FORMATION AND MULTIPLICATION OF SPHEROPLASTS OF ESCHERICHIA
COLI IN THE PRESENCE OF LITHIUM CHLORIDE

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Recent years have brought a better understanding of the relationships between such seemingly diverse phenomena as formation of protoplasts, the antibacterial action of penicillin, and L-type or pleuropneumonialike type of growth. All these phenomena appear to involve a common mechanism, impairment in the synthesis of the bacterial cell wall. In view of the highly hypertonic condition inside the organism, loss of the cell wall or decrease in its rigidity would be expected to result in burgeoning of the cell into a sphere and bursting, unless the hypertonicity of the surrounding medium were appropriately increased or the remaining cell envelope retained enough strength to confine the expanded and thus diluted protoplasm.

The literature describing morphological changes in bacteria which can be ascribed to the mechanism outlined above is enormous and dates back to the last century. This subject has been reviewed in the recent essay of Lederberg and St. Clair (1958). The dysmorphic effect of LiCl on bacteria which is evaluated in the present study, was described very early by Gamaleia (1900), whose work represents a comprehensive study of the morphological and chemical basis of this phenomenon. Diverse interpretations of these anomalous forms described as Pettenkoferie were reviewed by Kuhn and Sternberg (1931), and by Seppilli and Tolentino (1938). Recently, Pitzurra (1955) and Pitzurra and Mori (1957) reinvigated the effect of lithium ions on Escherichia coli and compared it with the "protoplasting" effect of penicillin (Pitzurra, 1956).

The present study was motivated by a desire to evaluate quantitatively certain properties of proplast-like, spherical cells of genetically marked strains of E. coli prepared under the action of hypertonic concentrations of LiCl. The choice of this inducing agent was based upon the studies of Pitzurra and Mori (1957) and especially of Ragni (1957), who has demonstrated a high efficiency of conversion of normal rodlike cells of E. coli to protoplast-like forms in the presence of LiCl. Their findings suggested the utilization of such "spheroplasts" in investigation of the mechanism of chemically induced mutation in streptomycin-dependent mutants of E. coli (Iyer and Szybalski, 1958; Szybalski and Pitzurra, 1959).

MATERIALS AND METHODS

Strains. Several strains of E. coli were employed in the present studies: a threonine-, leucine- and thiamin-deficient mutant (W-1) derived from strain K-12; a thymine-requiring mutant (15-T); and a wild-type prototroph (146). These were received from Dr. J. Lederberg, Dr. F. Forro, Jr., and the University of Perugia Institute of Hygiene Collection, respectively. In most of the experiments the streptomycin-dependent strain Sd-4, kindly supplied by Dr. M. Demerec of the Cold Spring Harbor Laboratories, was employed.

Media and methods of cultivation. The liquid medium was prepared from nutrient broth (Difco) (8 g/L). For the solid medium, 1.5 per cent agar was added. Streptomycin (SM) was employed in a concentration of 100 µg per ml in the solid media or 20 µg per ml in nutrient broth, to support the growth of the Sd-4 strain. The protoplasting medium was composed of nutrient agar (Difco) with or without SM, 2.5 per cent

1 This work was supported in part by grant CY-3492 from the National Institutes of Health, United States Public Health Service.

LiCl, and 10 μg of leucine per ml (Pitzurra and Mori, 1957). The cells were grown in broth over-
night at 37 C with agitation by means of a wrist-
action shaker. An aliquot of the cell suspension
was spread either directly on the surface of
freshly poured protoplasting agar, or on a 5 by 5
cm sheet of dialyzing membrane resting on the
surface of the agar. This last procedure was the
method of choice when quantitative removal of
the resulting L growth was intended, since the
L-type colony tends to imbide itself in the agar.
The cells were scraped off the surface of the agar
or cellophane membrane by means of a glass
spreader into a small volume of one of the follow-
ing suspending and diluting media: distilled
water, 0.7 per cent NaCl solution, and 20 pwr
cent sucrose.

**Smear microculture.** In addition to making
direct observations with the phase microscope of
cells multiplying on the agar surface, smear
microcultures (*microculture per atriacio—Pitzurra,
1954*) were employed to study cell behavior. A
heavy inoculum of bacteria was mixed with a
small volume of protoplasting agar (melted and
cooled to 50 C) and smeared very thinly over the
surface of a 3 by 1 inch microscope slide by means
of the edge of another slide. A number of slides
were incubated in a moist chamber and, at speci-
ﬁed intervals, removed one by one for microscopic
inspection after flame fixation and staining with a
saturated aqueous solution of methylene blue.

**RESULTS**

**Formation and multiplication of spheroplasts on
agar.** When an overnight culture of *E. coli* strain
Sd-4 is plated on streptomycin-containing proto-
plasting agar and incubated at 37 C, the follow-
ing sequence of events can be observed: Within
the first hour, the normal rodlike cells (ﬁgure 1)
begin to swell, first assuming spindle-like shapes
and later rounding to spheres. Multiplication by
binary fission takes place (ﬁgures 2 and 3), and
in 6 to 7 hr microcolonies of perfectly round
bodies are established (ﬁgures 4–7). Macropoc-
scopically visible colonies (up to approximately
0.5 mm in diameter) develop after 1 to 2 days
of incubation. These colonies, made up of proto-
plast-like cells, seem to be analogous to the
L-type colonies (named after the Lister Institute)
ﬁrst described by Klienemberger (1935) and re-
cently identiﬁed with protoplast growth by
Lederberg and St. Clair (1958). Not all the
altered cells produce macroscopically visible
growth. The colony size ranges from aggregates

![Figures 1 to 3. Bacillary forms (figure 1) and spheroplasts (figures 2 and 3) of *Escherichia coli* strain Sd-4. Cells grown for 5 (figures 1 and 2) or 10 (figure 3) hr at 37 C on the surface of 1-mm thick nutrient agar containing 100 μg streptomycin per ml and 10 μg leucine per ml, with (figures 2 and 3) or without (figure 1) 2.5 per cent LiCl. Blocks of agar placed on microscope slide, covered with thin cover glass, and observed under 100 × oil immersion phase objective and 12.5 × ocular (American Optical Company). Pictures, taken with Polaroid camera, are reduced 4 per cent for reproduction here.](http://jb.asm.org/ on September 23, 2017 by guest)
Figures 4 to 8. L-type colonies of *Escherichia coli* formed on protoplasting agar. 18-hr- (figures 4 and 5) and 38-hr- (figures 7 and 8) old growth prepared as described for figures 2 and 3, but photographed through $25 \times$ (figures 4, 5, and 8) or $5 \times$ (figure 7) ocular. Figure 6 represents a fixed and stained “smear microculture” (see Materials and Methods), 6 hr old. (Figures reduced 9 per cent for reproduction here)

of only a few cells to visible L-type colonies of 0.1 to 0.5 mm diameter. Each fully developed L-type colony is composed of a multilayered mass of round cells of variable size, intermixed with granular material (cellular debris?) buried in the agar. The spherical cells, some of which are several microns in diameter, are clearly visible only at the edge of the colony where they are arranged in a monolayer (figure 8). Figure 9 represents electron micrographs of round cells derived from an L-type colony.

The efficiency of L-type colony formation depends upon the particular strain or mutant employed and on the medium. The highest plating efficiency is obtained with freshly poured, moist nutrient agar containing 2.5 per cent LiCl and 10 $\mu$g of leucine per ml, seeded with strain 146 of *E. coli*. Under these conditions, up to 80 per cent of the cells form visible L-type colonies. With strain 15T, yields up to 50 per cent are obtained, whereas strain Sd-4 produces not over 5 per cent visible colonies. However, the total colony count for strain Sd-4 by microscopic inspection is approximately five times higher than the macroscopic count. Streptomycin is required for the formation of visible L-type
colonies by the Sd-4 (streptomycin-dependent) strain, although well-washed cells are able to form small clusters of spherical cells on protoplasting agar, even in the absence of streptomycin. In the case of strain Sd-4, the yield of visible L-type colonies increases with the inoculum size, indicating dependence of this type of growth upon the concentration of some intermediates released into the medium by metabolizing cells. The addition of leucine to the medium was based upon the finding that a substantially higher number of L-type colonies were formed on media supplemented with this amino acid; e.g., the yield of L-microcolonies produced by strain Sd-4 on protoplasting agar containing added leucine was 18 times higher than without the supplement, as assessed by the smear microculture technique. On a dry agar surface or a cellophane membrane, the yield of L-type colonies is lower by a factor of 5 to 10 than that obtained on a moist agar surface. In L-type colonies of all the above-mentioned strains, no bacillary forms are observed, provided the incubation period is more than 7 hr. This is not the case with the W-1 strain of E. coli, which has a plating efficiency on protoplasting agar of less than one colony from 3000 cells, and produces minute L-type colonies composed of approximately 10 per cent bacillary-type cells among spheres, long filaments, and spindle-like cells.

During the formulation of the protoplasting agar, the effect of diaminopimelic acid (DAP) was tested because of the known role of this intermediate in the synthesis of cell walls (Meadow et al., 1957). At concentrations of 10 and 100 μg per ml, DAP had no apparent effect on the formation of spheroplasts and L-type colonies.

Regeneration of normal bacillary growth from spheroplasts. When cells from 6- to 24-hr cultures are transferred from protoplasting agar to LiCl-free nutrient agar, they revert to normal bacillary forms which grow and multiply, producing normal colonies. The morphological aspects of this reversion, described by Piturra (1957), roughly resemble the conversion of penicillin protoplasts to bacilli observed by Lederberg and St. Clair (1958). By one mode of reversion, the large spherical cell produces a thin, sausage-like protrusion which, in turn, segments into normal bacilli. In other instances, the spheroplast divides into several sections, one or more of which have a roughly bacillary shape and by subdivision produce normal bacillary growth.

For the quantitative measurement of the viability of the spheroplasts and their capacity to form normal and L-type colonies, it was necessary to evaluate several factors: (a) the effect of the method used for harvesting the cells from the agar surface, (b) the influence of suspending, diluting and counting media, and (c) the percentage of colony-forming units in the suspension of spheroplasts.

A number of suspending media were evaluated, employing spheroplasts washed off the surface of dialyzing membranes over protoplasting agar (see Methods). Surprisingly, the viability (colony-forming capacity) of the LiCl spheroplasts is best preserved in distilled water, indicating a high osmotic stability. In table 1, the survival of spheroplasts during prolonged storage in distilled water, 0.7 per cent saline, and 20 per cent sucrose solution is compared with the survival of
normal bacillary cells under identical conditions. Stability in these suspending media was not significantly different for the two cell forms.

As a means of testing the viability of the spheroplasts, a direct comparison was made between the microscopic cell count obtained with a Petri-F-Hausser counting chamber (A. H. Thomas Co., Philadelphia, Pennsylvania) and the plate count. For the latter, a 7- to 18-hr growth on protoplasting agar was washed off into various suspending media (table 2) and appropriate dilutions were plated on nutrient agar. In the experiments summarized in table 2, no bacillary forms were observed among the spherical cells counted in the chamber, except in the case of strain W-1, which produces many bacillary forms on protoplasting agar as discussed earlier.

The results presented in table 2 indicate that spherical cells produced by growth in the presence of LiCl possess a remarkable capacity to regenerate; viability ranges from 20 to 80 per cent. The use of 20 per cent sucrose solution as a suspending and diluting medium has little, if any, effect on the viability of LiCl spheroplasts. How-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Bacillary forms*</th>
<th>Suspending and Diluting Medium</th>
<th>Surviving Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>0 hr</td>
</tr>
<tr>
<td>0.7% NaCl</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>20% Sucrose</td>
<td>100</td>
<td>74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protoplast-like forms†</th>
<th>Suspending and Diluting Medium</th>
<th>Surviving Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>0 hr</td>
</tr>
<tr>
<td>0.7% NaCl</td>
<td>100</td>
<td>108</td>
</tr>
<tr>
<td>20% Sucrose</td>
<td>100</td>
<td>37</td>
</tr>
</tbody>
</table>

* A heavy inoculum of *Escherichia coli* strain Sd-4 was spread on the surface of a cellophane membrane resting on nutrient agar containing 100 μg of streptomycin per ml. After incubation overnight at 37°C, the cells were washed off the membrane with 10 ml of the suspending medium by means of a glass spreader. Colonies were made in the same medium which was used to prepare the cell suspension. Colonies were scored on streptomycin-containing nutrient agar.

† The procedure was the same as for bacillary forms, except that protoplasting agar was employed as the growth medium.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Suspending and Counting Medium</th>
<th>Direct Count</th>
<th>Colony Count</th>
<th>Percentage of Colony-Forming Spheroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sd-4*</td>
<td>0.7% NaCl</td>
<td>2.5 X 10⁸</td>
<td>1.3 X 10⁹</td>
<td>52</td>
</tr>
<tr>
<td>Sd-4†</td>
<td>0.7% NaCl</td>
<td>3.9 X 10⁹</td>
<td>2.7 X 10⁹</td>
<td>69</td>
</tr>
<tr>
<td>Sd-4*</td>
<td>20% Sucrose</td>
<td>1.1 X 10⁹</td>
<td>0.9 X 10⁹</td>
<td>82</td>
</tr>
<tr>
<td>Sd-4†</td>
<td>20% Sucrose</td>
<td>2.5 X 10⁹</td>
<td>1.3 X 10⁹</td>
<td>52</td>
</tr>
<tr>
<td>146†</td>
<td>0.7% NaCl</td>
<td>6.6 X 10⁴</td>
<td>3.3 X 10⁴</td>
<td>50</td>
</tr>
<tr>
<td>146†</td>
<td>0.7% NaCl</td>
<td>6.6 X 10⁴</td>
<td>2.6 X 10⁴</td>
<td>39‡</td>
</tr>
<tr>
<td>W-1†</td>
<td>0.7% NaCl</td>
<td>2.0 X 10⁸</td>
<td>3.3 X 10⁷</td>
<td>16</td>
</tr>
<tr>
<td>W-1†</td>
<td>20% Sucrose</td>
<td>1.5 X 10⁶</td>
<td>4.1 X 10⁷</td>
<td>27</td>
</tr>
<tr>
<td>15 T*</td>
<td>0.7% NaCl</td>
<td>3.0 X 10⁶</td>
<td>2.2 X 10⁶</td>
<td>74</td>
</tr>
<tr>
<td>15 T†</td>
<td>20% Sucrose</td>
<td>2.1 X 10⁶</td>
<td>8.5 X 10⁵</td>
<td>40</td>
</tr>
</tbody>
</table>

* Washed off the surface of protoplasting agar.
† Washed off the surface of a cellophane membrane resting on protoplasting agar.
‡ Colony-forming capacity scored on protoplasting agar. The number of colonies forming under this condition amounts to 80 per cent of a similar count on nutrient agar.
ever, preliminary experiments indicate that addition of 20 per cent sucrose to the nutrient agar results in a decrease in the viable cell count. Spheroplasts can also initiate growth of L-type colonies on protoplasting agar (table 2).

**Formation and multiplication of spheroplasts in liquid media.** Exploratory experiments have been performed on the growth of *E. coli* strains in nutrient broth containing LiCl. Turbidity determinations and viable cell counts were carried out through a 24-hr incubation at 37°C, using an inoculum of 10^7 cells per ml. Although some increase in turbidity occurred, no increase in cell count was perceptible during this period. Visible growth appeared in the form of slimy streaks in the medium. The undesirable character of the growth, together with the availability of a satisfactory method of preparing spheroplasts on solid medium, discouraged further experiments.

**Streptomycin sensitivity of spheroplasts.** It was observed in the course of these experiments that the streptomycin-sensitive strains W-1, 15-T, and 146 are able to grow and form fully developed, viable L-type colonies on protoplasting agar containing up to 40 μg of streptomycin per ml. The same strains are inhibited by a minimum concentration of 0.8 μg of streptomycin per ml on nutrient agar. The presence of these relatively high concentrations of streptomycin in the protoplasting agar does not appear to influence any obvious property of the spheroplasts produced from streptomycin-sensitive bacteria. The antagonistic effect of high concentrations of salts on the antibacterial activity of streptomycin was frequently reported (Waksman, 1949).

**Discussion**

A protoplast, by definition, is a cell devoid of a wall. The structures produced under the action of LiCl seem to possess a modified cell envelope. Rigidity is strongly impaired, resulting in a rounding-up of the cell, but osmotic stability is not greatly affected. These protoplast-like forms (spheroplasts) seem to be analogous to those produced under the action of penicillin (Ledberg and St. Clair, 1958), in which residual cell wall material was demonstrated (Weibull, 1958; Salton and Shafa, 1958; Brenner et al., 1958). In this respect, they differ from the true protoplasts of *Bacillus megaterium* produced by the enzymatic action of lysozyme (Weibull, 1953).

The mechanism of the “protoplasting” effect of lithium ions is not obvious from these studies. It is not based upon the nonspecific effect of high osmotic pressure, because addition of NaCl or KCl of similar ionic strength results in the formation of neither spheroplasts nor L-type colonies. According to Gamaleia (1900), small crystals accumulate in the spherical cells, which contain LiCl as shown by qualitative flame spectroscopy. LiCl does not act by interfering with the synthesis of diaminopimelic acid (DAP), an important cell wall constituent, since high concentrations of DAP do not reverse the effect.

The quantitative aspects of the formation of the spheroplasts or L-type colonies, and especially the regeneration of normal cells, were the prime considerations of this study. With the majority of the mutants tested, only a small fraction of the cells are able to form fully developed L-type colonies; the growth of the other cells ceases earlier. The plating efficiency on protoplasting agar seems to be characteristic for a strain, and is not modified when descendants derived from a single L-type colony are tested. This last observation points to the fact that the capacity to form L-type colonies on LiCl agar is not a heritable property of a particular “mutant,” but is distributed by chance among the whole population.

Since the spheroplasts retain many of the properties of the normal cells of *E. coli*, they represent useful experimental tools. They appear to be remarkably stable to osmotic shock, and on LiCl-free agar form colonies composed of normal bacillary forms with an efficiency approaching 100 per cent. The properties which distinguish them from normal cells, in addition to their distinct morphology are: a slow rate of multiplication, especially in liquid media, and lowered susceptibility to streptomycin (up to 50 times more resistant than the parent cells). It is perhaps not surprising to note that the more morphologically oriented earlier microbiologists considered the LiCl-produced forms as separate organisms (*Pettenkoferie*) living in symbiosis with normal bacteria, but obvious only when growth of the bacilli is suppressed by LiCl, which in turn favors the propagation of the *Pettenkoferie*. This theory of Kuhn and Sternberg (1931), however, is incongruous with our present knowledge of L-type growth.

It is hoped that the present studies on the quantitative aspects of L-type growth performed with several mutants of *E. coli* will facilitate the use of these protoplast-like cells, produced in the
presence of LiCl, in various phases of biochemical and genetic research.

SUMMARY

Escherichia coli, when grown on enriched nutrient agar containing 2.5 per cent LiCl produces protoplast-like cells (spheroplasts) which are capable of further multiplication by binary fission. This growth results in the formation of L-type colonies composed of spherical bodies and granular forms. The percentage of cells able to form L-type colonies on LiCl agar depends upon the strain, varying from 0.01 per cent to as high as 80 per cent. Spheroplasts, when transferred to LiCl-free medium, exhibit a high osmotic stability, and on nutrient agar revert to normal bacilli, forming colonies with an efficiency approaching 100 per cent. The spheroplasts differ strikingly from the normal bacillary forms in their morphology, rate of multiplication, and sensitivity to streptomycin, while preserving unchanged many other properties characteristic of bacillary forms.

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