkage resulted from 5 M NaCl. The effect of the TCA in 5 M NaCl included an abrupt cytoplasmic banding and 4% shrinkage. Expansion did not precede or accompany the TCA effect, further implying that the expansion seen in LA 43-1 had been osmotic swelling.

In LA 46-4, 5 M NaCl caused a slow 23% shrinkage. Then 1 mg/ml aqueous polymyxin was added. Expansion (13%) began as the NaCl concentration apparently declined, followed by a cell-by-cell 7% abrupt shrinkage which prevented further expansion to the original size. The net effect was a 16% shrinkage from the initial size which compared with

12% when polymyxin was added singly. The impression conveyed by viewing this sequence was that osmotic turgor was only partially restored when the polymyxin concentration became sufficient to disrupt the osmotic barrier, relieving turgor. The fact that the net shrinkage was greater than that exhibited by polymyxin alone suggests that cell wall stretching due to turgor is required for the complete reversal of the NaCl shrinkage.

Effect of TCA and Polymyxin on Gram-Negative Bacteria

Because the gram-negative bacteria are thought to have perhaps 12-fold less turgor than gram-positive species, the effects of TCA and polymyxin on E. coli B and P. fluorescens NND were filmed in the wick slide culture system. LA 43-6 showed that 1% TCA caused a very gradual 10% shrinkage of E. coli B over an 18 minute period. TCA produced a similar gradual 10% shrinkage with P. fluorescens NND (LA 43-5). Polymyxin at 1 mg/ml caused a similar gradual shrinkage of E. coli B (LA 45-3, 13% during 30 minutes) and P. fluorescens NND (LA 45-2, 16% during 30 minutes).

A lower turgor pressure in these gram-negative species may indeed contribute to the relatively subdued shrinkage observed, yet other comparative properties may also have influenced this result. The complex gram-negative bacterial envelope probably retards the penetration of polymyxin, delaying its effect on the osmotic barrier. The action of TCA on the gram-negative surface is conjectoral, but the relatively thinner peptidoglycan region might be expected to display less contraction. That the percent shrinkage obtained with polymyxin exceeded that

of TCA was surprising. However, polymyxin has been shown to induce structural changes in the outermost layer of the gram-negative envelope (Koike, Iida, and Matsuo, 1969), and these effects may contribute to dimensional change. Thus, even though the shrinkage seen with the gram-negative species was rather slower and weaker than that observed with B. subtilis W 23, the extensive differences besides turgor which distinguish these kinds of bacteria render this result rather inconclusive.

Studies on the Release of Substances During Shrinkage

The pronounced dimensional changes observed during shrinkage implied very significant decreases in cell volume. For an 0.8 x 3.0 μm cell with the shape of the test organism, calculations showed that a 10% shrinkage of both length and diameter resulted in a 33% decrease in volume, 15% shrinkage gave 49% less volume, 20% shrinkage gave 57% less volume, and 25% shrinkage yielded 65% less volume. Since liquids are essentially non-compressible, considerable leakage was assumed to occur during shrinkage. Nothing was ever seen leaving the cell during shrinkage even at the 120 fpm rate employed in one experiment using TCA (LA 35-3). Attempts to detect leakage by surrounding cells with washed india ink particles revealed no convincing disturbance during shrinkage induced by vancomycin or TCA. This method may not have been sufficiently sensitive, however, because the carbon particles usually seemed immobilized due to adherence to the agar gel or cover slip. If more liquid was introduced during inoculation, the particles then exhibited constant Brownian motion which, as would be expected, appeared completely random

in the time-lapse films. Using a different approach, the incorporation of the reduction indicator 2,3,5-triphenyl tetrazolium chloride at 0.25% w/v in the glucose salts agar block failed to reveal any local zones of color surrounding cells during shrinkage. The inability to detect leakage during shrinkage by these two rather unconventional methods was interpreted to indicate inadequate methodology rather than the absence of leakage. Translational movements of entire single cells was almost never noted during shrinkage. Nor was shrinkage seen to disturb surrounding debris. These observations suggest that the hypothetical leakage accompanying shrinkage was either too gradual or too diffuse to develop a force sufficient to disturb debris or to shift the entire cell. No particles or jets of refractile substances could be seen exiting the cell during shrinkage, suggesting that only water and small molecular weight solutes comprised the hypothetical efflux. Of course, such negative evidence was inconclusive, but the general hypothesis of the gradual leakage of soluble substances during shrinkage was consistent with the available data.

Correlation of Shrinkage with Lethality

Beyond characterizing the physical mechanism of shrinkage, it was also of interest to determine the effect of this intriguing event on the potential for cellular growth. Although no growth was ever observed after the definite shrinkage of a cell, most of the agents used to cause shrinkage were of themselves known to be bactericidal. With the sealed slide culture technique, the shrinkage agent remained in the cellular environment exerting continued biological stress which would preclude the possibility of growth. A reasonable opportunity for growth after

shrinkage was not afforded by the conditions of the typical wick slide culture experiment either, owing to the severity of the chemical insult that the cells received and to the rather brief term of these experiments, which did not allow much time for recovery. Because several features of the wick slide culture technique made it unsuitable for long experiments, sealed slide cultures were used in further efforts to promote shrinkage under circumstances which would not necessarily preclude growth.

Several attempts were made to induce temporary microaerophilic conditions in the hope that minor autolytic events would include shrinkage, followed by growth instead of lysis. A heavy inoculum was employed to consume most of the dissolved oxygen of the medium, as described earlier. A Teflon cover slip was used, but its surface was completely covered with immersion oil to limit the infusion of atmospheric oxygen. To restore aeration, the excess oil was removed by blotting, exercising great care not to shift the field. Because neither shrinkage nor growth rate could be assessed during the experiment, the timing of the oil removal was largely a matter of trial and error. A spectrum of results was obtained from these experiments, ranging from normal growth to the absence of growth, followed by autolysis. Cells which shrank were rare, and subsequent growth of these was never observed. Although this method was promising, many more such experiments might have to be conducted to ascertain the possibility of non-lethal shrinkage achieved via redox potential manipulation.

Another approach was taken to diminish the long-term effects due to the shrinkage agent. The liquid inoculum was briefly treated with a relatively mild dose of a cell wall active antibiotic to permit its

adsorption, then a glucose salts agar block without the drug was inoculated. The rationale was that much of the antibiotic might elute from the cell after inducing minor wall lesions and shrinkage, thus enhancing the prognosis for repair and growth. Several exposures to vancomycin were tried: 30 μg/ml for 5 minutes, 5 μg/ml for 30 seconds, 1 μg/ml for 60 seconds, and 0.5 μg/ml for 15 seconds. The results ranged from normal growth to shrinkage and lysis. No cell was ever seen to undergo shrinkage followed by growth, however, even though some experiments were allowed to incubate overnight. In similar trials, cells were exposed to penicillin for 1 minute at either 35, 50, 75, or 100 μg/ml. At the higher penicillin dosages growth subsided within an hour, followed by shrinkage and lysis of most cells. In one of the trials (LA 48-1, which used a one minute exposure to penicillin at 50 μg/ml), the centermost cell pair grew normally, abruptly shrank 8% at frame 47, and then immediately resumed growth to the end of the sequence. Although the upper cell of this pair became somewhat swollen, the growth rate of both cells appeared normal. All of the other cells on the field exhibited growth without shrinking. This single example demonstrated that shrinkage is not always lethal, and that resumption of growth can occur quite soon. Conversely, the fact that growth after shrinkage was noted only once in many trials implies that shrinkage is an insult from which recovery is difficult under the experimental conditions employed.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Each of the two slide culture methods used in this study had both advantages and shortcomings. Except for gaseous exchange through the Teflon cover slip, the sealed slide culture was a closed system. Because test agents were of necessity incorporated in the agar medium, cells were exposed to them from the moment of inoculation, precluding filmed observations of their effects during the initial several minutes. Moreover, test agents could not be added or removed from sealed slide cultures during an experiment, a technical limitation which curtailed experimental design. On the other hand, the static aspect of the sealed slide cultures was a virtue in terms of better focus stability. When ambient temperature control was adequate, the focus on sealed slide cultures ordinarily required adjustment only at intervals of 10-15 minutes. Thus this method was more practical for long term observations over several hours, and was chosen for most studies of phenomena suspected to involve growth-related events.

In contrast, the wick slide culture technique afforded much experimental flexibility. It was possible to film the growth of cells followed by their response to several test agents in sequence. Because many variables influenced the concentration of wick-added agents reaching the cells under observation, relatively high concentrations were applied in order to ensure that the cells being filmed would receive an almost

simultaneous exposure to an effective dose. The enhanced experimental flexibility afforded by the wick slide culture system was obtained only through considerable sacrifice in focus stability. The same properties of this open system which facilitated the perfusion of substances through the agar block also permitted mechanical instability. Various influences likely contributing to this instability included evaporative loss of liquid, hydrolic pressure developed within the entrance reservoir, thermal fluctuation during additions, and the effect of test agents on the physical properties of the agar gel. Occasionally an experiment had to be terminated when such instability allowed the cells to become loose and drift out of the field. The principal disadvantage, however, was the inconvenience of constantly maintaining focus, typically adjusting each 10 seconds. Also the entrance reservoir required irrigation at frequent intervals. Because this method severely taxed the endurance of the operator and had an unknown effect on the autolytic mechanisms of cells, wick slide culture experiments were usually terminated within one hour. This method was considered best for assessing immediate effects of test agents on cell size and appearance, but was not judged ideal for filming protracted phenomena which depend on growth or autolysis.

The wick slide culture technique was original and was invented after a literature survey revealed no available microscopic method suitable for varying the bacterial environment which was sufficiently stable to allow time-lapse cinephotomicrography. Subsequently a related system was described which perfused a paraffin-sealed agar wedge with agents pumped through capillary tubing (Poos et al., 1972). The wick system would seem superior, since it is simpler to construct, allows a

thinner agar block and consequently better optical properties, and is not subject to the focus instability which would likely accompany pump pressure fluctuation. Thus the wick slide culture seems the best device for studies of this type, and indeed it may be the only such device with sufficient stability to support time-lapse cinephotomicrography. This development in methodology was therefore considered a quite important discovery issuing from this dissertation research. By permitting time-lapse cinephotomicrography of cells while their chemical environment is varied, the wick slide culture system could be an asset to many types of biological research.

All of the initial experiments in this study used sealed slide cultures, however, and much valuable information was gained from this approach. A survey established that a wide variety of antibiotics (vancomycin, ristocetins A and B, penicillin, D-cycloserine, polymyxin, circulin, and chloramphenicol) led to abrupt shrinkage and lysis. The general pattern was that cells underwent a single abrupt (less than 1 minute) shrinkage of approximately 15% (length and width) followed eventually by either point or diffuse lysis. The intervals before shrinkage and between shrinkage and lysis varied widely among cells. This variation in the timing of these events, together with the variety of antibiotics which could elicit these responses, suggested that shrinkage and lysis were secondary consequences of some primary event triggered by physiological stress. This hypothesis was augmented by demonstrating that shrinkage and lysis ensued if air was suddenly withheld from a dense exponential phase slide culture growing in the absence of any antibiotics. Thus, shrinkage appeared to be an autolytic event precipitated by physiological stress rather than by any specific agent.

Lysis, but not shrinkage, was inhibited by prior incubation of cells with chloramphenicol to suppress autolysin formation before exposure to vancomycin in the slide culture. This result suggested that shrinkage was less dependent upon autolytic enzymes than was lysis.

Because, like the autolytic enzymes, lysozyme catalyzes peptidoglycan degradation, its effect was examined in sealed slide cultures. Lysozyme caused brief cellular expansion, slight but abrupt shrinkage, then further expansion and gradual fading. Recognizing that lysozyme results in the cleavage of the glycan chains, the fact that this agent caused greater expansion of the diameter than the length suggests that the glycan chains may have a circumferential orientation in the cell wall, one of three structural models proposed by Kelemen and Rogers (1971). Shrinkage was not seen if the cells were first treated with formalin or heat, but in these experiments shrinkage likely had occurred before exposure to the lysozyme. $MgCl_2$ delayed the initiation of lysozyme action, enhanced the detection of shrinkage, and promoted a more explosive lysis. Although this result was subject to several interpretations it seemed possible that the cell membrane had a direct involvement in shrinkage, and that temporary stabilization of the membrane by Mg^{++} potentiated the shrinkage.

To further explore the possibility that shrinkage was a manifestation of cell membrane perturbation, the wick slide culture was employed to survey the effect of a variety of membrane active compounds. These included organic solvents, protein fixatives, strong acids and an alkali, detergents, and antibiotics. The organic solvents, acids, anionic and cationic detergents, as well as polymyxin and circulin all caused a concerted, rapid shrinkage of the cells. A concerted but gradual

shrinkage resulted from the addition of protein fixatives or non-ionic detergents. The antibiotics vancomycin and penicillin caused a rapid shrinkage of cells which occurred at random intervals, indicating that shrinkage due to these agents was a secondary effect which followed the development of cell wall lesions. The least amount of shrinkage resulted from $HgCl_2$ and the non-ionic detergents. The greatest amount of shrinkage occurred with acetone, formaldehyde, TCA, and HCl. Although the reliability of the percent shrinkage measurements was somewhat compromised by the limited number of cells which were measurable, the general trends were considered significant. Although the agents in this group were all expected to cause membrane perturbation, additional sites of action were indicated, and these may largely account for the differences noted in the rate and amount of shrinkage.

Unlike the other shrinkage agents, the strong acids caused cytoplasmic banding which appeared just prior to shrinkage and persisted afterward. This cue, which was visible during the experiment, permitted a more detailed study of the TCA shrinkage seen at a lower rate of acceleration. The TCA shrinkage was thus revealed to actually require about 4 seconds per cell unit. No loss of material from the cell could be detected.

To explore a possible relationship between osmotic turgor and shrinkage, plasmolysis of the cells was attempted. Although 2 M sucrose appeared to cause plasmolytic shrinkage, its syrup-like consistency was not well-suited to the wick system. Another potential osmotic effector, 5 M NaCl, apparently also caused plasmolysis; under its influence the cells slowly shrank 20%, and their protoplasts assumed a shriveled look within the cell walls. Alternate treatment with 5 M NaCl and water

caused repeated shrinkage and expansion of the cells typical of an osmotic response. Spontaneous shrinkage of a cell greatly attenuated--yet did not completely prevent--dimensional changes due to the salt. Isolated cell walls, autolyzed cells and autoclaved cells also displayed gradual shrinkage in response to the salt, but the percent shrinkage was half or less than half that seen with intact cells. These results suggested that the total amount of shrinkage in the presence of 5 M NaCl was perhaps equally due to plasmolytic relief of turgor and to an electrostatic cell wall contraction such as that reported by Marquis (1968). Similar controls indicated that approximately half the amount of the abrupt shrinkage resulting from TCA was due to cell wall contraction. Polymyxin caused essentially no shrinkage of autolyzed cells.

Experiments in which shrinkage agents were added in sequence further indicated that both relief of turgor and cell wall contraction contributed to the shrinkage observed with some agents. Following an 8% shrinkage due to polymyxin, TCA caused only a 10% additional shrinkage. After an initial 22% shrinkage due to TCA, 5 M NaCl caused only a minor 2% shrinkage. After the slow 20% shortening due to 5 M NaCl, 1% TCA caused a slight expansion, then 5% shrinkage. When aqueous polymyxin was introduced following shrinkage due to 5 M NaCl, slow expansion began, then abrupt shrinkage prevented further expansion.

Taken collectively, these results suggest that NaCl causes both plasmolysis and cell wall contraction, TCA causes disruption of the osmotic barrier and cell wall contraction, while polymyxin causes disruption of the osmotic barrier but not cell wall contraction. It seems probable that the cell surface is normally somewhat distended by osmotic turgor and that a sudden cell membrane perturbation could cause sufficient

leakage to relieve this turgor, allowing cell surface relaxation. This mechanism is offered for membrane perturbants such as polymyxin. Simple relief of turgor via cell membrane leakage could also account for shrinkage after physiological stress, since autolytic enzyme activity is thought to produce local lesions in the peptidoglycan network which may promote leakage. In addition to the rather passive shrinkage resulting from relief of turgor via either plasmolysis or membrane damage, actual cell wall contraction was indicated for certain ionic agents. When such agents also disrupt the permeability barrier, this cell wall contraction apparently supplements the shrinkage due to the loss of osmotic turgor. Non-ionic agents may also promote contraction via mechanisms other than electrostatic influence: acetone may dehydrate expanded structures, and formaldehyde might act through covalent cross-linking. Thus, the array of possible mechanisms for dimensional change probably accounts for the qualitative and quantitative differences observed in the shrinkage obtained with various agents applied via the wick system.

During sealed slide culture experiments in which physiological stress was imposed to arrest growth, shrinkage commonly preceded lysis, suggesting that shrinkage is a normal early event during autolysis. The paucity of time-lapse microscopy studies and the inability to detect shrinkage by other methods would account for this phenomenon being widely overlooked. Weakened regions in the peptidoglycan network due to excess autolysin activity may cause local stresses on the cell membrane which alter its permeability properties and permit the leakage of soluble substances until turgor is relieved. The cell membrane, meanwhile, apparently retains some structural integrity, since the shrinkage requires several seconds, and the subsequent restoration of turgor was

occasionally demonstrated by eventual forceful lysis or the extrusion of a protoplast. Particulate substances were never seen leaving the cell during shrinkage, and the cell usually appeared darker afterward, suggesting an increased concentration of such refractile substances.

Whether shrinkage might confer an enhanced prospect for survival is not known. When the wall became dangerously weakened, shrinkage would certainly seem a less catastrophic means than lysis for relieving turgor and thus ameliorating a potentially explosive situation. Yet such a drastic change in cell volume, likely accompanied by the loss of essential "pool" substances, would seem quite detrimental and often lethal. Growth was seen to follow definite shrinkage only once, although such negative results may reflect the allowance of inadequate recovery conditions rather than a general inability to recover. In any case, the strong impression remains that shrinkage is usually a lethal event, the initial overt stage in an avalanche of autolytic events, and more a consequence of structural and physiological properties than a design for survival.

LITERATURE CITED

Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in Bacillus subtilis. J. Bacteriol. 81: 741-746.

Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger. 1965. Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide: presumed membrane transport intermediates in cell wall synthesis. Proc. Nat. Acad. Sci. U. S. 53: 881-889.

Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. II. Phospholipid carriers in the reaction sequence. J. Biol. Chem. 242: 3180-3190.

Anderson, J. S., P. M. Meadow, M. A. Haskin, and J. L. Strominger. 1966. Biosynthesis of the peptidoglycan of bacterial cell walls. I. Utilization of uridine diphosphate acetylmuramyl pentapeptide and uridine diphosphate acetylglucosamine for peptidoglycan synthesis by particulate enzymes from Staphylococcus aureus and Micrococcus lysodeikticus. Arch. Biochem. Biophys. 116: 487-515.

Best, G. K., and N. N. Durham. 1965. Vancomycin adsorption to Bacillus subtilis cell walls. Arch. Biochem. Biophys. 111: 685-692.

Brown, W. C., and F. E. Young. 1970. Dynamic interactions between cell wall polymers, extracellular proteases and autolytic enzymes. Biochem. Biophys. Res. Commun. 38: 564-568.

Chin, T., M. W. Burger, and L. Glaser. 1966. Synthesis of teichoic acids. VI. The formation of multiple wall polymers in Bacillus subtilis W-23. Arch. Biochem. Biophys. 116: 358-367.

Corner, T. R., and R. E. Marquis. 1969. Why do bacterial protoplasts burst in hypotonic solutions? Biochim. Biophys. Acta 183: 544-558.

Durham, N. N., E. C. Noller, M. W. Burger, and G. K. Best. 1967. Time-lapse cinematography of vancomycin-treated microbial cells. Can. J. Microbiol. 13: 417-421.

Ellwood, D. C., and D. W. Tempest. 1972. Effects of environment on bacterial wall content and composition. Advan. Microbial Physiol. 7: 83-117.

Fan, D. P. 1970. Autolysin(s) of Bacillus subtilis as dechaining enzyme. J. Bacteriol. 103: 494-499.

French, D., and J. T. Edsall. 1945. The reactions of formaldehyde with amino acids and proteins. Advan. Protein Chem. 2: 277-335.

Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in metabolism. Bacteriol. Rev. 32: 425-464.

Ghuyson, J. M., J. L. Strominger, and D. J. Tipper. 1968. Bacterial cell walls, p. 54-104. In M. Florkin and E. H. Stotz (ed.), Comprehensive biochemistry, vol. 26A. American Elsevier Publishing Co., New York.

Gilby, A. R., and A. V. Few. 1960. Lysis of protoplasts of Micrococcus lysodeikticus by ionic detergents. J. Gen. Microbiol. 23: 19-26.

Grula, E. A., T. F. Butler, R. D. King, and G. L. Smith. 1967. Bacterial cell membranes. II. Possible structure of the basal membrane continuum of Micrococcus lysodeikticus. Can. J. Microbiol. 13: 1499-1507.

Grula, E. A., G. L. Smith, and M. M. Grula. 1968. Cell division in a species of Erwinia. X. Morphology of the nuclear body in filaments produced by growth in the presence of D-serine. Can. J. Microbiol. 14: 293-298.

Hamilton, W. A. 1971. Membrane active antibacterial compounds, p. 77-93. In W. B. Hugo (ed.). Inhibition and destruction of the microbial cell. Academic Press, Inc., New York.

Harold, F. M. 1970. Antimicrobial agents and membrane function. Advan. Microbial Physiol. 4: 45-104.

Haslam, D. F. 1973. Studies on the biological action of a hydroxypyrazole compound. Ph.D. Dissertation, Oklahoma State University.

Hughes, R. C. 1970. Autolysis of isolated cell walls of Bacillus licheniformis N.C.T.C. 6346 and Bacillus subtilis Marburg strain 168. Biochem. J. 119: 849-860.

Hughes, R. C., J. G. Pavlik, H. J. Rogers, P.J. Tanner. 1968. Organization of polymers in the cell walls of some bacilli. Nature (London) 219: 642-644.

Hughes, R. C., P. J. Tanner, and E. Stokes. 1970. Cell wall thickening in Bacillus subtilis. Comparison of thickened and normal walls. Biochem. J. 120: 159-170.

Izaki, K., M. Matsuhashi, and J. L. Strominger. 1966. Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin sensitive enzymatic reactions. Proc. Nat. Acad. Sci. U. S. 55: 656-663.

Kelemen, M. V., and H. J. Rogers. 1971. Three-dimensional molecular models of bacterial cell wall mucopeptides (peptidoglycans). Proc. Nat. Acad. Sci. U. S. 68: 992-996.

Knaysi, G. 1930. The cell structure and cell division of Bacillus subtilis. J. Bacteriol. 19: 113-115.

Koike, M., and K. Iida. 1971. Effect of polymyxin on the bacteriophage receptors of the cell walls of gram negative bacteria. J. Bacteriol. 108: 1402-1411.

Koike, M., K. Iida, and T. Matsuo. 1969. Electron microscopic studies on the mode of action of polymyxin. J. Bacteriol. 97: 448-452.

Lopes, J., and W. E. Inniss. 1969. Electron microscopy of effect of polymyxin on Escherichia coli lipopolysaccharide. J. Bacteriol. 100: 1128-1130.

Marquis, R. E. 1968. Salt-induced contraction of bacterial cell walls. J. Bacteriol. 95: 775-781.

Matsumoto, A., N. Higashi, and A. Tamura. 1973. Electron microscope observations on the effects of polymyxin B sulfate on cell walls of Chlamydia psittaci. J. Bacteriol. 113: 357-364.

Mauck, J., L. Chan, L. Glaser. 1971. Turnover of the cell wall of gram positive bacteria. J. Biol. Chem. 246: 1820-1827.

Mitchell, P., and J. Moyle. 1956. Osmotic function and structure in bacteria. Soc. Gen. Microbiol. Symp. 6: 150-180.

Neuhaus, F. C., and J. L. Lynch. 1964. The enzymatic synthesis of D-alanyl-D-alanine. III. On the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. Biochemistry 3: 471-480.

Newton, B. A. 1956. The properties and mode of action of the polymyxins. Bacteriol. Rev. 20: 14-27.

Noller, E. C., and N. N. Durham. 1968. Sealed aerobic slide culture for photomicrography. Appl. Microbiol. 16: 439-440.

Poos, J. C., F. R. Turner, D. White, G. D. Simon, K. Bacon, and C. T. Russell. 1972. Growth, cell division, and fragmentation in a species of Flexibacter. J. Bacteriol. 112: 1387-1395.

Pulvertaft, R. J. V. 1952. The effect of antibiotics on growing cultures of Bacterium coli. J. Pathol. Bacteriol. 64: 75-89.

Quesnel, L. B. 1969. Methods in microculture, p. 365-425. In J. R. Norris and D. W. Ribbons (ed.) Methods in microbiology, Vol. 1. Academic Press, Inc., New York.

Reaveley, D. A., and R. E. Burge. 1972. Walls and membranes in bacteria. Advan. Microbial Physiol. 7: 1-18.

Rogers, H. J. 1970. Bacterial growth and the cell envelope. Bacteriol. Rev. 34: 194-214.

Rogers, H. J., and C. W. Forsberg. 1971. Role of autolysins in the killing of bacteria by some bactericidal antibiotics. J. Bacteriol. 108: 1235-1243.

Salton, M. R. J. 1967. Structure and function of bacterial cell membranes. Annu. Rev. Microbiol. 21: 417-442.

Salton, M. R. J. 1968. Lytic agents, cell permeability, and monolayer penetrability. J. Gen. Physiol. 52: 2275-2525.

Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36: 407-477.

Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Science 175: 720-731.

Strominger, J. L. 1962. Biosynthesis of bacterial cell walls. Fed. Proc. 21: 134-143.

Strominger, J. L., and J. M. Ghuysen. 1967. Mechanisms of enzymatic bacteriolysis. Science 156: 213-221.

Strominger, J. L., E. Ito, R. H. Threnn. 1960. Competitive inhibition of enzymatic reactions by oxamycin. J. Amer. Chem. Soc. 82: 998-999.

Warth, A. D., and J. L. Strominger. 1971. Structure of the peptidoglycan from vegetative cell walls of Bacillus subtilis. Biochemistry 10: 4349-4358.

Whitfield, J. F., and R. G. E. Murray. 1956. The effects of the ionic environment on the chromatin structure of bacteria. Can. J. Microbiol. 2: 245-260.

Young, F. E. 1966. Fractionation and partial characterization of the products of autolysis of cell walls of Bacillus subtilis. J. Bacteriol. 92: 839-846.

APPENDIX

Figure 3. LA 23-2, Frame 27. Normal growth, 2 fpm. Figures 3-6 illustrate three generations of cell a.

Figure 4. LA 23-2, Frame 141.

Figure 5. LA 23-2, Frame 243.

Figure 6. LA 23-2, Frame 380.

Figure 7. LA 9-5, Frame 70. Effect of vancomycin, 15 µg/ml, 2 fpm. Figures 7, 8 show shrinkage of cell a. Figures 9, 10 show point lysis of cell a with material ejected at the indicated locus.

Figure 8. LA 9-5, Frame 71.

Figure 9. LA 9-5, Frame 584.

Figure 10. LA 9-5, Frame 587.

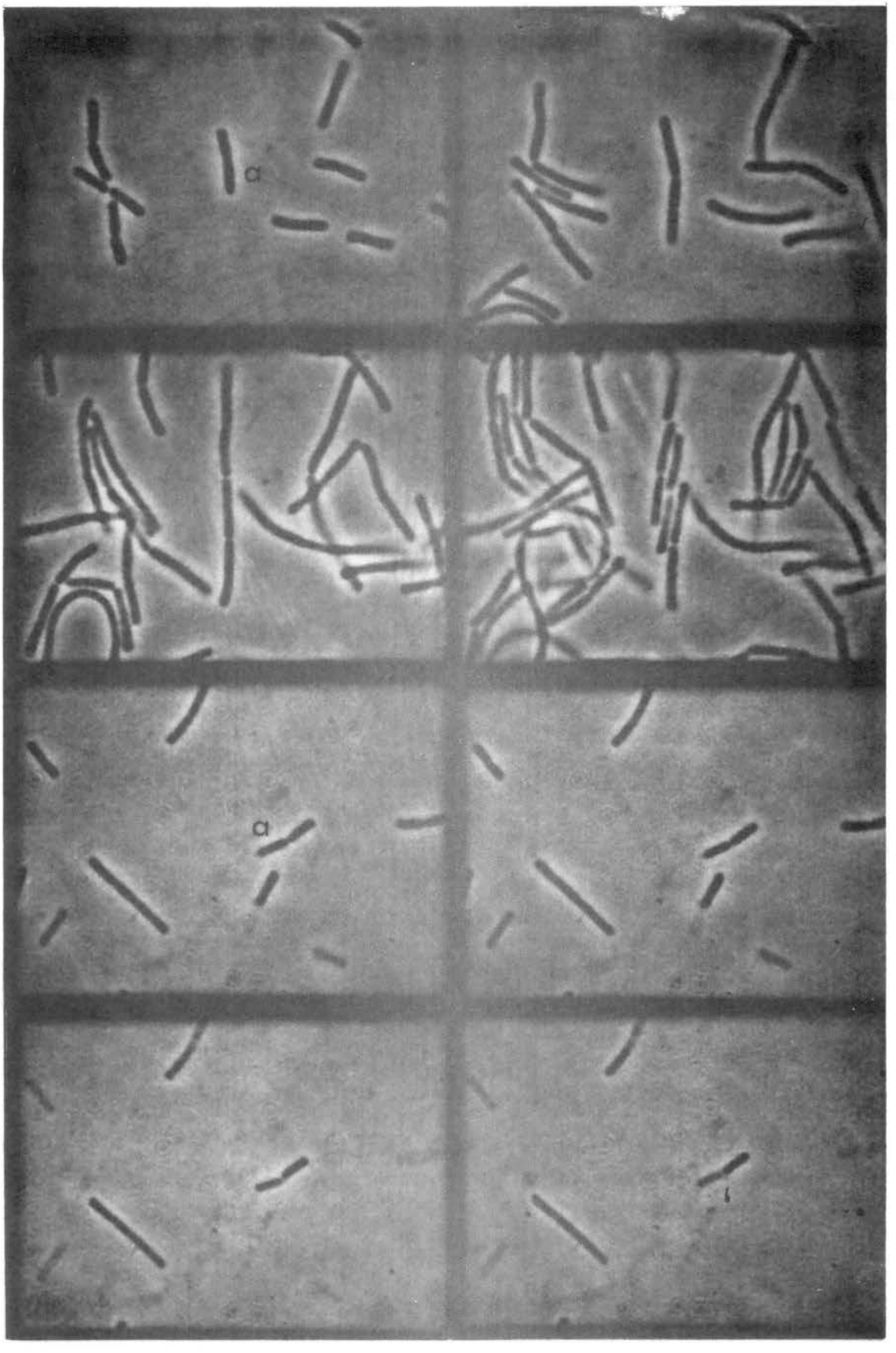

a
a

Figure 11. LA 6-3, Frame 99. The effect of penicillin, 100 μg/ml, 2 fpm, is shown in Figures 11-18. Selected cells are identified.

Figure 12. LA 6-3, Frame 100. Cell b has shrunk.

Figure 13. LA 6-3, Frame 174.

Figure 14. LA 6-3, Frame 175. Cell c has apparently just lysed, yet retains some darkness.

Figure 15. LA 6-3, Frame 399.

Figure 16. LA 6-3, Frame 400. Formation of a protoplast from cell c.

Figure 17. LA 6-3, Frame 744. Appearance of the field after 6.5 hours.

Figure 18. LA 6-3, Frame 776. Pleomorphic growth of cells from the region d is shown after filming was resumed at approximately 12 hours.

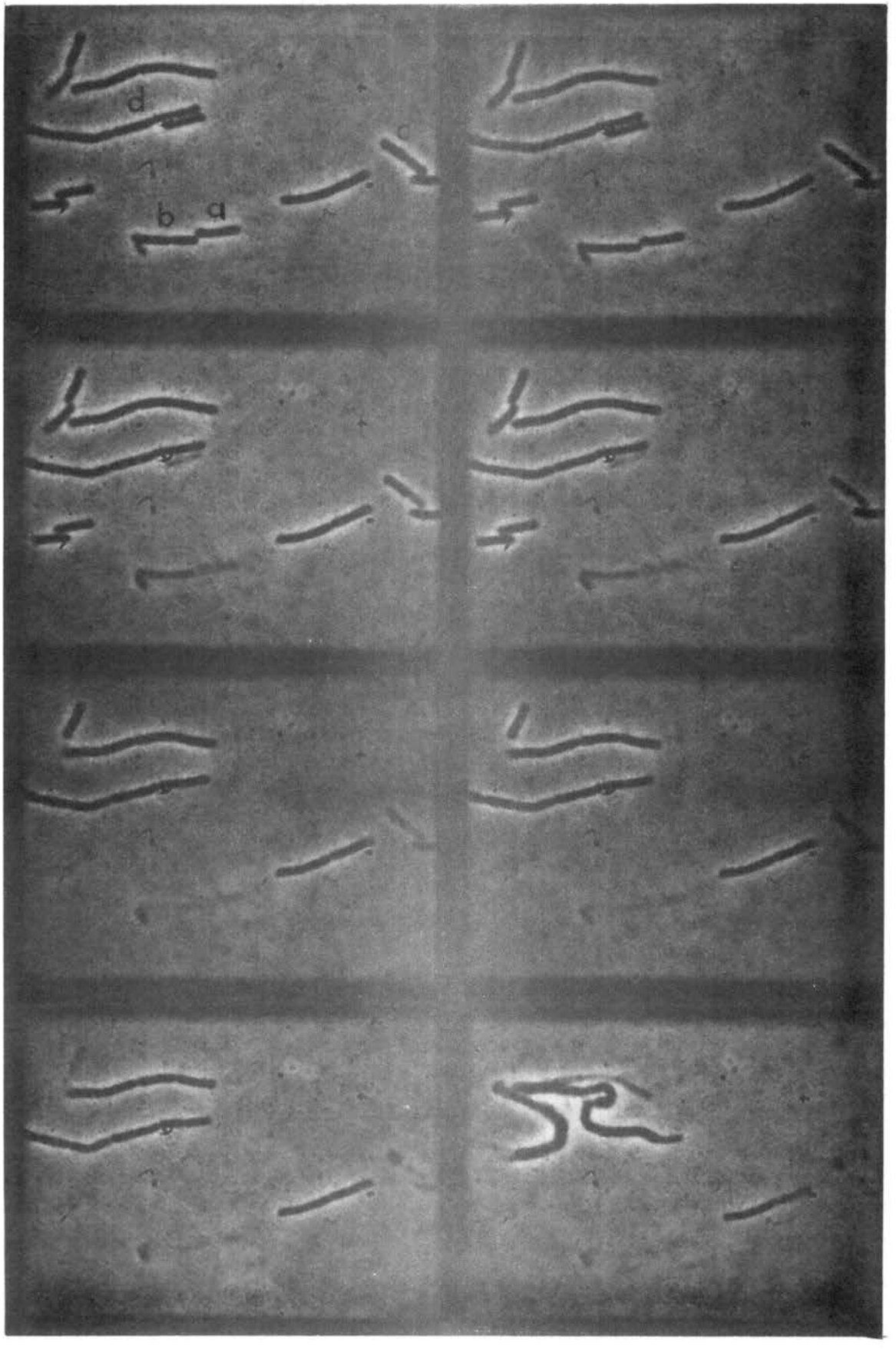

d
c
b
a

Figure 19. LA 22-2, Frame 1. Effect of D-cycloserine, 25 µg/ml, 2 fpm. Cells a and b are identified before shrinkage.

Figure 20. LA 22-2, Frame 160. Cell b has shrunk but retains some density.

Figure 21. LA 22-2, Frame 193. The field has been moved downward. The formation of a protoplast from cell b is indicated. Cell d, which later undergoes point lysis, is identified.

Figure 22. LA 22-2, Frame 370. The appearance of swollen cell c immediately prior to shrinkage.

Figure 23. LA 11-1, Frame 300. Effect of polymyxin, 200 µg/ml, 3 fpm. A protoplast was slipping from the end of cell a. Cell b, which may shrink twice, is identified.

Figure 24. LA 11-1, Frame 1260. The protoplast of cell a is still intact after more than 5 hours.

Figure 25. LA 10-5, Frame 2 The effect of limited aeration is shown in Figures 25-28, 2 fpm.

Figure 26. LA 10-5, Frame 167. Growth has ceased.

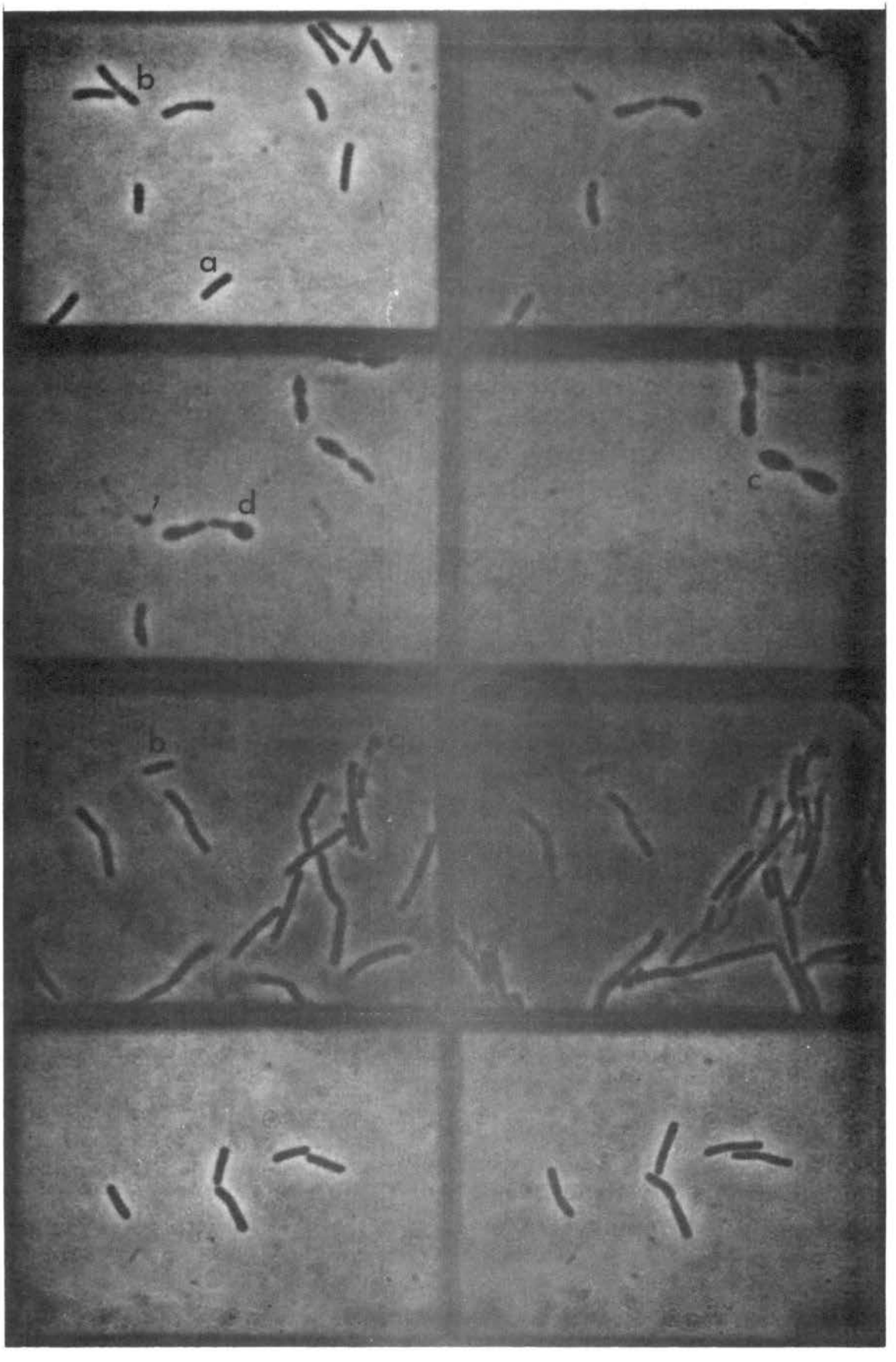

b
a
d
c
b

Figure 27. LA 10-5, Frame 219. All cells have shrunk.

Figure 28. LA 10-5, Frame 300. All cells have undergone diffuse lysis.

Figure 29. LA 13-3, Frame 1 Effect of lysozyme, 100 µg/ml, 6 fpm, is illustrated in Figures 27-32 at 2700 X. When this frame was taken, about 5 minutes had elapsed after exposure to the enzyme.

Figure 30. LA 13-3, Frame 35.

Figure 31. LA 13-3, Frame 70.

Figure 32. LA 13-3, Frame 105.

Figure 33. LA 17-1, Frame 16. Effect of lysozyme, 75 µg/ml, 20 fpm. Cell a shrank by frame 18, 6 seconds later.

Figure 34. LA 17-1, Frame 18. Note that cell a is slightly darker after shrinking. Cell b has not yet shrunk.

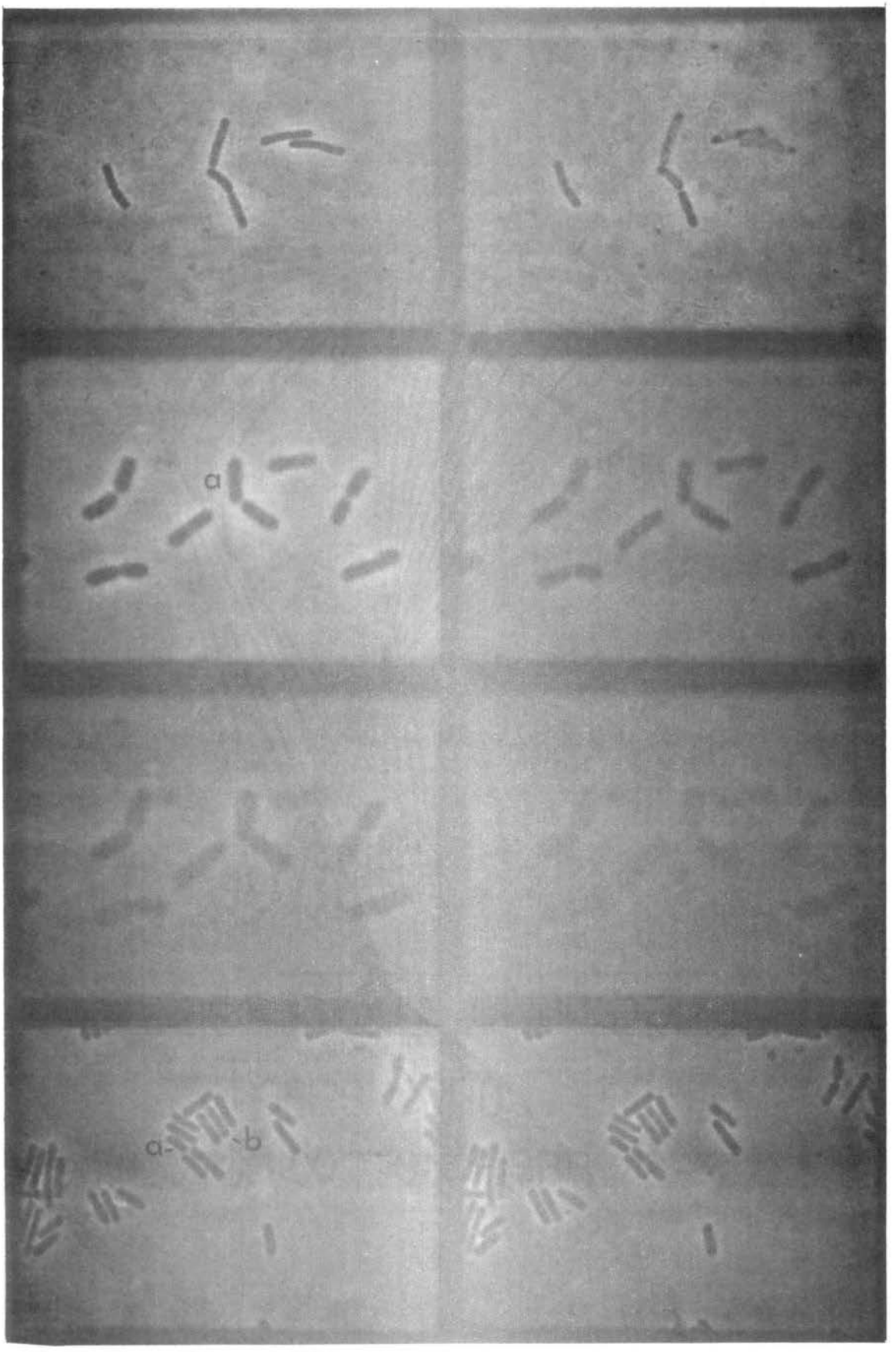

a
a-
-b

Figure 35. LA 18-5, Frame 1. Effect of lysozyme, 75 µg/ml on formalin-treated cells, 5 fpm.

Figure 36. LA 18-5, Frame 650. Note the expansion of cells.

Figure 37. LA 18-6, Frame 5. Effect of lysozyme, 75 µg/ml on autoclaved cells, 10 fpm.

Figure 38. LA 18-6, Frame 100. Note the separation of cells and the lack of expansion of the dark regions.

Figure 39. LA 35-3, Frame 1. The effect of TCA, 1% w/v, in the wick slide culture is shown in Figures 39-41, and 42-43. Before banding.

Figure 40. LA 35-3, Frame 23. After banding, but before shrinkage.

Figure 41. LA 35-3, Frame 200. After shrinkage.

Figure 42. LA 35-3, Frame 743. A new field on the same slide culture has been located. After banding, but before shrinkage. Note the possible correlation between dark zones and septal loci.

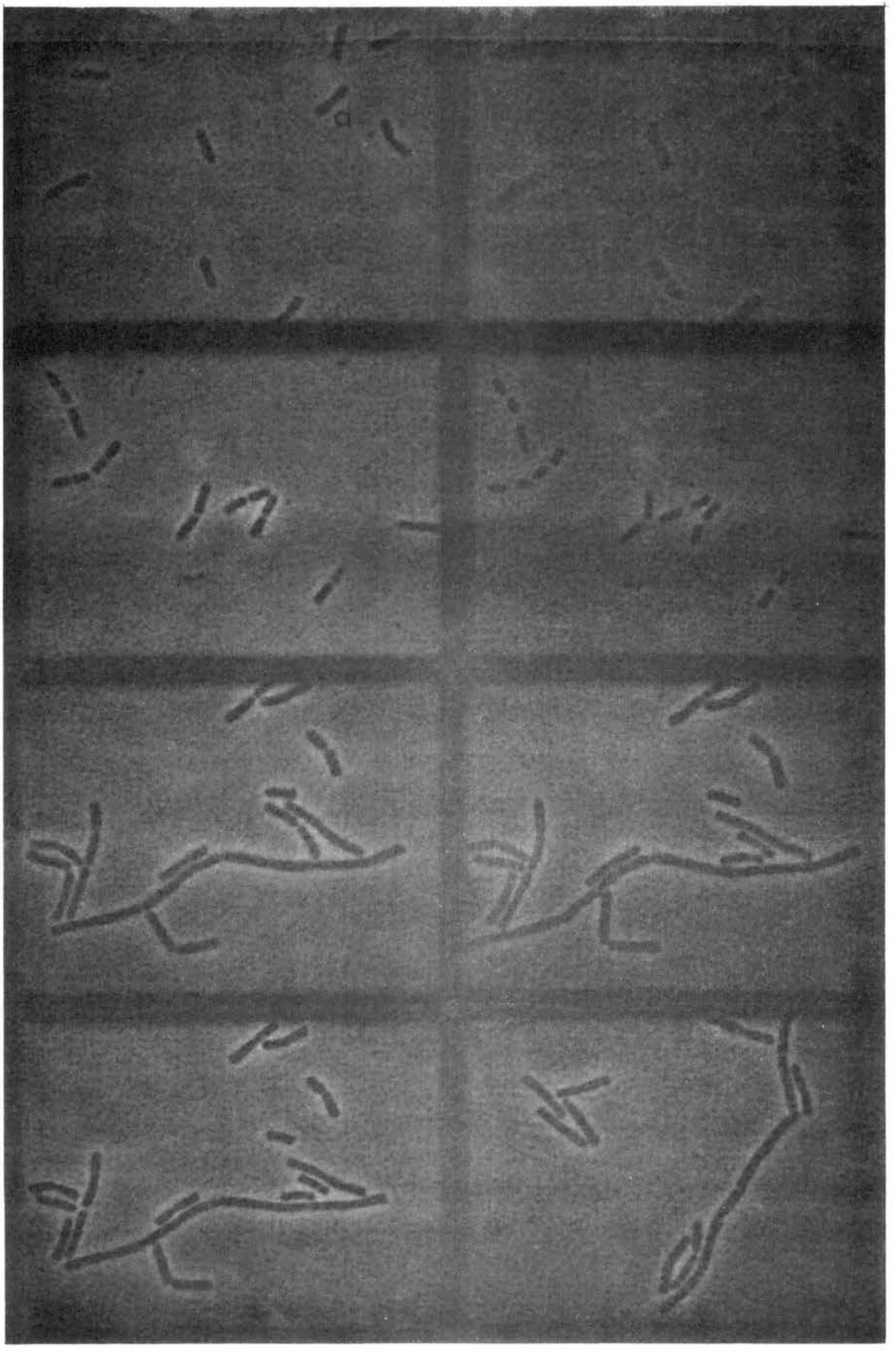


Figure 43. LA 35-3, Frame 843. Same field as Figure 42, after shrinkage.

Figure 44. LA 40-2, Frame 1. The series of Figures 44-50 shows the effects of sequential additions in the wick slide culture system at 6 fpm.

Figure 45. LA 40-2, Frame 180. Water irrigation has permitted growth since Figure 44, except for cell c which has shrunk.

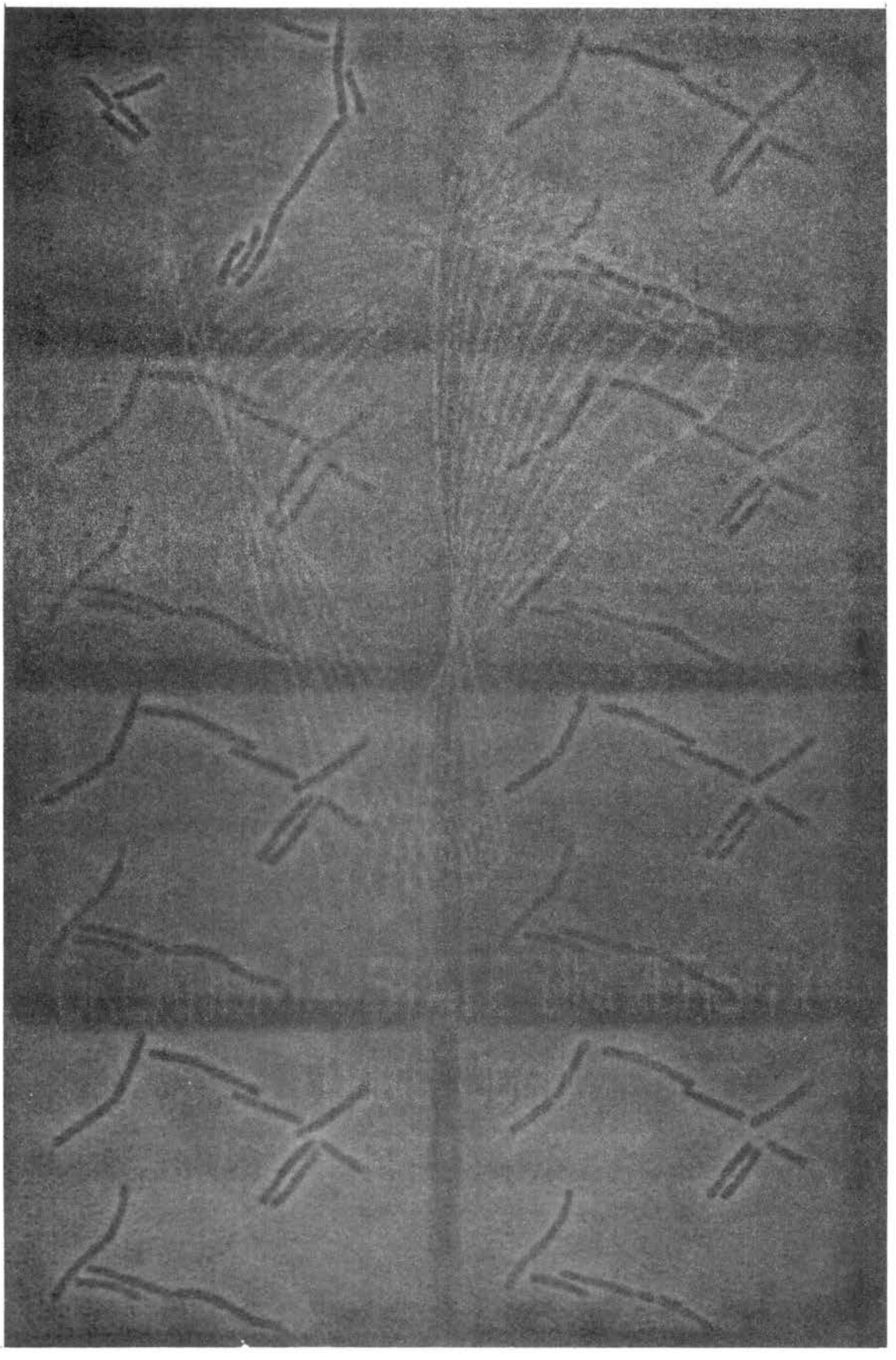
Figure 46. LA 40-2, Frame 320. Appearance after 5 M NaCl. Slow shrinkage and the shriveled appearance of protoplasts indicate plasmolysis.

Figure 47. LA 40-2, Frame 520. Appearance after water has caused expansion.

Figure 48. LA 40-2, Frame 620. Appearance after the second application of 5 M NaCl.

Figure 49. LA 40-2, Frame 920. Appearance after the second application of water.

Figure 50. LA 40-2, Frame 985. Appearance after the shrinkage due to 1% TCA.



VITA

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