Fleming's Lysozyme

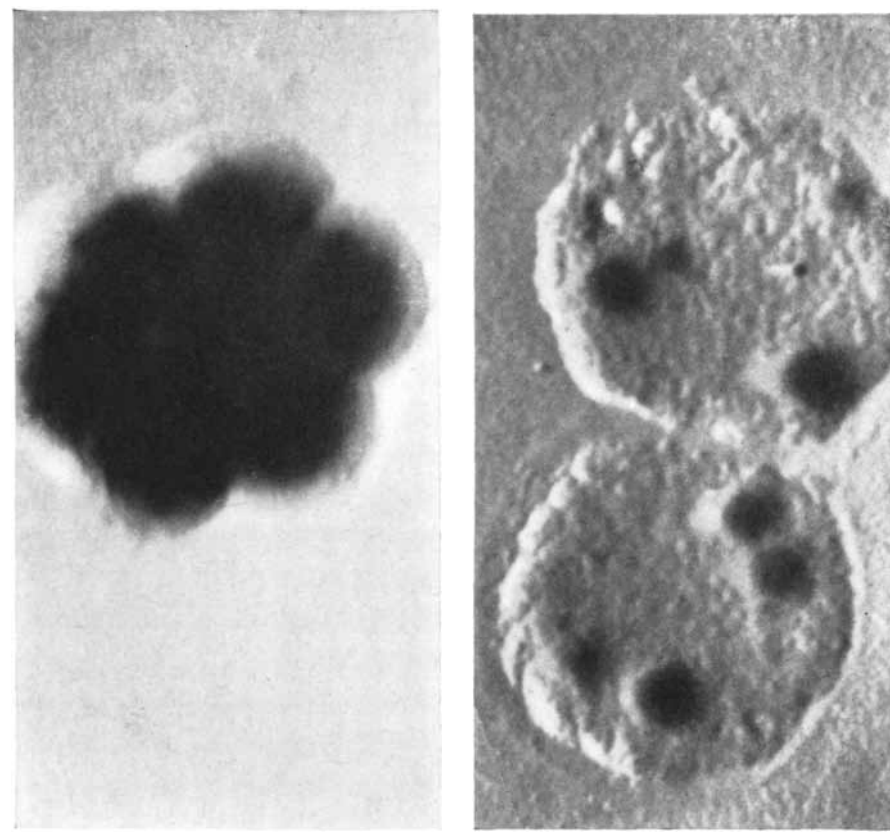
The discoverer of penicillin also found a substance that dissolves bacteria. Occurring in many human tissues and secretions, lysozyme is presently used to investigate the structure of the bacterial cell

by Robert F. Acker and S. E. Hartsell

The several major classes of bacteria have characteristic shapes: spherical, rodlike or spiral. From these shapes the bacterial classes get their names: cocci, bacilli and spirilla. Although nonbacterial cells have recognizable shapes, most of them are soft and pliable. The individual bacterial cell usually holds its shape with considerable rigidity. This feature of bacterial anatomy long ago attracted the curiosity of microbiologists. They soon discovered the reason for it: In bacteria the soft outer cell-membrane is enclosed in a tough, thick wall, which is often enclosed in turn in an even thicker gummy or slimy capsule. Together the wall and capsule may constitute as much as 45 per cent of the total mass of the cell. Plainly such an important structure must serve a purpose more significant than the conferring of rigid shapes for ready recognition by microbiologists. The cell wall, in fact, insulates bacteria from the vagaries of the environment, especially during their passage from host to host. In distilled water, for example, the osmotic pressure exerted by the bacterial cell would cause it to draw in water, swell up and burst; it has been estimated that the cell wall must in some species withstand an internal pressure of 20 atmospheres (300 pounds per square inch).

Bacterial anatomists are indebted to the late Sir Alexander Fleming for a sensitive chemical tool with which they have been studying bacteria, dissolving away the cell wall and exposing the cell body, or cytoplasm, within. In 1922 at St. Mary's Hospital in London, six years before his epochal discovery of penicillin, Fleming found "a substance present in the tissues and secretions of the body, which is capable of rapidly dissolving certain bacteria." Because of its resemblance to enzymes and its capacity to dissolve, or lyse, the cells, he called it "lysozyme." Fleming's lysozyme has not joined his penicillin in the armamentarium of medicine, but it undoubtedly renders all the service that could be expected of it as an element in the natural defenses of the body. In the hands of Fleming and his successors, moreover, lysozyme has helped to develop significant new understanding of the bacterial cell wall in its relationship to the processes that go on in the cytoplasm it otherwise protects so well.

Fleming isolated both lysozyme and the bacterium (*Micrococcus lysodeikticus*) that has been found to be most susceptible to it from the nasal secretion of a patient suffering from acute catarrh. A few simple experiments soon demonstrated the remarkable properties of the



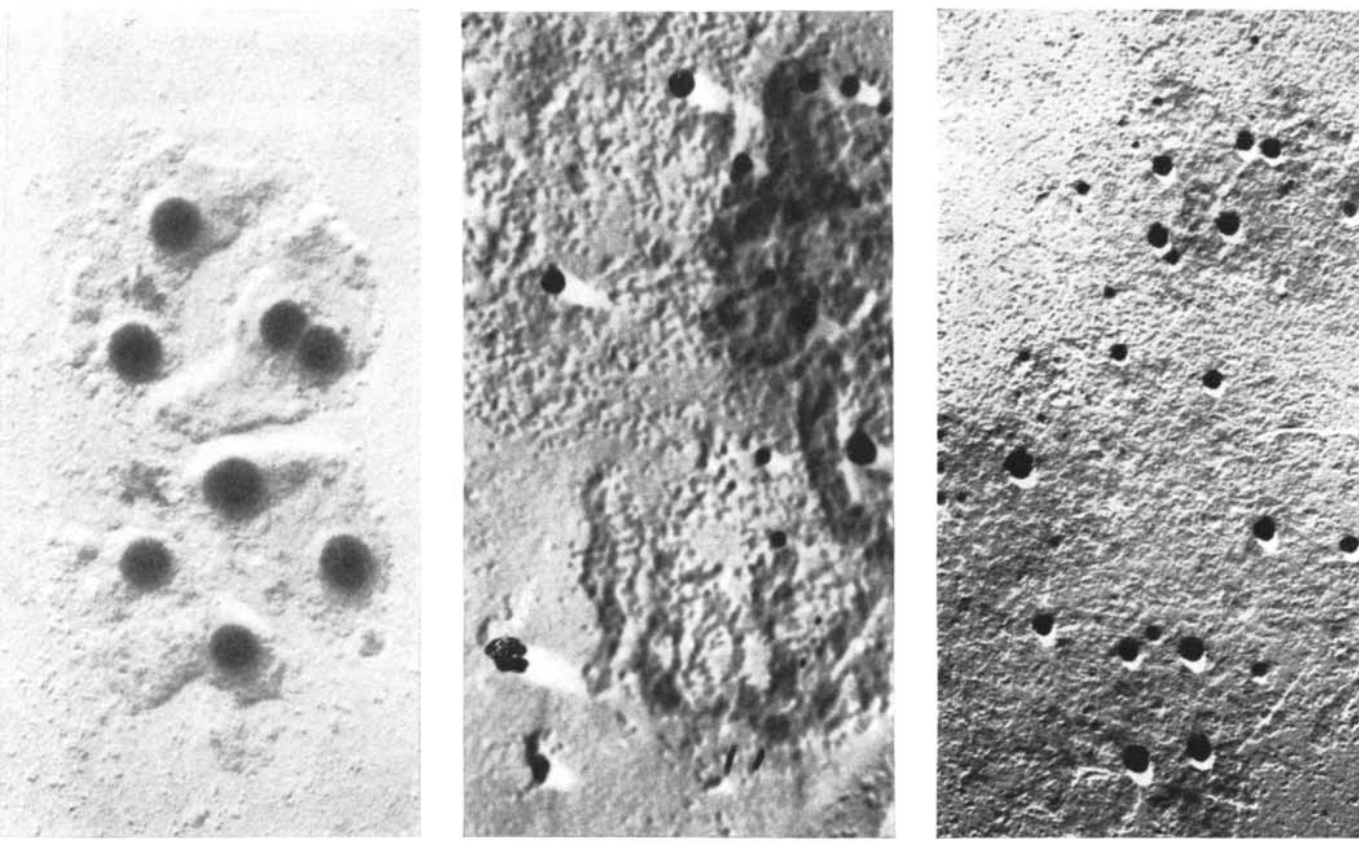
DISSOLUTION OF BACTERIAL CELLS with lysozyme treatment is shown in this series of electron micrographs. At far left is a clump of normal cells of *Micrococcus lysodeikticus*. About 15 seconds after the addition of lysozyme (*second picture*), the cell wall starts to

substance. In one experiment Fleming seeded a dish of solid culture medium with *Micrococcus lysodeikticus* and placed a droplet of mucus at the center of the dish. The bacterial cells multiplied and covered the surface with a cloudy continuous film, except in the area surrounding the mucus droplet. Here a clear zone appeared, created by the dissolution of the cells. Under the microscope Fleming observed that bacteria exposed to lysozyme undergo a series of characteristic changes. First the cells begin to swell, both spherical and cylindrical cells becoming transformed into large spheres. These soon lyse and lose their visible outline. After a time the only visible remains are a scattering of dark granules.

Lysozyme has been found in tears, nasal mucus, saliva and the exudate from infections; in extracts from the spleen, kidney, liver, lungs and lymph; and, in especially high concentration, in cartilage. It is not detectable in urine, cerebrospinal fluid or sweat. Lysozyme is not restricted, however, to tissues and fluids of animal origin; plants such as turnip, cabbage and cauliflower and even certain bacteria contain it. Egg white is the best source of lysozyme in quantity. Good yields are obtained by adding a small quantity of salt to egg white and homogenizing and acidifying it, whereupon the enzyme crystallizes out in pure form.

Chemical analysis of lysozyme has shown it to be a protein with the relatively low molecular weight of 14,700, of the same order as that of ribonuclease (13,895) and insulin (6,000), which are proteins simple enough to have yielded the principal features of their structure to investigation. The elucidation of lysozyme is not yet complete, but it is known that some 130 amino acid units, comprising 18 different amino acids, make up its structure. It is unique in that it contains very little of the amino acid tyrosine; the amino acid lysine, on the other hand, occurs characteristically at the ends of its amino acid chains. Since lysine occupies the same position in the structures of other proteins, particularly those found in wheat grains, it may be that lysozyme has a significant place in the evolution of proteins that accompanied the evolution of the organisms composed of them.

Like other enzymes, lysozyme is highly specific in its activity. In susceptible bacteria it is the capsule and cell wall that are broken down by lysozyme. The complete susceptibility of the cell wall of *Micrococcus lysodeikticus* has been demonstrated in experiments with purified preparations of its cell wall. M. R. J. Salton of the University of Manchester ruptured these cells by shaking them at high frequency in the presence of tiny glass beads, and then separated the walls from their contents by repeated centrifugation. The entire substance of this preparation was dissolved when it was mixed with lysozyme. Upon analysis, the susceptible material proved to be half polysaccharide (sugar units forming a chain) and half peptide (short amino acid chains) in composition. The polysaccharide portion breaks down into simpler sugar units such as glucose, and the peptide portion into a half dozen or so amino acids. In addition to the major components, substances classed as amino sugars have been found. As the name implies, these compounds are the result

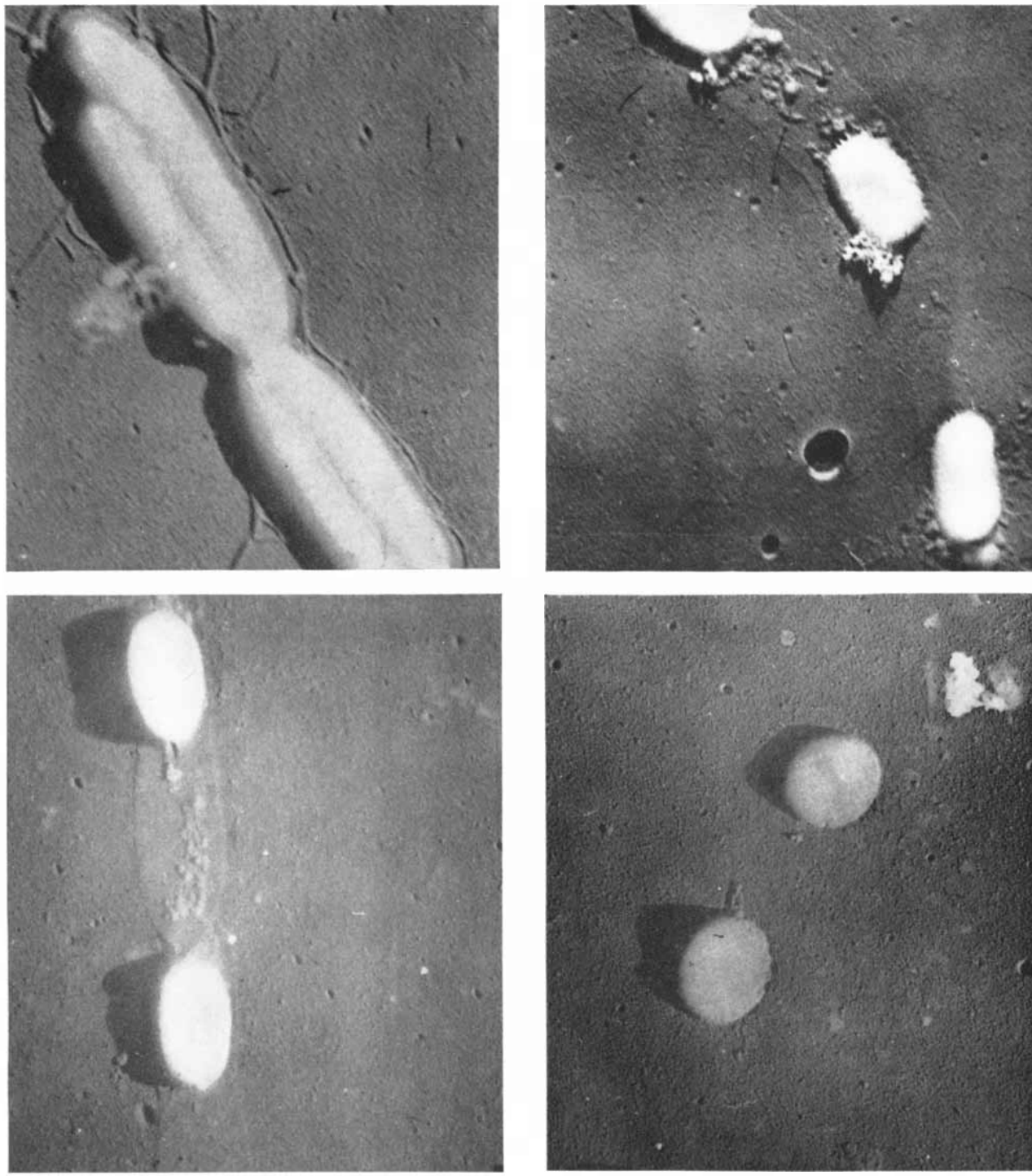


dissolve and the cell material spreads out. At 60 seconds (*third*) the cell wall disappears and at 90 seconds (*fourth*) cell organization is almost lost. At 120 seconds (*fifth*) nothing remains but granules. The granules in the last two micrographs appear to be holes because of a special shadowing technique. All these structures are enlarged 1,800 diameters. The micrographs were provided by Hartsell.

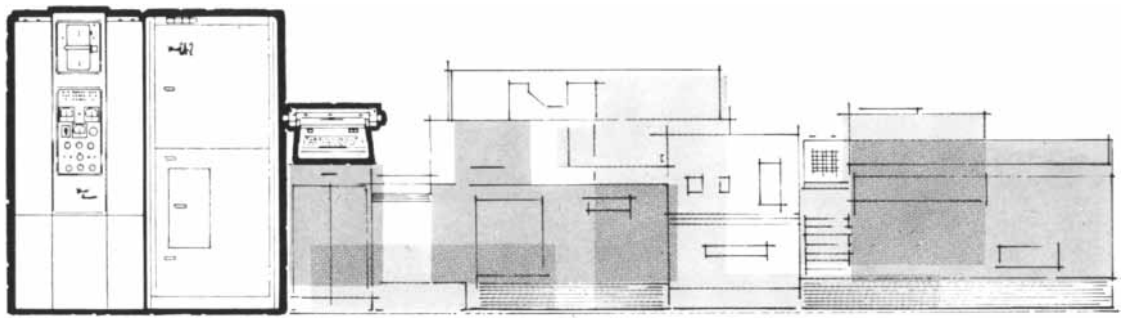
of chemical attachment of amino groups (NH_2) to sugar molecules.

The selective action of lysozyme fills a long-felt want of the microbiologist, permitting detailed study of the cell wall and a new approach to investigation of the cytoplasm. Other agents fail to segregate one from the other or tend to disrupt and destroy both together. When bacteria are placed in strong salt solution, for example, the cytoplasm shrinks away from the cell wall into rounded bodies called protoplasts. The opposite reaction, swelling of the cytoplasm, may be induced by certain toxic or growth-promoting substances. The cell wall now splits open. The protoplast may be visible momentarily as a large sphere of cytoplasm enclosed in a thin flexible membrane, but it disintegrates rapidly.

Investigators tried for many years without success to secure protoplasts in stable form in the hope of using them to



SPHERICAL FORMS OF BACTERIA, called spheroplasts or protoplasts, result from lysozyme treatment in a sucrose solution. Cells of *Bacillus subtilis*, which are normally rod-shaped (*first picture*), upon treatment gradually lose their cell walls and become rounded. The structures are enlarged some 15,000 diameters. The electron micrographs on this page and on page 142 are reproduced from an article by J. M. Wiame, R. Storck and E. Vanderwinkel that appeared in *Biochimica et Biophysica Acta*, Vol. 18, page 353.



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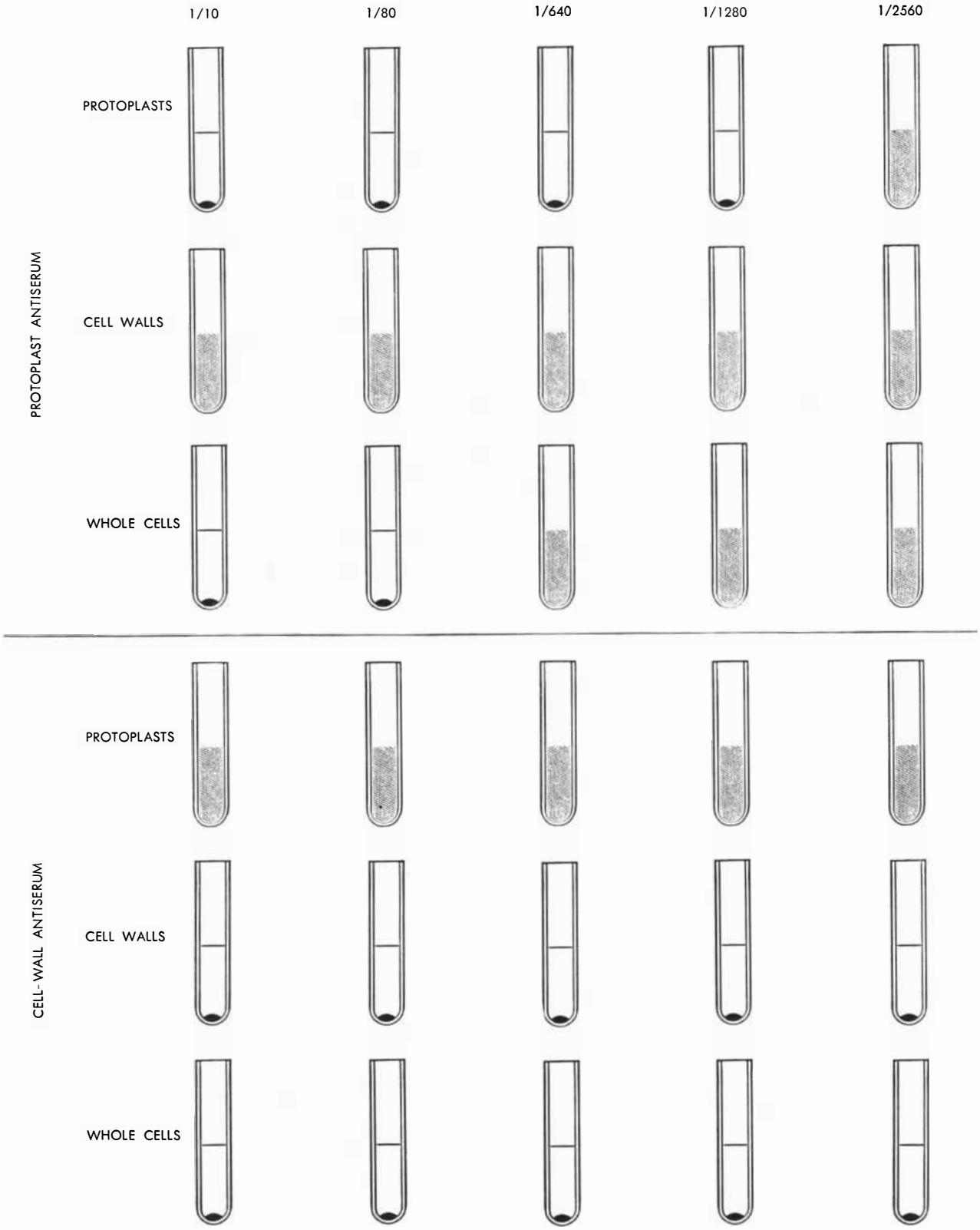


SEROLOGICAL TESTS show that protoplasts of *Bacillus megaterium* are free of cell-wall material. Serum containing antibodies to the protoplasts (*upper group*) causes the protoplasts to clump together and settle out of suspension, but does not react with isolated cell walls of the same organism. Serum containing antibodies to the cell walls (*lower group*) reacts with cell walls but not with protoplasts. Whole cells are clumped by cell-wall antiserum and, at low dilutions, by protoplast antiserum. Figures at top indicate serum dilution. Data are based on a study by John W. Vennes and Philipp Gerhardt at the University of Michigan Medical School.

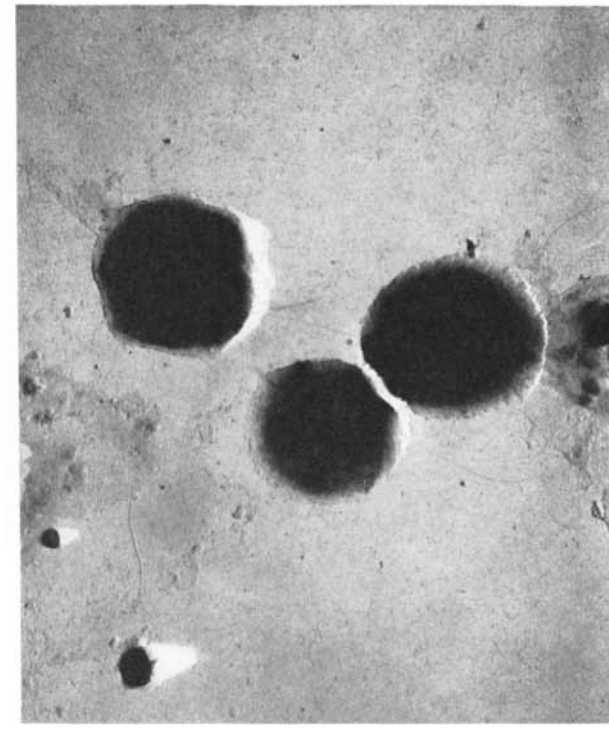
study the properties of bacterial cytoplasm. This objective was achieved in 1953 when Claes Weibull of the University of Uppsala in Sweden developed a simple technique employing lysozyme to isolate and preserve protoplasts. Weibull found that a sufficiently low concentration of lysozyme would dissolve the cell walls of the giant *Bacillus megaterium* slowly enough to permit experimental control of the process. He was able to keep the resulting protoplasts from breaking down by conducting the process in a dilute solution of sucrose, which balanced the osmotic pressure across the exposed cell membrane, and by maintaining a low level of oxygen in the medium to inhibit the metabolism of the protoplasts.

That this treatment achieves complete separation of the cell wall and protoplast has been demonstrated by serological means. Protoplasts, like other protein-containing substances, are antigenic; when they are injected into the bloodstream of an experimental animal, they induce the formation of antibodies that react with them. Blood serum taken from the animal and mixed with the same type of protoplasts will cause the protoplasts to clump together. The serum will also cause clumping of whole bacteria of the same type, but will not clump isolated cell-walls. On the other hand, antiserum prepared by injecting an animal with purified cell walls will cause whole bacteria as well as cell walls to form clumps, but will not affect the protoplasts of the same bacteria. The smallest contamination either way would blur these clear-cut results.

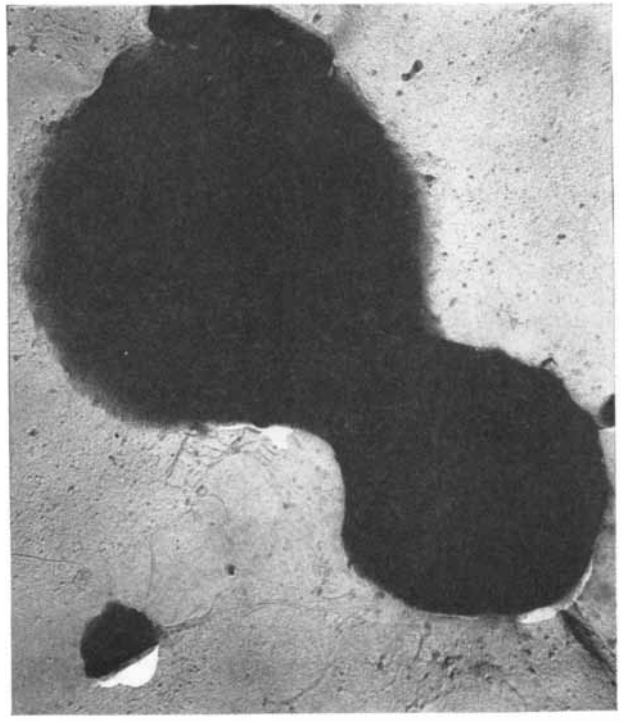
With protoplasts available in stable form for experiment, investigators have found that many activities of the cell remain unaffected when the cell wall has been stripped away. For example, the respiration rate—the intake of oxygen and elimination of carbon dioxide—appears to be much the same in protoplasts as in intact cells. This is particularly the case when the respiration rate is throttled down by culturing the cells or protoplasts in a medium devoid of energy-yielding nutrients. When nutrients are added to the medium in which cells or protoplasts are suspended, the protoplasts take up the nutrients with about 75 per cent the efficiency of whole cells. Interestingly enough, they do not assimilate certain compounds that are normal constituents of cell-wall substance, although they may incorporate their precursors. Accordingly it seems that those systems that function within the cyto-

plasm of the cell do so as effectively in protoplasts as in intact cells, and so are not dependent on the presence of the cell wall.

The next question is whether protoplasts can also grow and divide like whole cells. The protoplasts of *B. megaterium* swell and increase in weight and volume, much as whole bacteria do between cell divisions. Under certain conditions they even exhibit a phenomenon suggestive of division. In the presence of glucose and selected amino acids they increase in size, produce budlike protrusions and become dumbbell-shaped. But unlike the intact cells, which undergo division at a sufficiently rapid rate to form visible colonies on a solid growth-medium, protoplasts do not succeed in carrying through the process of normal division. Though a single protoplast can



NEWLY FORMED PROTOPLASTS of *B. megaterium* at right have the same volume as the rod-shaped cells from which they came. Normal cells are shown at left. Kenneth McQuillen of the University of Cambridge provided these electron micrographs.



PROTOPLASTS AFTER NINE HOURS in a nutritive medium have increased in size and weight. Some appear to be budding (*left*) or dividing (*right*). These structures, which are enlarged about 15,000 diameters, are shown at the same scale as those at top.

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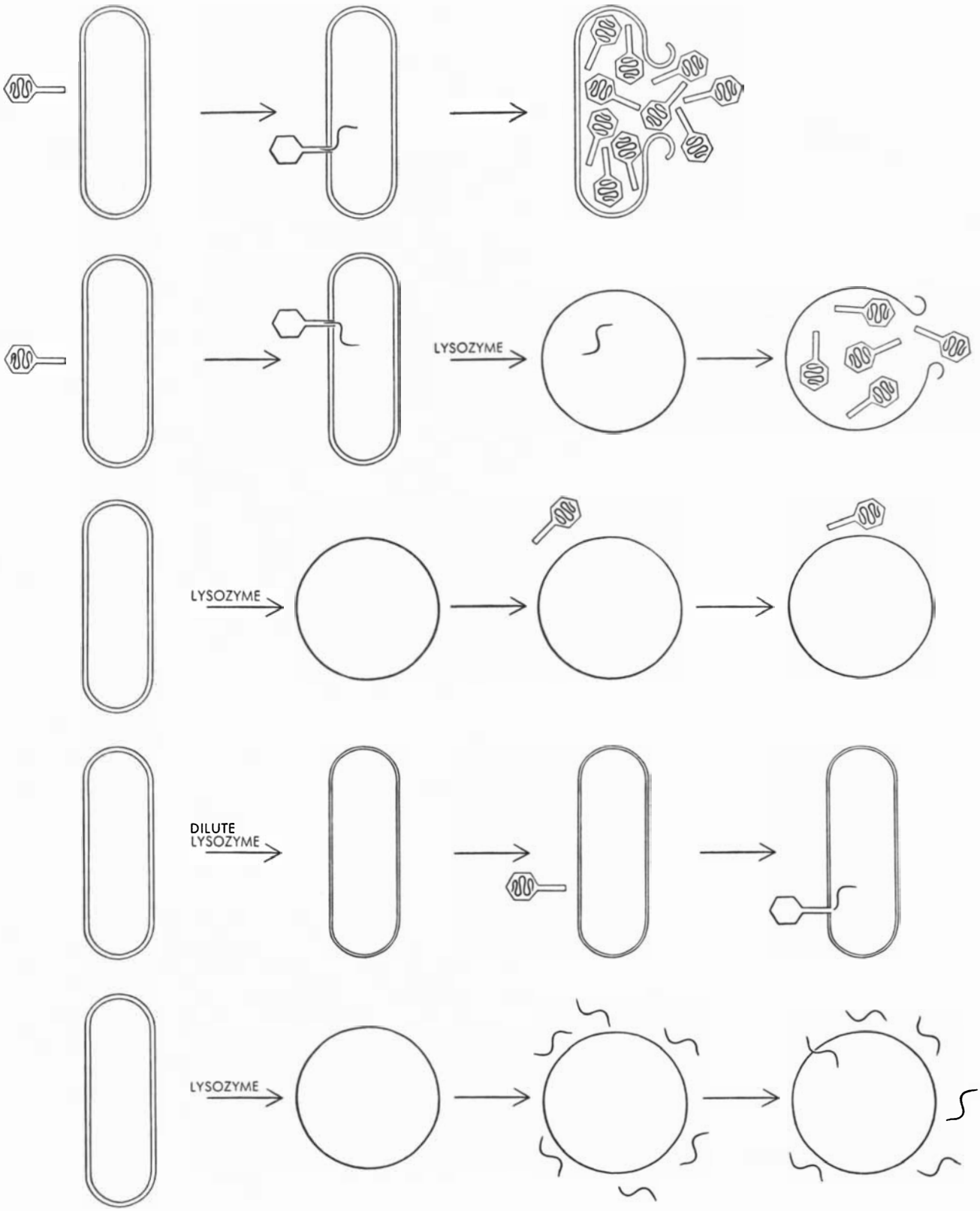


VIRUS INFECTION OF BACTERIA has been studied by means of protoplasts. Normally a virus particle attaches itself to a bacterium and injects its DNA (*thread*) into the cell; later the cell bursts to release many new virus particles (*first row*). A cell converted to a protoplast by lysozyme after it is already infected produces viruses in reduced number (*second row*). Viruses cannot attach themselves to protoplasts (*third row*), but can attach to and infect cells having some cell-wall layers remaining after mild

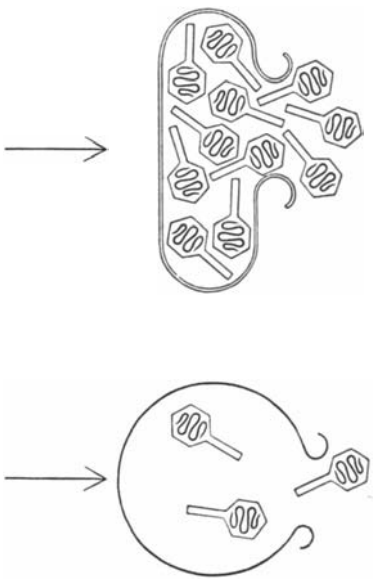
divide in two, neither it nor its offspring can form cell walls. This may be because protoplasts lack the ability to synthesize new cell-wall substance, or because they require special, and as yet undetermined, environmental conditions.

Some bacteria, such as *B. megaterium*, are equipped with whiplike appendages called flagella, the rhythmic motion of which propels the cells about when they are suspended in a fluid medium. Dissolution of the cell wall of these motile bacteria shows that the flagella are part of the cytoplasmic structure, for they remain attached to the protoplasts. The protoplasts are nevertheless incapable of motion. Probably some essential triggering mechanism associated with the cell wall is lacking. Or it may be that the protoplast structure lacks the rigidity necessary to make the thrust of the flagella effective. (An aircraft propeller would not work very well attached to a soft balloon!)

Virus infection represents one chink in the armor of the bacterial cell-wall. Investigators have found, however, that viruses which infect *B. megaterium* will not affect protoplasts of this bacterium. This supports other evidence that some chemical affinity between the virus and the cell wall makes a bacterium susceptible to infection by a particular virus. The submicroscopic, tadpole-shaped bacterial virus is known to be a quite complex structure, composed largely of virus genetic material (deoxyribonucleic acid, or DNA) enclosed in a protein coat. Ordinarily virus particles attach themselves tail-first to the cell wall, digest a hole in it and then inject their DNA into the bacterial cytoplasm. Inside the cell the virus DNA redirects the host's metabolism, causing it to produce virus material. The host cell then ruptures and frees several hundred newly formed virus particles into the surrounding medium. On the other hand, when a bacterial virus is cultured with protoplasts of a susceptible bacterium, it shows no affinity for them and does not infect them; it does, however, attach itself to the empty cell-walls of the same bacteria. Although the virus cannot attach itself to naked protoplasts, it can multiply in the protoplasts of bacteria that receive an infecting dose of viral DNA before their cell walls are removed. The process differs from that in normal whole cells only in that the protoplasts yield somewhat fewer viruses than whole cells do.

Must the entire cell-wall be present, or will the virus attach itself to cells with some layers of wall removed? A dilute solution of lysozyme, added to a suspension of *B. megaterium*, will remove small amounts of cell-wall material instead of the whole wall. When bacteria so treated are exposed to virus, they succumb to infection as readily as do intact cells. This indicates that the lower levels of cell-wall structure exposed by the lysozyme digestion are sufficiently like the surface for viruses to take hold. Either the configuration of the chemical bonds on the surface of the cell is not essential for phage attachment or it is somehow duplicated at lower levels as cell-wall material is removed. The lysozyme-treated cells produce a larger yield of virus than do normal cells, implying that the thinned-down cell wall is more readily penetrated by the virus.

The experience gained in the production and handling of protoplasts of organisms susceptible to lysozyme has encouraged investigators to extend this kind of study to other organisms and to adapt other agents, such as penicillin, to the production of protoplasts. The important and ubiquitous colon bacillus (*Escherichia coli*), extensively used in

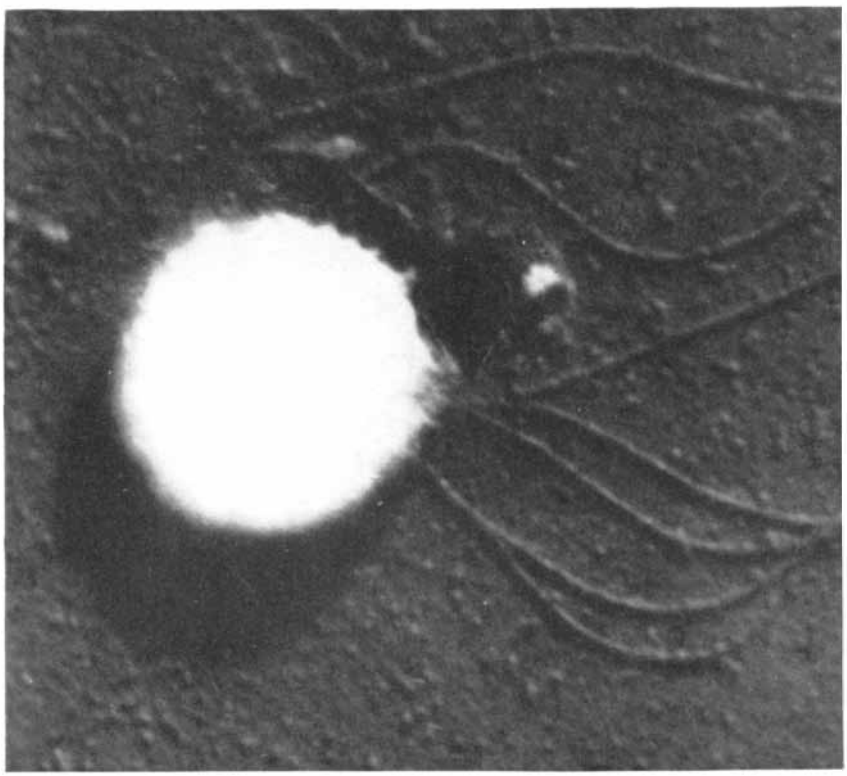


lysozyme treatment (*fourth row*). In one experiment spheroplasts, possibly still retaining some cell-wall material, were infected by free virus DNA (*fifth row*).

studies of bacterial biochemistry, bacterial genetics and virus infection, is resistant to lysozyme under the usual laboratory conditions, but is susceptible under special conditions. One of us (Hartsell) has discovered that dead cells of *E. coli* respond to lysozyme in an acid environment at high temperature to form stable protoplast-like spheres. Other workers have succeeded in removing the cell-wall material from living *E. coli* by employing lysozyme in combination with an alkaline solution or a chelating (metal-binding) agent. The cells thus treated lose their characteristic lozenge shape and form protoplast-like spheres. They can be preserved for extended periods in solutions containing sugar, salts or serum albumin in proper concentrations to balance the osmotic pressure on either side of the cell membrane. There is some question, however, whether these protoplasts are really completely free of cell-wall material, since their behavior sometimes differs from that observed in protoplasts of more sensitive strains that can be stripped of their cell walls by lysozyme alone. Pending the outcome of serological, chemical and other tests to prove the complete absence of cell-wall material, they are designated as spheroplasts.

Spheroplasts of *E. coli* have recently figured in a significant experiment involving DNA preparations from *E. coli* virus. Investigators at Indiana University and at Western Reserve University, working independently of one another, succeeded in separating most of the protein from the DNA fraction of this virus. Each group exposed a suspension of spheroplasts to their DNA preparation and secured a yield of viruses. Careful checking showed that the preparation contained no intact virus, although it was probably contaminated by a small amount of virus protein. Thus, although virus DNA by itself may not infect intact cells, the DNA in these preparations had penetrated the thin membrane enclosing the spheroplasts. The yield of virus from these experiments was small, but the findings are remarkable nevertheless. The synthesis of complete virus particles from DNA constitutes another piece of evidence that this molecule carries the important genetic properties usually associated only with intact organisms.

Fleming could not have anticipated the direction in which lysozyme would carry research in microbiology. The exploitation of lysozyme as a research tool shows how a simple discovery may open up vast new areas to investigation.



FLAGELLATED PROTOPLAST of *B. subtilis* shows that these whiplike swimming organelles are attached to the cell cytoplasm and not to the cell wall. This electron micrograph was obtained from J. M. Wiame. The structures are enlarged about 64,000 diameters.

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