STUDIES OF L FORMS AND PROTOPLASTS OF GROUP A STREPTOCOCCI*, ‡

I. Isolation, Growth, and Bacteriologic Characteristics

By EARL H. FREIMER, M.D., RICHARD M. KRAUSE, M.D., and MACLYN McCARTY, M.D.

(From The Rockefeller Institute)

Plates 89 to 91

(Received for publication, July 21, 1959)

The term "L form" has been applied to certain morphological variants of bacteria which grow in special media as minute colonies composed of pleomorphic globules that do not take the Gram stain (1, 2). These bacterial variants show a high degree of mechanical and osmotic fragility, and their properties suggest that they may lack a rigid bacterial cell wall.

The first direct evidence for the absence of a cell wall was obtained by Sharp *et al.* (3) who demonstrated by chemical and serological techniques that L forms of Group A streptococci lack the group-specific carbohydrate, a major component of the streptococcal cell wall. Chemical analysis of L forms of other species also indicates that important constituents of the cell wall are not present (4).

Enzymatic removal of the cell wall from living bacteria without disruption of the cell was described by Weibull (5). His initial studies involved the action of lysozyme on *B. megatherium* in a hypertonic environment provided by sucrose, and the spherical structures remaining after removal of the cell wall were termed protoplasts. Protoplasts have now been prepared by similar techniques from many bacterial species, and they share the property of requiring a hypertonic environment which can be supplied either by sucrose or salts (6). Dilution of a suspension of protoplasts with water results in their disruption (5). The protoplast membrane, which remains as a well defined structure after osmotic rupture, has been shown by morphological and chemical studies to be distinct from the bacterial cell wall (7–9). The viability of protoplasts is indicated by their metabolic activity and by the observation of "budding" and "dumbbell" forms (10, 11). However, reproduction of protoplasts in a nutrient medium has not been described.

Group A streptococci offer certain advantages for a study of the relationship of L forms and protoplasts to each other and to the parent bacterial forms,

* This investigation was supported in part by research grant H3919 from The National Heart Institute, Division of Research Grants, United States Public Health Service.

‡ Presented in part on May 11, 1959, at the 59th general meeting of the Society of American Bacteriologists in St. Louis.

since comparison of their composition and physiological activity is facilitated by the fact that a large number of different streptococcal components have been identified. In addition to several antigens known to occur in association with the cell wall, these organisms elaborate a wide variety of extracellular products for which specific tests are available (12). The present investigation is concerned with the isolation and production of L forms and protoplasts of Group A streptococci, and the comparison of these cell wall-free structures with their bacterial parents by morphological, serological, chemical, and physiological techniques.

Materials and Methods

Strains of Streptococci.—Group A streptococcal strains T1/119 (Type 1), S43/100 (Type 6), 1GL8 and J17D/90 (Type 19), T22/83 (Type 22), C98/90 (Type 24), and J17F/90 (Type 26) have been used to make L forms. Group A streptococcal strains T1/119 (Type 1), D58X/11 (Type 3), S43/100 (Type 6), T12/36 (Type 12), T14/46 (Type 14), J17D/90 (Type 19), T22/83 and B547 (Type 22), T25/41 (Type 25), J17F/90 (Type 26), and D24/94 (Type 30) have been used to make protoplasts and protoplast colonies. These strains were all from The Rockefeller Institute collection.

Serological Identification.—The serological identification of the strains used in these experiments was confirmed with the capillary tube precipitin test using streptococcal group and type-specific rabbit antisera (13).

Modified Proteose Peptone-Beef Heart Infusion Agar.—Infusion agar for the growth of L forms and protoplasts of hemolytic streptococci was prepared by a modification of a previously described medium (14). This modified nutrient agar contained 4 per cent sodium chloride, 1.5 per cent agar, and 10 per cent horse serum. In most instances crystalline potassium penicillin G was added at a final concentration of 1000 units/ml. to prevent reversion of L forms and protoplasts to characteristic hemolytic streptococci.

Phage-Associated Lysin.—The partially purified phage-associated lysin, which was used to make protoplasts in all of the critical chemical and serological experiments, was prepared as described by Krause (14). A crude phage lysate of Group C streptococci was utilized as the source of lysin in many of the preliminary experiments. Both the partially purified and the crude lysin preparations have been stored indefinitely in a dry ice box without deterioration.

Analytical Methods.—Rhamnose was determined by the method of Dische and Shettles (15). Desoxyribonucleic acid was determined by the Burton modification of the diphenylamine reaction (16). Quantitative glucosamine determinations were done by a modification of the Elson and Morgan procedure (14).

Preparation of the Streptomyces albus Enzyme.—This material was prepared by methods previously described (17).

Precipitin Analysis.—Quantitative precipitin tests for the group-specific carbohydrate were done by a spectrophotometric procedure employed previously in immunochemical studies of the carbohydrate (18).

EXPERIMENTAL

Streptococcal L Forms

Isolation of Streptococcal L Forms.—L forms were isolated by a modification of the penicillin gradient technique described originally by Sharp (19–21). In

this method bacteria are streaked over the surface of a modified nutrient agar plate containing penicillin in a ditch at one end. The penicillin freely diffuses into the agar, and inhibits streptococcal growth in an area several centimeters from the penicillin depot. After several days of anaerobic incubation, small L form colonies appear near the margin of the zone of bacterial inhibition.

The plates for these experiments were prepared as follows: Horse serum was filtered through a Coors p 3 filter in order to ensure sterility. The filtered serum was added in a final concentration of 10 per cent to proteose-beef heart infusion agar which contained 4 per cent NaCl. Thirty ml. volumes of this mixture were dispensed into standard Petri dishes. After solidification of the medium, a 40 × 5 mm. groove was cut to a depth of several millimeters near the edge of the plate and filled with 0.2 ml. of aqueous potassium penicillin G, 1000 units/ml., freshly prepared in sterile distilled water.

Several loopfuls of an overnight blood broth culture of Group A streptococci were streaked on each plate at right angles to the penicillin ditch. Ten plates were placed in a desiccator and the air was displaced by nitrogen. To accomplish this, each desiccator was connected by a Y-tube containing a cotton filter to both a vacuum line and a tank of technical grade nitrogen. By alternately evacuating the desiccator and refilling it with nitrogen three times, anaerobiosis was achieved and could be confirmed by observing the color loss of an indicator tube containing methylene blue and dextrose. After 72 hours of incubation at 37°C., the cultures were examined at a magnification of 20 using a binocular microscope with reflected light.

The small colonies visible in the region of inhibition of streptococcal growth were similar in morphology to the colony described for L forms of other bacteria (2). While the streptococci grew as raised colonies on the surface of the plate, the L forms grew down into the agar. Repeated attempts to Gram-stain the L forms were unsuccessful, and the usual technique of staining L colonies with methylene blue was not suitable for adequate resolution of the microscopic details of colonial structure. However, excellent detail was obtained by examination of coverslip preparations of unstained L colonies by phase contrast microscopy. At the periphery of these colonies, it was possible to observe aggregates of phase dense spherical structures which varied considerably in size. Streptococcal colonies examined in a similar manner consisted of the usual chains of cocci of almost uniform size.

Only four of nine streptococcal strains produced L forms on penicillin gradient plates, and the yield varied with the strain used. The failure to obtain L forms from five strains was probably related to the poor growth of these streptococci in high concentrations of sodium chloride. This suggested that selection from the bacterial population of those organisms which are able to grow well in hypertonic media might be of value in order to increase recovery of L forms from streptococcal strains.

The nine cultures were transferred into fresh salt broth daily. By gradually increasing the concentration of sodium chloride, streptococci were finally obtained which grew well in a medium containing 6 per cent sodium chloride. When penicillin gradient plates were streaked with organisms from the cul-

tures that had grown in a hypertonic medium, L forms were obtained from seven of the nine strains, and the yield was increased for each of the four strains that had previously produced L forms.

Subculture of L Forms.—The L form colonies were successfully subcultured to modified nutrient agar with penicillin. Subculture was accomplished by cutting agar blocks containing surface colonies, and sliding the blocks face down over the new agar surface. The colonies in the upper block grew down into the new agar. Occasionally, colonies appeared along the path of placement, but the number of new colonies was usually dependent on the growth in the transferred block. While anaerobic conditions were required for the initial isolation of streptococcal L forms, subcultures grew very well aerobically. The protective effects of albumin or serum, and at least 2 per cent sodium chloride, were essential for the growth of subcultures of L forms. Sharp has grown several strains of streptococcal L forms in a liquid medium (3). For the strains tested here, a culture medium containing as little as 0.4 per cent agar supported excellent growth of L forms, but repeated attempts to obtain growth with lower concentrations of agar, or in a liquid medium were unsuccessful. However, when an agar block containing several L colonies is transferred into a liquid culture medium, the colonies grow until they fill the entire block with confluent growth.

Protoplasts of Group A Streptococci

Studies of the chemical and serological properties of L forms have been hindered by the limited quantity of L forms that can be obtained with present techniques. Since the rigid cell walls of bacteria are absent in protoplasts as well as L forms, investigations were begun to obtain streptococcal protoplasts for chemical and serological examination, and for comparison with the L forms. The usual technique for obtaining protoplasts from other bacteria depends upon the removal of the cell wall material by lysozyme (22). However, lysozyme does not appreciably affect the cell walls of Group A streptococci, and the enzymes of *Streptomyces albus* which lyse streptococci are not well suited to the preparation of protoplasts because of the large amount of proteolytic enzyme present in usual preparations of this material (17). On the other hand, the absence of proteolytic activity had been demonstrated in preparations of a phage-associated lysin which was being investigated in our laboratory (23). This lysin, obtained from fresh phage lysates of Group C streptococci, dissolves Group A streptococcal walls with the release of group-specific carbohydrate and M protein, thus suggesting that protoplasts of Group A streptococci might be obtained with this material. In the present study, protoplast formation was in fact accomplished by enzymatic removal of the cell wall with this lysin in the presence of a hypertonic sodium chloride solution which was used to prevent rupture of the osmotically fragile protoplasts. Gooder and Maxted have also

obtained streptococcal protoplasts by means of a similar phage-associated lysin (24, 25).

The Preparation of Protoplasts.—When phage lysin was added to a streptococcal culture in the log phase of growth and the mixture was incubated at 37°C., the streptococcal cell wall was dissolved with almost complete lysis of the organisms within 10 minutes. The mixture became viscous because of the release of intracellular desoxyribonucleic acid (DNA), and a phase contrast microscopic examination of this mixture revealed cellular debris. Only partial clearing of the suspension occurred, however, when the lysin and the streptococcal culture were incubated in a solution containing 4 per cent sodium chloride, suggesting that protoplasts had been formed. The procedure employed in these studies was as follows.

A subculture from the overnight growth in blood broth of Type 6 streptococci was made in Todd-Hewitt broth and incubated at 37°C. for 4 hours. The bacteria were centrifuged, washed with saline, and resuspended in one-tenth of their original volume with saline buffered by M/100 phosphate at pH 6.5. One ml. of the same phosphate buffer with a sodium chloride concentration of 8 per cent was mixed with 0.5 ml. of crude lysin and 0.5 ml. of the streptococcal suspension, and the mixture incubated at 37°C. for 40 minutes.

Examination of a drop of this suspension with the phase microscope disclosed individual phase dense spherical structures with some variation in size and complete absence of the typical chains of cocci present in the original culture. This suspension of single spherical structures rapidly cleared and became viscous when its salt concentration was lowered by dilution with distilled water. The development of single osmotically fragile spherical structures from streptococcal chains by the use of an enzyme capable of dissolving the streptococcal cell wall suggested that protoplasts had been produced, and had been protected from rupture by the hypertonicity of the medium. Evidence presented below demonstrates that these structures are free of cell wall carbohydrate.

Protoplast Membranes.—The rupture of protoplasts with the release of intracellular contents by the addition of distilled water was observed under the phase microscope. When this occurred there was a sudden loss of phase contrast; but discrete disc-like structures of low phase density remained. These are the protoplast membranes which have been described for other bacteria as "protoplast ghosts." The morphology of streptococcal protoplast membranes was visualized with the electron microscope.

Protoplasts of strain S43FL were prepared as described, centrifuged, and ruptured in distilled water. In order to remove the DNA which was adsorbed to the membranes, the suspension was incubated at 37°C. with magnesium-activated DNase for 30 minutes. The membranes were collected by centrifugation, washed with saline, followed by distilled water, and resuspended in distilled water. This preparation was mounted on carbon-coated No. 150 mesh copper grids, and shadowed with metallic chromium at an angle of 16°. The specimens were examined and photographed in a Phillips EM100 electron microscope.

An electron micrograph of protoplast membranes is shown in Fig. 1. The membranes are delicate structures which are much thinner than the cell walls shown in Fig. 2. These cell walls, isolated from streptococci by a modification of the method of Salton in which bacteria are disrupted in a Mickle disintegrator (26), were mounted on copper grids by a method similar to that used for the membranes.

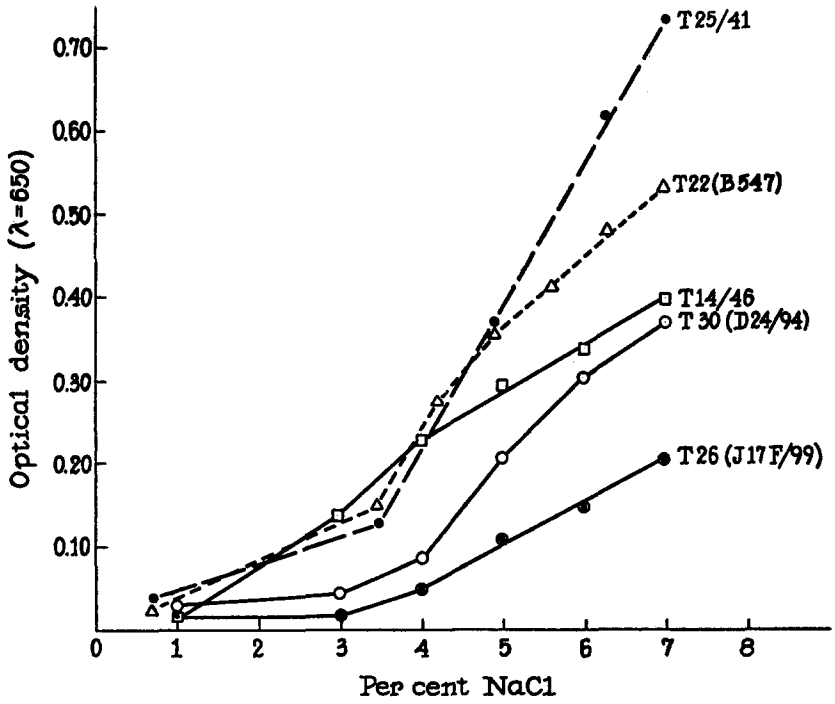
Effects of Salt Concentration on Protoplast Formation.—In the previous experiments, protoplasts were obtained from streptococci suspended in solutions containing 4 per cent sodium chloride. However, the suspensions partially cleared after lysin was added. This suggested that, after their cell walls had been removed, some of the streptococci had completely lysed at this salt concentration instead of remaining as protoplasts. The concentration of sodium chloride that would preserve the greatest number of intact protoplasts was studied in the following experiments.

A series of seven solutions with increasing concentrations of sodium chloride was prepared in M/100 phosphate buffer, pH 6.8. One ml. of each of the salt solutions was placed in a separate 75 by 12 mm. cuvette; 0.5 ml. of phage lysin was added together with 0.5 ml. of a 10-fold concentrated bacterial suspension prepared from streptococci in the log phase of growth. This series of tubes with the final salt concentration ranging from 1 to 7 per cent, was incubated for 40 minutes in a 37°C. water bath. The optical density of each tube was measured at 650 mμ in a Coleman Jr. spectrophotometer, and each suspension was examined under the phase microscope. The presence of single spherical protoplasts and the absence of streptococcal chains indicated that conversion of streptococci to protoplasts was completed. The experiment was performed with six different strains of Group A streptococci.

Text-fig. 1 shows the results of treating streptococci with lysin in the presence of various concentrations of sodium chloride. The optical density of each suspension is plotted against the final salt concentration. The low optical density in the lower concentrations of salt suggests that more of the streptococci have completely lysed, and that fewer have merely lost their cell walls and remained in the suspension as intact protoplasts. The results with several strains are consistent in indicating that at higher salt concentration an appreciably greater number of undisrupted protoplasts remain. However, even at concentrations of sodium chloride as high as 7 per cent there is some decrease from the optical density of the untreated streptococcal suspension. For example, the action of lysin on T14/46 in 7 per cent sodium chloride resulted in a fall of optical density from 0.70 to 0.40 in 40 minutes. Part of this fall in optical density may be due to disruption of chains and dissolution of the cell wall, but experiments described in the next section indicate that complete lysis of a portion of the streptococcal suspension has occurred.

Quantitative Studies of the Conversion of Streptococci to Protoplasts.—The previous experiments have shown that as the salt content of the streptococcal mixture to be converted to protoplasts was lowered, the optical density of the

final suspension of protoplasts also decreased. To determine the proportion of organisms that withstand removal of the cell wall without rupture, quantitative measurements of the amount of lysis were desirable. Since little or no DNA is found in the cell wall or in the protoplast membrane, the DNA in solution after the treatment of streptococci with lysin is a measure of the streptococci lysed during the procedure. The following experiments were designed to deter-



TEXT-FIG. 1. The preparation of protoplasts from streptococci in hypertonic concentrations of sodium chloride. The optical density of each culture at the various salt concentrations was measured after 40 minutes of incubation at 37°C. with the lysin.

mine the percentage of intact protoplasts remaining at two concentrations of salt by the measurement of DNA released during removal of the cell wall by lysin.

Three liters of Todd-Hewitt broth were inoculated with sufficient actively multiplying streptococci of the Type 6 strain S43FL so that after 4 hours of incubation at 37°C. the culture had reached an optical density of 0.25, measured at 650 mμ in a Coleman Jr. spectrophotometer in a 19 by 105 mm. cuvette. The culture was centrifuged, and the bacteria were washed with 1 per cent sodium chloride prepared in M/100 phosphate buffer, pH 6.5. The bacteria were resuspended in 75 ml. of the same buffer in sufficient salt to give a final concen-

tration of either 5.2 or 7 per cent sodium chloride, and incubated with phage-associated lysin at 37°C. for 40 minutes.

At the end of the incubation period, the resulting protoplast suspension was examined under the phase microscope. Conversion of bacteria to protoplasts was indicated by complete disruption of the streptococcal chains into single spherical units. The protoplasts were collected by centrifugation at 4000 R.P.M. in a refrigerated angle head centrifuge, and the supernatant was saved (supernatant of protoplast formation). The protoplasts were washed with 10 ml. of cold M/100 phosphate buffer containing the concentration of NaCl used in their preparation, and recentrifuged. The wash was combined with the first supernatant. The protoplasts were resuspended in distilled water and rupture resulted. The protoplast membranes remaining after this procedure were collected by centrifugation at 10,000 R.P.M. in the angle head of a high speed centrifuge, and the supernatant was saved (supernatant

TABLE I

Distribution of Streptococcal DNA and Rhamnose in Fractions Obtained during Protoplast Formation at Two NaCl Concentrations

	NaCl concentration	Supernatant of protoplast formation	Supernatant of protoplast rupture	Protoplast membranes
	per cent	*per cent*	*per cent*	*per cent*
DNA	5.2	42	54	2
	7.0	29	67	2
Rhamnose	5.2	97	1	0.4
	7.0	96	3	0.6

The streptococcal suspension of S43FL had 153 μg./ml. of desoxyribonucleic acid (DNA) and 407 μg./ml. of rhamnose.

of protoplast rupture). The membranes were washed with distilled water, and recentrifuged. This wash was combined with the supernatant of protoplast rupture. The membrane pellet was resuspended in distilled water, and stored in the cold. To reduce viscosity which might prevent clean separation of fractions, an effective concentration of magnesium-activated DNase was present in all stages of the procedure.

As a control for determination of the total DNA, an aliquot of the original streptococcal suspension was lysed by the enzyme in buffer without added salt so that the unprotected osmotically fragile streptococcal protoplasts would be ruptured as soon as they formed. Each of the fractions was precipitated with five volumes of alcohol. The precipitates were washed with alcohol and ether, and extracted with 5 per cent trichloracetic acid, first in the cold and finally at 90°C. for 30 minutes. DNA determinations were carried out on the trichloracetic acid extracts.

Table I gives the results of a typical experiment. Of the 153 mg. of DNA present in the original suspension of streptococci, 54 per cent was recovered on rupture of the washed protoplasts formed in 5.2 per cent NaCl and 67 per cent in the case of those formed in 7 per cent NaCl. Thus, in the higher salt concentration two-thirds of the protoplasts remained intact after removal of the cell wall. The remainder of the DNA was found in the supernatant fluid after enzyme treatment (supernatant of protoplast formation), and represents

material released from cells which had completely lysed. Measurement of ribonucleic acid indicated a comparable amount of cell disruption during protoplast formation. These findings on the distribution of cellular contents in the various fractions stand in contrast to the distribution of cell wall components, as described in the next section.

Quantitative Studies on Removal of Cell Wall.—All of the preliminary studies suggested that the osmotically fragile spherical structures released from chains of Group A streptococci by phage-associated lysin are protoplasts. However, further evidence for the absence of the cell wall is important since protoplasts have been defined as structures which are completely free of cell wall material (27). The group-specific carbohydrate is the major structural component and comprises approximately 50 per cent of the streptococcal cell wall. Rhamnose represents almost 50 per cent of this carbohydrate (17). In order to demonstrate that streptococcal protoplasts prepared with phage-associated lysin do not contain cell wall carbohydrate, the same fractions of Type 6 streptococcal protoplasts which were prepared in 5.2 and 7 per cent sodium chloride for analysis of DNA were analyzed for rhamnose before alcohol precipitation and trichloracetic acid extraction. The rhamnose content of the original streptococcal suspension, and the distribution of this rhamnose in the several fractions are recorded in Table I. Of the 407 μg./ml. of rhamnose present in the original streptococcal suspension, more than 96 per cent was recovered in solution during the action of lysin (supernatant of protoplast formation). Only very small amounts of rhamnose were found in association with the protoplasts. The figures recorded in Table I for the protoplast contents (supernatant of protoplast rupture) and protoplast membranes are almost certainly high, since the rhamnose determinations on these materials did not have absorption maxima in the range of 396 to 400 mμ characteristic of rhamnose. Therefore, some of the absorption at this wave length was not due to rhamnose, and the actual per cent of rhamnose remaining in these protoplast fractions is lower than that recorded in Table I.

An additional cell wall component, type-specific M protein, was also studied. In this case, methods for quantitative measurement are not available, and the presence of M protein was determined by the capillary precipitin reaction. Type 6 M protein was predominantly present in the supernatant of protoplast formation, indicating that this cell wall component had been almost quantitatively released into solution by the phage-associated lysin. The possibility of occurrence of M protein in the protoplast membrane was tested by preparing extracts at pH 2 and 100°C. by the standard method for obtaining M extracts. These extracts showed no reaction with any of the type-specific rabbit antisera including the homologous Type 6 antiserum. These results indicate that as the cell wall is dissolved during protoplast formation, the M protein is released into solution, and is not serologically detectable in the protoplast membrane.

Further studies on the protoplast membranes were carried out with another streptococcal strain, T25/41. The cells from 4 liters of broth were converted to protoplasts as described, and more than 98 per cent of the rhamnose of the original streptococci was recovered in the supernatant of protoplast formation. The washed protoplasts were ruptured, and the bulk of the intracellular material was removed from the membranes by washing with water, saline, phosphate buffer pH 8, and finally three times with water. The yield of protoplast membranes was 86 mg. These membranes contained less than 0.5 per cent rhamnose. Analysis for glucosamine, the other major sugar in the streptococcal cell wall carbohydrate, also gave values of less than 0.5 per cent of the dry weight of the membranes. In addition to the chemical studies, the protoplast membranes were checked serologically for the presence of residual Group A cell wall carbohydrate by quantitative precipitin analysis. A 9 mg. sample of T25/41 membranes was treated with *Streptomyces albus* enzyme to dissolve the residual carbohydrate. After centrifugation, the carbohydrate in the supernatant was precipitated with Group A rabbit antiserum, and this precipitate was compared with precipitates obtained from solutions with known concentrations of Group A carbohydrate. As measured by this technique, cell wall carbohydrate comprised less than 0.05 per cent of the protoplast membrane.

The results of these chemical and serological analyses suggest that the cell wall of the streptococcus is removed by phage-associated lysin during the formation of protoplasts, and that both the intact protoplast and its isolated membrane are essentially free of cell wall material.

Growth and Appearance of Protoplast Colonies.—Minute colonies similar to L forms were grown from protoplasts in the same modified nutrient agar with penicillin that supported growth of the L forms. Protoplast suspensions of strains S43FL and T25, shown to be essentially free of cell wall carbohydrate as described in the previous section, were used in the following experiments on growth. One ml. of the protoplast suspension was added to 250 ml. of previously liquefied agar, and the mixture was poured into Petri dishes. After incubation at 37°C. for 24 hours very small colonies appeared throughout the agar and these continued to enlarge during additional incubation. At 72 hours these protoplast colonies, when viewed with the colony microscope, closely resembled streptococcal L form colonies. Examination of a wet coverslip preparation of an individual protoplast colony with the phase microscope, disclosed an aggregate of spherical units similar to the round phase-dense structures in a freshly prepared protoplast suspension. The microscopic appearance of the protoplast colony was identical with that previously described for the L form colony.

Protoplasts have been prepared from strains of Group A streptococci representing twelve different types, and protoplast colonies have been grown from each strain by the use of the pour plate technique. The yield of growing protoplast colonies varies with the strain and with the salt concentration at the time

the cell wall is removed. In order to estimate the number of streptococcal protoplasts that grew into colonies, protoplasts of S43FL were prepared in 4 per cent NaCl. The streptococci in the suspension of S43FL prior to the addition of lysin were estimated by the pour plate technique. The number of protoplasts that formed colonies was determined by plating 10-fold dilutions of the protoplast suspension in soft agar layers of modified nutrient agar, and counting after 3 days of incubation. In addition, in order to determine the number of viable streptococci that remained after treatment with lysin, dilutions of the protoplast suspension also were plated in nutrient agar which did not contain penicillin or added NaCl. The number of streptococci prior to the addition of lysin was 4.4×10^8 per ml., while the number of intact streptococci still surviving after incubation with lysin was only 1×10^4 per ml. Each ml. of this suspension yielded 5.4×10^7 protoplast colonies. These counts suggest that 12 per cent of the streptococci had been converted to viable protoplasts. However, this percentage is somewhat high because each streptococcal colony represents an entire chain of cocci, while each protoplast colony probably represents a single viable protoplast. These colonies of protoplasts could not have grown from any residual streptococci in the protoplast suspension, which may have been converted to L form colonies by the penicillin in the modified nutrient agar, because the number of protoplast colonies was 5,000 times greater than the number of residual streptococci.

Subculture of Protoplast Colonies.—Surface growth of protoplast colonies was obtained by layering a mixture of 0.1 ml. of a protoplast suspension and 2 ml. of modified nutrient medium containing 0.7 per cent agar on plates containing 30 ml. of previously hardened nutrient agar with penicillin. Subcultures were easily obtained by transfer of blocks of agar containing surface growth to fresh modified nutrient agar plates, a technique described in detail for the L forms. Subcultures into a liquid medium have not yet been successful, although when protoplasts are transferred into salt broth containing as little as 0.2 per cent agar, good colonial growth develops.

Antibiotic Sensitivity of Protoplasts.—The available evidence indicates that penicillin acts by inhibiting formation of the bacterial cell wall (28), and it is thus significant that L forms are highly resistant to the action of penicillin (29). In the present studies it has been found that streptococcal protoplasts also grow in the presence of a high concentration of penicillin, and appear to be resistant to this antibiotic. Certain other antibiotics which inhibit streptococcal growth do not interfere with cell wall formation. Therefore, several strains of streptococci and the protoplasts derived from them were compared for sensitivity to streptomycin, tetracycline, and chloramphenicol, as well as to penicillin. Varying concentrations of each antibiotic were mixed with a suitable dilution of either streptococci or protoplasts derived from the strain, and three modified nutrient agar pour plates were made for each mixture. In

this experiment 5 units/ml. of penicillin was also incorporated in the protoplast plates to prevent reversion to the bacterial forms. Table II compares the minimal concentration of each antibiotic that inhibited the growth of strain S43FL and the protoplasts derived from it. The streptococci were highly sensitive to penicillin while the protoplasts were resistant to 1000 units/ml. Both the streptococci and the protoplasts of this strain were very sensitive to low concentrations of tetracycline and chloramphenicol, and although both were sensitive to streptomycin, the protoplasts were sensitive to slightly smaller concentrations. Similar sensitivities were obtained with three other strains of streptococci and their protoplasts.

TABLE II

The Concentration of Antibiotics Which Inhibits the Growth of Streptococci and Their Protoplast Colonies

Streptococcal strain	Antibiotic	Concentration of antibiotics	
		Bacteria	Protoplasts
S43FL	Penicillin	<0.05 U/ml.	>1000 U/ml.
	Streptomycin	<5 but >10 U/ml.	>0.5 but <2.5 U/ml.
	Tetracycline	<1 μg./ml.	<1 μg./ml.
	Chloromycetin	<1 μg./ml.	<1 μg./ml.
B547	Penicillin	<0.05 U/ml.	>1000 U/ml.
	Streptomycin	>1000 U/ml.	>1000 U/ml.

For these tests the streptococci and protoplast colonies were grown in modified nutrient agar to which the antibiotics had been added.

The fact that bacteria and protoplasts have the same sensitivity to antibiotics which do not interfere with cell wall synthesis is further illustrated by an experiment in which a laboratory selected streptomycin-resistant strain of Group A streptococcus, B547, was used. As shown in Table II, protoplast colonies derived from this strain have the same high level of resistance to streptomycin as the parent bacterial strain.

Interrelationships between L Forms, Protoplasts, and Bacterial Forms

In studying derivative forms, such as L forms and protoplasts, it is important to obtain confirmatory evidence for their biological relationship to the original bacterial strain in order to eliminate the possibility that chance contaminants are involved. In the present studies, evidence of this sort was obtained by comparison of antigenic constituents and certain of the extracellular products of Group A streptococci with those produced by derivative forms, and by testing the capacity of the derivative forms to revert to the parent bacterial form under appropriate conditions.

Extracellular Enzyme Production.—The ability to produce a variety of substances such as streptolysin, streptokinase, and desoxyribonuclease (DNase) which are released into the extracellular environment by living bacterial cells is characteristic of hemolytic streptococci (12). The production of hemolysin by L forms of Group A streptococci has been demonstrated by the following technique. Ten ml. of 0.75 per cent proteose agar containing 4 per cent rabbit blood were layered on top of an agar plate containing a surface subculture of L forms that had grown for 72 hours. Hemolytic zones appeared above each L colony after 15 minutes of incubation. A similar technique was used to demonstrate hemolysin production by protoplasts. Protoplast colonies were grown in a thin modified nutrient agar layer for 72 hours, and then blood agar was layered over the colonies. After 15 minutes of incubation at 37°C., hemolytic zones appeared over each protoplast colony. An example of the hemolysis that develops around a protoplast colony is illustrated in Fig. 3. Such rapid hemolysis suggests the presence of a preformed hemolysin. When a large number of colonies were present on the plate, the entire blood layer was hemolyzed after several hours. Hemolysin was released from each of the four strains of streptococcal L forms and each of the five strains of streptococcal protoplasts that were tested. After repeated subculture, L form and protoplast colonies continued to produce hemolysin. This hemolysin has not yet been identified as one of the known extracellular streptococcal hemolysins, streptolysin O or S.

Because sufficient growth of L form colonies has not been obtained, the production of DNase has only been demonstrated for the protoplast colonies. The following procedure was employed.

Protoplast colonies were grown in modified nutrient agar for 72 hours. The agar was frozen and thawed, and the fluid released by this procedure was dialyzed to remove the salt. The DNase activity of the fluid was measured by its ability to depolymerize a substrate of calf thymus DNA (1 mg./ml.) in veronal buffer pH 7.5 containing 0.005 M Mg^{++}. In this method depolymerization is measured by the absence of a fibrous precipitate when absolute alcohol is added to the substrate enzyme mixture after 30 minutes in a 37°C. water bath (30).

The DNase activity of the fluid extracted from the agar culture of protoplasts varied with the extent of growth of colonies, and the salt concentration of the medium. However, under the optimal conditions for good protoplast growth, fluids obtained from two protoplast strains and diluted as high as 1:100 still retained DNase activity. Hemolytic streptococci produce three immunologically and electrophoretically distinct desoxyribonucleases which have been designated as DNase A, B, and C (31). As a strain grows in a given medium, the relative amount of each enzyme produced by the strain usually is constant, and one of the three enzymes is often produced in larger amounts than the other two. The DNase contained in the extracts of protoplast colonies growing in agar, and the DNase present in the supernatants of broth cultures of the parent bacteria were identified by the following serological technique.

Unabsorbed rabbit sera containing antibody specific for DNase A, B, or C[1] were diluted 1:2 with saline and heated at 65°C. for 30 minutes to destroy serum nuclease activity. Various mixtures of the three antisera were also used to test for the possible presence of more than one of the enzymes. After the sera were diluted 1:10 with saline, 0.1 ml. of each dilution was mixed with 0.1 ml. of an appropriate broth dilution of the solution containing the unknown DNase. After the mixtures were incubated for 30 minutes at 37°C., 0.5 ml. of the DNA substrate was added, and the new mixtures were reincubated for 30 minutes at 37°C. One ml. of ethyl alcohol was then added to precipitate any intact residual DNA.

Table III records the results obtained by testing the supernatant of two S43 streptococcal strains and the fluid extracted from the agar growth of protoplast

TABLE III

Serological Comparison of DNase Production by Hemolytic Streptococci and Their Protoplasts

Source		Specific DNase antisera			Mixtures of specific DNase antisera			
		A	B	C	AB	AC	BC	ABC
S43/100 broth culture Supernatant diluted	1:30	0	+	++++	0	++++	++++	++++
S43FL broth culture Supernatant diluted	1:20	0	0	+++	0	++++	++++	++++
Protoplasts of S43FL Extract of agar culture diluted	1:10	0	0	+++	0	++++	++++	++++

++++, intact fibrous precipitate (complete inhibition of enzymatic activity).
0, no fibrous precipitate (no inhibition of enzymatic activity).

colonies derived from one of these strains. It is apparent that DNase C is the predominant enzyme produced by all three organisms. In a similar experiment DNase B was found to be the major nuclease obtained from both the bacterial and the protoplast growth of D58X/11.

Production of Type-Specific Antigen.—The M protein is the cell wall component of Group A streptococci upon which serological type specificity is based. Preliminary evidence suggests that L form and protoplast colonies elaborate a similar serologically type-specific antigen which is released into the agar. This is in contrast to the behavior of intact streptococci, in which the M protein is firmly attached to the cell wall and only traces are released into the medium. Initial evidence for production of type-specific antigen was obtained by the formation of concentric rings of precipitate around colonies growing in homologous type-specific antiserum. L forms or protoplasts, after

[1] These antisera were prepared by Dr. L. W. Wannamaker in this laboratory (31).

several subcultures, were transferred to penicillin proteose agar plates containing 10 per cent rabbit immune serum of the type of Group A streptococcus from which they were originally derived. Control plates were prepared in an identical way except that heterologous immune serum was used. The plates were incubated for 72 hours at 37°C.

A typical experiment is illustrated in Fig. 4. In the plate on the right the L forms of strain S43/100 were grown in a medium containing homologous antiserum. Zones of precipitate are clearly visible around each colony. When incubation was continued beyond 72 hours, each zone of precipitate increased, and in some instances became confluent over large areas of the plate. In the control plate on the left, L forms of the same strain were grown in a medium containing the heterologous Type 26 antiserum and no precipitate is visible. Additional control plates, not pictured here, in which Types 1, 3, and 30 or Group A antiserum were incorporated into the medium did not contain a precipitate. Experiments using Type 1 and Type 26 L forms and homologous antisera also demonstrated zones of type-specific precipitate.

Similar zones of precipitate developed around protoplast colonies growing in type-specific antiserum. Although the protoplast colonies grew as well in the control plates which contained heterologous type or Group A antisera, no precipitate appeared around any of these protoplast colonies. When intact streptococci were grown in a medium containing homologous type-specific antiserum, no zones of precipitate appeared indicating that intact streptococci did not release an appreciable amount of M protein into the medium.

Further evidence for the type specificity of the material released by protoplast colonies was obtained by the following technique:

Protoplast colonies of Type 6 streptococci were grown in modified nutrient agar for 72 hours. The agar was cut into small cubes and frozen and thawed. The fluid extracted by this procedure was dialyzed against saline to lower the salt concentration, and tested serologically in capillary precipitin tubes with the anti-group and the anti-M immune rabbit sera.

The agar extract produced a definite precipitin reaction with the Type 6 rabbit antiserum while no cross-reactions occurred with forty-five heterologous antisera. In addition, no reactions were noted with group-specific antisera. The material in the extract responsible for this type-specific reaction was sensitive to trypsin and resistant to boiling at pH 2 for a short period, two characteristic properties of M protein. The agar extracts of Type 1 and Type 3 protoplast colonies also gave type-specific precipitin reactions with homologous type antisera.

A convincing demonstration of the release of type-specific material by protoplast colonies was obtained by the use of modified Ouchterlony plates (32).

Lucite cylinders, 9 mm. high, were placed on a solidified thin layer (10 ml.) of clear agar in Petri dishes and 15 ml. of supporting agar were poured around them. One per cent agar

containing 3 per cent sodium chloride and 1000 units of penicillin per ml. was employed. Modified nutrient agar containing protoplasts was poured into a large center cylinder (diameter 40 mm.), and the plates were incubated for 3 to 5 days until good growth of protoplast colonies had occurred. At this time 0.25 ml. of an immune serum was placed in each small peripheral cylinder (diameter 9 mm.), and the plates were reincubated for an additional 72 hours.

The results of a typical experiment can be seen in Fig. 5 in which Type 6 protoplasts had been placed in the large center cylinder. A single band of antigen-antibody precipitate formed in the bottom agar layer between the large cylinder and each of the two small cylinders that contained Type 6 rabbit antiserum. Cylinder 4 contained absorbed antiserum while cylinder 1 contained unabsorbed antiserum. The bands of precipitate curved towards the smaller cylinders that contained antiserum. No bands appeared with the unabsorbed heterologous Type 30 antiserum in cylinder 2 or with Group A antiserum in cylinder 3. Control plates which employed absorbed and unabsorbed Types 1, 3, 22, and 26 antisera revealed no bands of precipitate. The protoplasts from streptococcal Types 1, 3, and 30 were grown in the central cylinders. With each of these strains bands of precipitate occurred with homologous type antiserum but not with heterologous type antisera or with Group A antiserum.

Agar diffusion studies also provide additional evidence for the relationship of the type-specific material released by protoplast colonies to M protein obtained by the classical method. Extracts of the agar medium in which protoplasts had grown were prepared by the method described above. These extracts were compared with acid-extracted M protein for their reactivity with rabbit antiserum in Ouchterlony plates. The results of a typical experiment are shown in Fig. 6. Cylinders 1, 3, and 5 contained an M extract of Type 6 streptococci, and Nos. 2, 4, and 6 contained extracts of Type 6 protoplasts. The central cylinder contained absorbed Type 6 rabbit antiserum. The bands of antigen-antibody precipitate have merged, and the presence of these "bands of identity" indicates that the extracts from Type 6 protoplasts contain a substance identical with Type 6 M protein.

These experiments strongly suggest that the type-specific substance produced by protoplast colonies is M protein. The data are less extensive in the case of L forms since adequate L form growth has not yet been obtained to apply the Ouchterlony technique. However, the development of zones of type-specific precipitate around L form colonies certainly suggests that they are similar to protoplast colonies in the production of a type-specific substance. It is of interest that both L forms and protoplasts continue to produce this antigen, and that it is released into the medium instead of being incorporated into a surface structure as in the case of intact Group A streptococci. However, there is no evidence for the production of the group-specific carbohydrate which is a major structural component of the cell wall.

Reversion of L forms and Protoplasts to Streptococci.—A common property of streptococcal L forms and protoplasts is their reversion to the original organism in the absence of penicillin. The L forms and protoplasts used in these reversion experiments were well established strains since they had been subcultured several times in an agar medium containing 1000 units of penicillin per ml. Bacterial forms were recovered by incubation of agar blocks containing L colonies or protoplast colonies for several days in a penicillin-free liquid medium that contained 5 to 10 units of penicillinase per ml. Although reversion occurred without penicillinase, the use of this penicillin-inactivating enzyme greatly accelerated the process. In the absence of penicillinase, agar blocks with 1000 units of penicillin/ml. still contained 50 units/ml. after incubation for 7 days at 37°C. and therefore several weeks elapsed before the subcultures were free of active penicillin. Since streptococcal growth is inhibited by less than 0.01 units of penicillin/ml., reversion was slowed by this persistence of traces of penicillin in the subcultures.

By this technique, L forms of protoplasts of strains S43/100, S43/155, and D24 have reverted to morphologically typical Group A streptococci of the same serological type from which they were originally isolated. The M protein in streptococci that had reverted from L form or protoplast colonies of S43 and the M protein of the parent strain are identical when compared by an agar diffusion technique using absorbed Type 6 rabbit antiserum. An interesting characteristic of reverted streptococcal strains is the property of yielding a greater number of L forms or viable protoplasts than the strain from which they were derived. For example, in parallel tests 1 per cent of the streptococci of strain S43/100 grew as protoplast colonies while the strain S43/100FL obtained by reversion of L forms yielded 12 per cent. This 12-fold difference may represent the selection of a strain possessing a membrane with greater osmotic stability.

DISCUSSION

Detailed information on the nature of the bacterial surface has been accumulating rapidly during the past several years. An important step in defining the cell surface was the isolation and characterization of the rigid cell wall which has now been accomplished in the case of a large number of bacterial species. The occurrence of closely related mucopeptides, made up of muramic acid and a limited number of amino acids, has been shown to be a unique feature of the composition of the bacterial cell wall.

These mucopeptides, in combination with carbohydrate, form the rigid framework of the wall (26, 33). Recent investigations indicate that penicillin probably acts by interfering with the incorporation of the mucopeptides into the cell wall structure (28). Bacteria actively metabolizing in the presence of penicillin accumulate uridine nucleotides that contain muramic acid and the characteristic amino acids in the same proportion as they are found in the bacterial cell wall.

The study of certain derivative forms of bacteria has contributed to the understanding of the cell surface. For example, the evidence suggests that the osmotically fragile L forms, isolated from many species by growth in the presence of penicillin, are bacterial variants growing without a cell wall (34). They are highly resistant to penicillin and other substances that act by interfering with cell wall formation (35, 36). Finally, enzymes, such as lysozyme and phage-associated lysin, which dissolve cell walls, have uncovered a previously unknown spherical structure, the protoplast. This subcellular unit, enclosed in a distinct membrane, but without a cell wall, is capable of many of the biological functions of the intact bacterial cell (37, 38).

A group of investigators interested in the bacterial surface has suggested that the term protoplast be used only to describe osmotically fragile spherical structures free of cell wall material (27). The term has also been applied to apparently analogous spherical structures obtained from Gram-negative bacilli after exposure to penicillin. However, the cell wall of Gram-negative bacteria contains polysaccharide-lipid-protein complexes as well as a mucopeptide structure analogous to the cell wall of Gram-positive species. Penicillin apparently interferes only with the synthesis of the mucopeptide portion which is the rigid structure responsible for the shape of the cell. The chemical demonstration of residual cell wall substances, the persistence of phage receptors, and the presence of cell wall antigens with serological reactivity suggest that the spherical structures obtained from these Gram-negative bacilli have retained part of the complex cell wall (39).

The spherical structures obtained by treatment of Group A streptococci with phage-associated lysin do meet the proposed criteria of protoplasts. Streptococcal protoplasts are sensitive to osmotic changes in their environment, and both chemical and serological studies indicate that these protoplasts are free of cell wall carbohydrate. The protoplasts are bounded by a distinct envelope, the protoplast membrane, which is appreciably thinner than the bacterial cell wall, and comparable to protoplast membranes of other species. The streptococcal protoplast membrane has been isolated free of other cellular components, and cell wall carbohydrate has not been detected in significant amounts by chemical or serological tests. The chemical composition and serological properties of this membrane are now being investigated.

Although the growth of L forms has been described for many species, the reproduction and growth of protoplasts free of cell wall material has not been reported (37). However, both protoplasts and L forms derived from Group A streptococci grow as colonies in concentrations of penicillin 100,000-fold greater than that known to inhibit growth of whole streptococci. Although the structural unit of protoplast or L form colonies is distinctly different from that of streptococcal colonies, a close bacteriologic relationship has been demonstrated. Both release similar enzymes into their environment, and both produce a similar type-specific protein antigen.

While the M protein is confined to the cell wall of streptococci, a unique feature of the multiplying protoplast is the release of this substance into the medium. This suggests that in the absence of a cell wall, the M protein is not bound to the organism. The absence of serologically detectable Group A carbohydrate either in growing protoplasts or in the medium, may indicate that penicillin is suppressing synthesis of the cell wall before a serologically active material has been formed. It is not known whether the monosaccharide constituents of the cell wall polysaccharide, rhamnose and *N*-acetylglucosamine, are synthesized by the protoplast and released as serologically inactive oligosaccharide. However, the protoplasts retain the potential for cell wall synthesis since, after many transfers, protoplast colonies revert to the original Group A streptococcal type when penicillin is removed from the nutrient medium.

The close similarity of L forms and protoplasts of Group A streptococci has become apparent in the course of these studies. Both are osmotically fragile spherical structures without cell walls, and both grow into colonies of identical appearance. These colonies grow in the presence of concentrations of penicillin in which the original streptococci do not grow because penicillin interferes with synthesis of the bacterial cell wall. Serological evidence suggests that protoplasts and L forms produce the type-specific M antigen characteristic of the parent streptococcal strain. In addition both produce a similar hemolysin. These apparently identical streptococcal derivatives differ only in the methods used to remove the streptococcal cell wall. Enzymatic dissolution of the cell wall produces protoplasts while inhibition of cell wall synthesis by penicillin results in the appearance of L forms. These considerations suggest that protoplasts and L forms of Group A streptococci are identical.

SUMMARY

L forms of Group A streptococci have been isolated by the use of penicillin gradient agar plates. Osmotically fragile protoplasts of Group A streptococci have been obtained by the use of Group C phage-associated lysin which lyses Group A streptococci and their isolated cell walls. Membranes surrounding these enzymatically derived protoplasts have been isolated, and chemical and immunological studies indicate that they are free of cell wall carbohydrate and M protein. The streptococcal protoplasts reproduce as colonies which are morphologically indistinguishable from streptococcal L forms. Evidence is presented to show that these two streptococcal derivatives are serologically and physiologically related to each other as well as to the parent streptococcal strain from which they were isolated.

BIBLIOGRAPHY

1. Klieneberger-Nobel, E., Filterable forms of bacteria, *Bact. Rev.*, 1951, **15**, 77.
2. Dienes, L., and Weinberger, H. J., The L forms of bacteria, *Bact. Rev.*, 1951, **15**, 245.

3. Sharp, J. T., Hijmans, W., and Dienes, L., Examination of the L forms of group A streptococci for the group-specific polysaccharide and M protein, *J. Exp. Med.*, 1957, **105**, 153.
4. Weibull, C., Chemical analysis elucidating the structure of bacterial L forms, *Acta Path. et Microbiol. Scand.*, 1958, **42**, 324.
5. Weibull, C., The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme, *J. Bact.*, 1953, **66**, 688.
6. Mitchell, P., and Moyle, J., Liberation and osmotic properties of the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea*, *J. Gen. Microbiol.*, 1956, **15**, 512.
7. Weibull, C., The nature of the "ghosts" obtained by lysozyme lysis of *Bacillus megaterium*, *Exp. Cell Research*, 1956, **10**, 214.
8. Weibull, C., and Bergström, L., The chemical nature of the cytoplasmic membrane and cell wall of *Bacillus megaterium*, strain M, *Biochim. et Biophysica Acta*, 1958, **30**, 340.
9. Gilby, A. R., Few, A. V., and McQuillen, K., The chemical composition of the protoplast membrane of *Micrococcus lysodeikticus*, *Biochim. et Biophysica Acta*, 1958, **29**, 21.
10. McQuillen, K., Bacterial protoplasts. I. Protein and nucleic acid metabolism in protoplasts of *Bacillus megaterium*, *Biochim. et Biophysica Acta*, 1955, **17**, 382.
11. McQuillen, K., Bacterial protoplasts: growth and division of protoplasts of *Bacillus megaterium*, *Biochim. et Biophysica Acta*, 1955, **18**, 458.
12. McCarty, M., The hemolytic streptococci, *in* Bacterial and Mycotic Infections of Man, (R. J. Dubos, editor), Philadelphia, J. B. Lippincott Co., 3rd edition, 1958, 248.
13. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78**, 127.
14. Krause, R. M., Studies on the bacteriophages of hemolytic streptococci. II. Antigens released from the streptococcal cell wall by a phage-associated lysin, *J. Exp. Med.*, 1958, **108**, 803.
15. Dische, Z., and Shettles, L. B., A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination, *J. Biol. Chem.*, 1948, **175**, 595.
16. Burton, K., A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid, *Biochem. J.*, 1956, **62**, 315.
17. McCarty, M., The lysis of Group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. I. Production and fractionation of the lytic enzymes, *J. Exp. Med.*, 1952, **96**, 555. II. Nature of the cellular substrate attacked by the lytic enzymes, *J. Exp. Med.*, 1952, **96**, 569.
18. McCarty, M., Variation in the group-specific carbohydrate of Group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates, *J. Exp. Med.*, 1956, **104**, 629.
19. Sharp, J. T., L colonies from hemolytic streptococci: New technic in the study of L forms of bacteria, *Proc. Soc. Exp. Biol. and Med.*, 1954, **87**, 94.
20. Dienes, L., and Sharp, J. T., The role of high electrolyte concentration in the production and growth of L forms of bacteria, *J. Bact.*, 1956, **71**, 208.

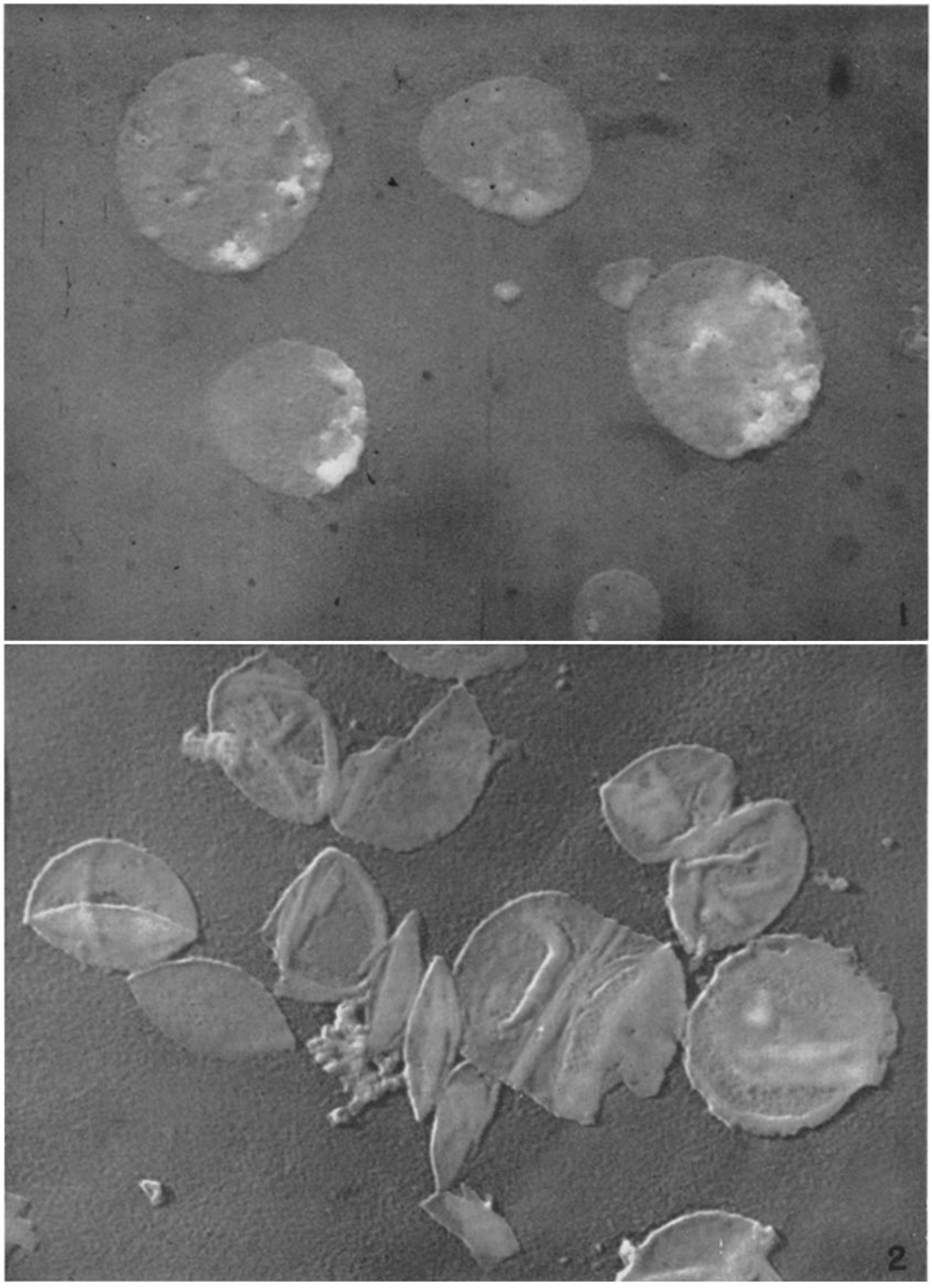
21. Crawford, Y. E., Frank, P. F., and Sullivan, B., Isolation and reversion of L forms of beta hemolytic streptococci, *J. Infect. Dis.*, 1958, **102,** 44.
22. Salton, M. R. J., The properties of lysozyme and its action on microorganisms, *Bact. Rev.*, 1957, **21,** 82.
23. Krause, R. M., Studies on bacteriophages of hemolytic streptococci. I. Factors influencing the interaction of phage and susceptible host cell, *J. Exp. Med.*, 1957, **106,** 365.
24. Maxted, W. R., The active agent in nascent phage lysis of streptococci, *J. Gen. Microbiol.*, 1957, **16,** 584.
25. Gooder, H., and Maxted, W. R., Protoplasts of Group A beta-haemolytic streptococci, *Nature*, 1958, **182,** 808.
26. Salton, M. R. J., Studies of the bacterial cell wall. IV. The composition of the cell walls of some Gram-positive and Gram-negative bacteria, *Biochim. et Biophysica Acta*, 1953, **10,** 512.
27. Brenner, S., et al., Bacterial protoplasts, *Nature*, 1958, **181,** 1713.
28. Park, J. T., and Strominger, J. L., Mode of action of penicillin, *Science*, 1957, **125,** 99.
29. Pierce, C. H., Streptobacillus moniliformis, its associated L_1 form, and other pleuropneumonia-like organisms, *J. Bact.*, 1942, **43,** 780.
30. McCarty, M., The inhibition of streptococcal desoxyribonuclease by rabbit and human antisera, *J. Exp. Med.*, 1949, **90,** 543.
31. Wannamaker, L. W., The differentiation of three distinct desoxyribonucleases of Group A streptococci, *J. Exp. Med.*, 1958, **107,** 797.
32. Ouchterlony, O., Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion, *Acta Path. et Microbiol. Scand.*, 1953, **32,** 231.
33. Cummins, C. S., and Harris, H., The chemical composition of the cell wall in some Gram positive bacteria and its possible value as a taxonomic character, *J. Gen. Microbiol.*, 1956, **14,** 583.
34. Bacterial Anatomy. The sixth symposium of the Society for General Microbiology, (E. T. C. Spooner and B. A. D. Stocker, editors), Cambridge, University Press, 1956.
35. Ward, J. R., Madoff, S., and Dienes, L., *In vitro* sensitivity of some bacteria, their L forms and pleuropneumonia-like organisms to antibiotics, *Proc. Soc. Exp. Biol. and Med.*, 1958, **97,** 132.
36. Park, J. T., Selective inhibition of bacterial cell wall synthesis: Its possible applications in chemotherapy, *in* The Strategy of Chemotherapy, The eighth symposium of the Society for General Microbiology, (S. T. Cowan and E. Rowatt, editors), Cambridge, University Press, 1958, 49.
37. Weibull, C., Bacterial protoplasts, *Ann. Rev. Microbiol.*, 1958 **12,** 1.
38. Kalina, G. P., Bacterial protoplasts (a review), *J. Microbiol. Epidemiol. and Immunobiol.*, 1958, **29,** 143.
39. Lederberg, J., and St. Clair, J., Protoplasts and L-type growth of *Escherichia coli*, *J. Bact.*, 1958, **75,** 143.

EXPLANATION OF PLATES

PLATE 89

FIG. 1. Electronmicrograph of trypsin treated protoplast membranes of streptcoccal strain S43FL. Approximately × 10,000.

FIG. 2. Electronmicrograph of trypsin treated cell walls of hemolytic streptococci. Approximately × 10,000.

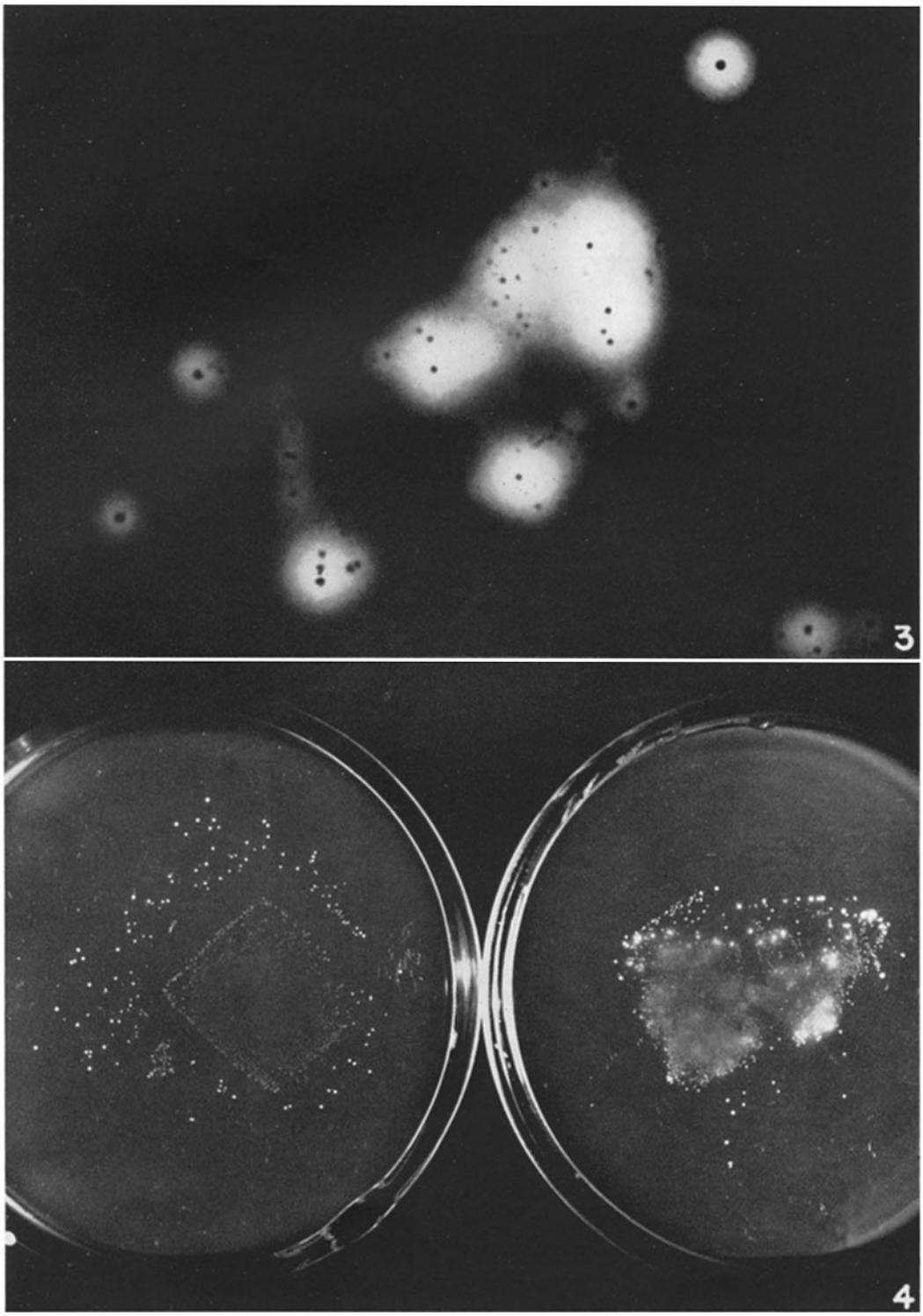


(Freimer *et al.*: L forms and protoplasts of Group A streptococci)

PLATE 90

FIG. 3. Hemolysis of rabbit blood by protoplast colonies. The photograph was taken with transmitted light at a magnification of 5.

FIG. 4. Demonstration of type-specific precipitate zones surrounding L form colonies grown in the presence of homologous type-specific antiserum. In the plate on the right, protoplast colonies of strain S43FL were grown in the presence of homologous Type 6 rabbit antiserum. In the plate on the left, they were grown in heterologous Type 26 antiserum. × 1.6.

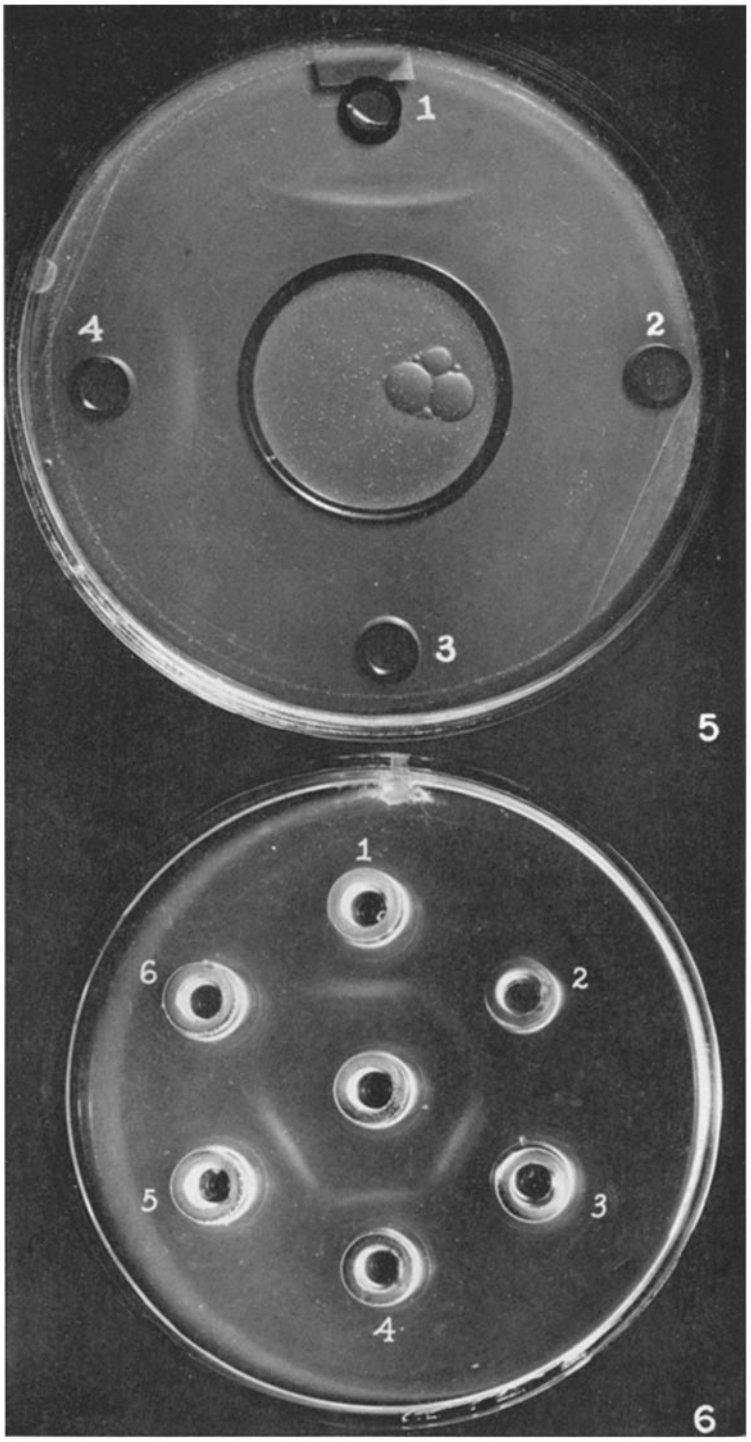


(Freimer *et al.*: L forms and protoplasts of Group A streptococci)

PLATE 91

FIG. 5. Diffusion of type-specific material from Type 6 protoplast colonies demonstrated by a modified Ouchterlony plate. The large central cylinder contains growing protoplast colonies while the small peripheral cylinders contain antisera. Cylinders 1 and 4 contain Type 6 antisera, unadsorbed and adsorbed respectively. Cylinder 2 contains Group A antiserum and cylinder 3 heterologous Type 30 antiserum.

FIG. 6. The production of "bands of identity" by Type 6 acid-extracted M protein and extracts of agar cultures of Type 6 protoplast colonies. The central cylinder contains absorbed Type 6 antiserum. Cylinders 1, 3, and 5 contain Type 6 M protein. Cylinders 2, 4, and 6 contain extracts of growth of Type 6 protoplast colonies.



(Freimer *et al.*: L forms and protoplasts of Group A streptococci)