Adaptation of an Osmotically Fragile L-Form of *Streptococcus pyogenes* to Physiological Osmotic Conditions and Its Ability to Destroy Human Heart Cells in Tissue Culture

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An osmotically fragile L-form of *Streptococcus pyogenes*, type 12, was quickly rendered osmotically stable by decreasing the sodium chloride content of the growth medium and with the temporary use of oleic acid. The change from osmotic fragility to stability was accompanied by changes in cell yield, generation time, saturated/unsaturated fatty acid ratio of the membrane, and cytoplasmic protein composition. Finally, this resulting osmotically stable L-form survived and was capable of rapidly destroying Girardi human heart cells in tissue culture.

Although much biochemical information is available about stabilized, osmotically fragile bacterial L-forms, nothing is known of structural or physiological changes that might occur when these organisms are adapted to grow in isotonic media. To our knowledge, only one report has appeared which indicates even the frequency of successive transfers for adaptation to a low-salt medium of an osmotically fragile L-form of *Streptococcus pyogenes* (13). However, more information is available dealing with latent infection or induction and reversion of L-forms from the group A cocci or *Streptococcus faecalis* when inoculated into human cell cultures (8, 22). Pertinent to our studies, Schmitt-Slomksa et al. (17, 18) were successful in induc- ing L-forms in human diploid cells infected by group A cocci and of establishing a chronically infected cell system for several months. Also, Clasener et al. (4) studied the persistence in mice of L-forms of three streptococcal strains adapted to physiological osmotic conditions. However, their results were at variance with those of others (19).

Detailed biochemical knowledge of any adaptive mechanism by which osmotically fragile L-forms or aberrant forms of bacteria might survive in vivo would add significantly to their speculative role in pathogenesis, either in persistence or relapse of infectious disease. Gallin et al. (6) had shown striking elevations in the concentration of serum lipids of patients with infection by gram-negative bacteria. The elevated levels of total lipid was due to marked increases in the serum of triglycerides, free fatty acids, or both. Also, it is known that the osmotic fragility of various mycoplasmas (Acholeplas- mas) is markedly decreased by incorporation into their membranes of preformed monoenoic acids absorbed from the growth medium (16; S. Razin, In J. F. Danielli, M. D. Rosenberg, and D. A. Cadenhead [ed.], *Progress in Surface and Membrane Science*, in press). This report presents a method in which oleic acid, an unsaturated fatty acid ubiquitous in human tissues, is used temporarily for the rapid adaptation of an osmotically fragile L-form of *S. pyogenes* to grow in physiological isotonic media. During this transition, concomitant biochemical and structural changes are quantitated, and the capability of this newly established L-form to rapidly destroy human heart cells in tissue culture is established.

(This investigation was presented, in part, in a preliminary form at the annual meeting of the American Society for Microbiology, New York, 27 April–2 May 1975.)

**MATERIALS AND METHODS**

Organism, media, and growth conditions. A stabilized (i.e., nonreverting) but osmotically fragile L-form of *S. pyogenes*, type 12, was used. The organism was cultured in the regular brucella broth (Pfizer Diagnostics, Brooklyn, N.Y.) with bovine serum albumin, fraction V (8 g/liter; Armour Pharmaceutical Co., Chicago, Ill.) and a total sodium chloride content of 3.5% (wt/vol). For certain experiments this medium was rendered "lipid free" as detailed for tryptose elsewhere (9), whereas the albumin was treated with charcoal according to the method of Chen (3). The final volume of the albumin solution was adjusted to 1 liter with distilled water and frozen. This stock solution (10% [wt/vol]) was thawed and sterilized by filtration (Hormel filters, Hormann & Co., Milldale, Conn.) before use. Additions to the
medium, where indicated, included potassium tellurite and oleic acid. A 1% (wt/vol) aqueous solution of potassium tellurite was sterilized by filtration. Its final concentration in the growth medium was 0.001%. Oleic acid, in 75% methanol, was added (2 μg/ml of medium) so that the final concentration of alcohol in the medium never exceeded 0.1%.

Viable counts (colony-forming units) were performed in duplicate on an agar medium prepared by the addition of 1.5% agar (Difco) to the regular medium above. Similarly, beta-hemolysis was detected by plating the L-form on this same agar medium containing 5% fresh sheep erythrocytes and the sodium chloride and oleic acid concentrations required for growth of the particular L-form being studied.

Adaptation to growth at lower sodium chloride concentrations. The adaptation of an osmotically fragile L-form to grow at decreasing concentrations of sodium chloride, as indicated in Table 1, was performed in Erlenmeyer flasks (50 ml) containing 10 ml of liquid medium. An 18-h inoculum (1 ml) was used. Each culture was transferred in medium with a given concentration of sodium chloride until adapted (maximal viable count and optical density at 680 nm at the stationary phase of growth) before serving as the inoculum for medium with the next lower salt content. The number of transfers at each concentration of sodium chloride is given in Table 1. Use of oleic acid began when the sodium chloride content of the medium reached 1.3% (wt/vol).

Generation times. Growth curves of the L-form adapted to grow at concentrations of 3.5, 1.2, and 0.85% sodium chloride and 0.5% sodium chloride with oleic acid were obtained using Erlenmeyer flasks (125 ml) containing 50 ml of lipid-extracted medium and equipped with a side arm for optical density determinations. Each flask was inoculated with a 10% inoculum from a 16- to 18-h culture and incubated at 37 C. Optical density determinations were made at frequent intervals with a Bausch and Lomb Spectronic 20 at 660 nm. Protein, as an indication of cellular yields and expressed as milligrams of protein per 100 ml of culture medium, was determined on liter batches (2 by 5) of each culture grown in the lipid-extracted medium and harvested during the mid-logarithmic growth phase. Cells were obtained by centrifugation (9,000 x g, 20 min), washed once with the corresponding sodium chloride solution equal to the isotonicity of the growth medium used, lyophilized, and dried over P₂O₅-NaOH in vacuo. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard.

Assessing osmotic fragility of the L-form in tissue culture medium. Aliquots of tissue culture medium (see below) were inoculated (10% [vol/vol] inocula) with overnight cultures of the L-form, each adapted to grow at a different concentration of sodium chloride, and incubated at 37 C (see Fig. 3). Samples were removed periodically and viable counts were determined by plating in duplicate on the agar medium indicated above, but with the appropriate concentration of sodium chloride and oleic acid to which the particular L-form was adapted.

Tissue culture cells, medium, and infectivity. Because of the relationship between streptococcal infection and rheumatic heart disease, Girardi human heart cells (CCL2, American Type Culture Collection Repository, Rockville, Md.) were used. Cells were grown in flat, 250-ml plastic bottles (Falcon) with 20 ml of Eagle base medium (10×), diluted 1:10, and containing 10% inactivated fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.), 0.29 mg of glutamine per ml, pH 7.5 adjusted with sodium bicarbonate. Control cells were subcultured at 5-day intervals with a split ratio of 1:3 after trypsinization (0.25% solution). Viable cell counts were obtained by the dye-exclusion method with erythrocin B and a hemacytometer. For assessing cytopathological damage, 1-day-old tissue cultures were refed and infected with an 18-h culture of the L-form adapted to grow in medium with a final sodium chloride content of 0.85%, and the pH 7.5 was adjusted to 7.5 and incubated. The infectivity ratio of L-form to heart cells was 100:1. For correlating uptake of the L-form by heart cells with tissue culture destruction, 16-h cultures (10 ml) of this physiologically isotonic L-form were labeled by growing in the presence of 0.1 μCi of oleic acid. After centrifugation (4 C) and washing twice with physiological saline, the labeled L-form was resuspended in saline (10 ml) and aliquots (2 ml) were used to inoculate each tissue culture. After gentle shaking, 0.15-ml samples were removed when indicated for determining changes in radioactivity by liquid scintillation and viable counts with time in tissue culture supernatants (see Fig. 5b, c). Also, morphological changes of infected tissue cultures were followed by light microscopy in situ and after fixing with methanol and staining with the May-Grunwald and Giemsa blood stain.

The actual uptake and incorporation of the physio-

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### Table 1. Adaptation of the stabilized, osmotically fragile L-form to growth in lower concentrations of sodium chloride with and without oleic acid

<table>
<thead>
<tr>
<th>No. of transfers</th>
<th>Medium additions</th>
<th>With 2 μg of oleic acid per ml (CFU/ml)</th>
<th>Without oleic acid (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NaCl (%) (final concn)</td>
<td>3.5 x 10⁸</td>
<td>2.7 x 10⁸</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>2.6 x 10⁷</td>
<td>5.5 x 10⁷</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>2.3 x 10⁷</td>
<td>1.3 x 10⁸</td>
</tr>
<tr>
<td>3</td>
<td>1.7</td>
<td>1.7 x 10⁷</td>
<td>3.0 x 10⁷</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.5 x 10⁷</td>
<td>1.4 x 10⁷</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>3.8 x 10⁶</td>
<td>1.3 x 10⁸</td>
</tr>
<tr>
<td>3</td>
<td>1.1</td>
<td>8.1 x 10⁶</td>
<td>NGc</td>
</tr>
<tr>
<td>3</td>
<td>0.9</td>
<td>3.2 x 10⁶</td>
<td>NGc</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>5.0 x 10⁶</td>
<td>NGc</td>
</tr>
</tbody>
</table>

* All transfers were in liquid medium: regular brucella broth plus albumin with NaCl and oleic acid as indicated. Solid medium for plating was liquid medium with addition of 1.5% agar. Fatty acid added as methanolic (75% [vol/vol]) solution; final concentration of methanol less than 0.1%.

b CFU, Colony-forming units.

c NG, No growth.
logical isotonic L-form by tissue culture cells was established by using potassium tellurite (10, 20). The L-form was grown in the presence of this salt (0.001% [wt/vol]) for 1 or 2 days and a 10% inoculum was used to infect each tissue culture. After 2 days, tissue cultures were fixed with 3% (vol/vol) glutaraldehyde, postfixed with osmium tetraoxide (1% [wt/vol]), dehydrated with ethanol, embedded in Epon 812, thin sectioned, and double stained with uranyl acetate and lead citrate before viewing with the electron microscope.

Fatty acid studies. L-forms from lipid-extracted medium with 3.5, 1.2, and 0.85% sodium chloride and 0.5% sodium chloride plus oleic acid were harvested during their respective mid-logarithmic phases of growth, washed once with a sodium chloride concentration equivalent to that of the growth medium, and lyophilized. Methods for the extraction and analyses of the fatty acid content and composition of these cells, by highly resolving capillary gas chromatography with a 150-ft (4,572-cm) polar column, are detailed elsewhere (16). These analyses included the use of hydrogenation studies and infrared spectroscopy.

Isoelectric focusing. Isoelectric focusing in polyacrylamide gels was done as detailed elsewhere (1, 5). Two Ampholine carrier electrolytes (LKB-Produkter AB, Sweden), forming gradients of pH 3 to 10 and 4 to 6, were used. The cytoplasm of the L-form growing in lipid-extracted medium containing 3.5 and 0.85% sodium chloride and 0.5% sodium chloride plus oleic acid, respectively, was examined. Cells were centrifuged during their mid-logarithmic growth and ruptured with the Biotech X-Press (Biochemical Processes, Islip, N.Y.) at low temperature. After removal of membrane particles by centrifugation, each cytoplasm was desalted with G15 Sephadex and subjected to isoelectric focusing. Each gel contained approximately 250 µg of protein as determined by the method of Lowry et al. (11) as already indicated above. After electrophoresis for 4.5 h, gels were removed, their protein bands were precipitated, and the carrier ampholyte was removed (5). Gels were stained with Liessamine Green SF (1% [wt/vol]) in 7% (vol/vol) acetic acid and desitained nonelectrically with acetic acid (7% [vol/vol]). The size of each gel was approximately 3 mm by 7 cm.

RESULTS
Adaptation to physiological osmotic conditions. Table 1 compares the adaptability of the L-form to grow in decreasing concentrations of sodium chloride with and without oleic acid. As is apparent, decreasing the sodium chloride concentration below 1.3% without oleic acid was not possible. Also, multiple blind passages (i.e., passages without visible growth) to the 1.1% sodium chloride level proved unsuccessful. However, with oleic acid in the growth medium, attaining growth with a final concentration of 0.7% sodium chloride, was relatively easy. The smallest decreasing incremental change of sodium chloride used during adaptation was 0.2%. This transition from 3.5 to 0.7% sodium chloride, with the aid of oleic acid, required approximately 20 transfers. Although not indicated, attempting to further reduce the sodium chloride content below 0.7% proved difficult. However, this was accomplished by decreasing the salt content in increments of 0.01% in the presence of oleic acid; adaptation was stopped at a final level of 0.5% sodium chloride. Approximately 35 additional transfers were required for the adaptation of an L-form capable of growing under physiological conditions to grow in a medium with only 0.5% sodium chloride. The L-form adapted to growth at a level of 0.5% sodium chloride continued to require oleic acid after being transferred 16 times, at which point these experiments were terminated.

The L-form adapted to growth in medium with a final concentration of 0.85% sodium chloride is termed our physiological isotonic L-form and was used in all subsequent tissue culture experiments.

The omission of oleic acid from the growth medium once growth had been obtained at the 0.85% sodium chloride level was accomplished by transferring the culture in the presence of this acid 10 times before removing it, which was a one-step procedure. Excellent growth of an established L-form (i.e., no longer requiring added oleic acid) was always observed upon its return to the hypertonic growth medium (3.5% sodium chloride), if done for one transfer only, and then returned to the isotonic medium (0.85% sodium chloride). However, if the physiological isotonic L-form was kept for at least three transfers in the hypertonic medium (with or without oleic acid), the adaptation procedure indicated in Table 1 had to be repeated for growth to reoccur in isotonic medium.

The temporary need for oleic acid by the L-form growing with 1.2% NaCl and with new isolates of the physiological isotonic L-form in the lipid-extracted growth medium was quantitated. A typical concentration-dependent growth response curve, with the optical density (at 660 nm) being from less than 0.020 to 0.200, was obtained with the addition of form 0 to 3.0 µg of oleic acid per ml of medium. In contrast, oleic acid remained a permanent requirement when the L-form was adapted to grow in lipid extracted or nonextracted medium with a sodium chloride content of 0.5%. The temporary need for oleic acid by the physiological isotonic L-form could not be replaced by various concentrations of palmitic or myristic acids or by cholesterol. Also, no greater increase in growth (synergistic effect) was observed when various
mixtures of oleic and palmitic acids replaced this temporary octadecenoic acid requirement.

Chances in growth characteristics. Figure 1 shows changes in cell yields and generation times of established mid-logarithmically growing cells, and their maximal optical densities when entering the stationary phase of growth, with changes in the sodium chloride content of the lipid-extracted growth medium. As is apparent, protein content and optical densities decreased while the generation times increased as adaptation to growth at lower and lower levels of sodium chloride was realized. Growth of the physiological isotonic L-form in terms of colony-forming units was comparable to that of the osmotically fragile L-form from which it was derived (10^7 to 10^9 colony-forming units per ml). However, its cellular yields and the final optical density attained were lower (by approximately 20%).

Fatty acid changes. The fatty acid content of the L-form growing in the extracted medium with 3.5 (control), 1.2, and 0.85% sodium chloride and 0.5% sodium chloride plus oleic acid remained relatively constant, 4.0 to 4.5% of the dry weight of each intact organism. During adaptation, oleic acid incorporated from the growth medium accounted for 30 to 35% of the total fatty acid content of the L-form. However, once established, cultures of the physiological isotonic L-form growing in the absence of added oleic acid showed an octadecenoic acid content of approximately 15%. The predominant saturated fatty acid in the L-form remained palmitic acid during the adaptation from osmotically fragile to osmotic stability. Qualitatively, the fatty acid composition of the established L-form growing at various levels of sodium chloride was similar to that reported previously for this L-form from hypertonic medium (14), except that linoleic acid was absent. Our improved extraction procedure has eliminated most of this dienoic acid which is absorbed from the medium.

Although the fatty acid composition was similar, a change or shift in the saturated to unsaturated fatty acid ratio was observed. With the established L-form growing with sodium chloride concentrations of 3.5, 1.2, and 0.85%, the saturated to unsaturated fatty acid ratio was 0.74, 1.00, and 1.19, respectively. Therefore, as the sodium chloride content of the growth medium was reduced more saturated but less unsaturated long chain fatty acids were formed. The L-form growing at a sodium chloride concentration of 0.5% grew only in the presence of oleic acid (2 µg/ml of medium) and its saturated to unsaturated fatty acid ratio, for comparison only, was 0.89.

Cytoplasm changes and hemolysis. The protein profile of the cytoplasm of the L-form growing at 3.5 and 0.85% sodium chloride and at 0.5% sodium chloride plus oleic acid was significantly different when examined by isoelectric focusing. Figure 2 diagrammatically illustrates typical results obtained with a pH 3 to 10 ampholine gel system. Only the cytoplasm from the physiological isotonic L-form contained three distinct bands of approximate equal intensity after staining. All of the proteins observed in the three samples examined migrated towards the anode, indicating their basicity.

During this adaptation procedure, the resulting physiological isotonic L-form retained its ability to hemolysze blood (beta-hemolysis). This is in contrast to the findings of Clasener et al. (4), who reported that hemolysis production of an L-form from a group A coccus adapted to physiological osmotic conditions disappeared with successive transfers.

Survival and infectivity in tissue culture. Figure 3 compares the survival or osmotic fragility of the L-form in tissue culture medium alone. As is apparent, viability decreased fastest, a matter of minutes, when the osmotically fragile L-form (control, 3.5% sodium chloride) was suspended in tissue culture medium. Maximum survival time for the physiological isotonic L-form and the L-form capable of growth with 0.5% sodium chloride plus oleic acid was similar, 4.5 to 5.0 days. But, the relative plateauing of viability followed by a precipitous drop after 3 days for only the latter was reproducible.

Figure 4 illustrates a typical result of the uptake or infectivity and subsequent release of the physiological isotonic L-form by Girardi.
LEON AND PANOS

ANODE

CATHODE

A

B

C

microscope (Fig. 5). When inoculated immediately after trypsinization (splitting), either with a culture of the physiologically isotonic L-form or this same L-form washed with saline, heart cells failed to adhere to the surface of the bottle even after 2 to 3 days of incubation. Failure of these inoculated heart cells to reduce the pH of the growth medium, or to be subcultured, indicated that they were no longer viable. Likewise, inoculation of these heart cells with the L-form 16 h after trypsinization resulted in almost complete destruction of the tissue culture monolayer after 3 to 4 days. The few adhering cells remaining were conspicuous by their atypical morphology, which included large and deeply stained nuclei relative to their cytoplasmic volume as compared with the uninfected heart cell controls (Fig. 6). Refeeding of human heart cells in tissue culture. These results were always obtained but for variable periods of time, ranging from 6 days shown here to 10 days, with viable counts still detectable after day 13. Under these conditions, maximum destruction of the infected heart cells occurred after 3 to 4 days, with the control cells remaining in excellent condition for from 5 to 6 days. As is apparent, agreement between viable count and radioactivity for uptake and release of the L-form by the tissue culture cells is excellent. The final viable count observed never reached the initial inoculum size used for these infectivity studies, indicating survival rather than cell division. In contrast, the parental streptococcus rapidly overgrows the tissue culture when used as the inoculum.

Uninoculated heart cell tissue cultures exhibit a typical morphology under the light microscope (Fig. 5). When inoculated immediately after trypsinization (splitting), either with a culture of the physiologically isotonic L-form or this same L-form washed with saline, heart cells failed to adhere to the surface of the bottle even after 2 to 3 days of incubation. Failure of these inoculated heart cells to reduce the pH of the growth medium, or to be subcultured, indicated that they were no longer viable. Likewise, inoculation of these heart cells with the L-form 16 h after trypsinization resulted in almost complete destruction of the tissue culture monolayer after 3 to 4 days. The few adhering cells remaining were conspicuous by their atypical morphology, which included large and deeply stained nuclei relative to their cytoplasmic volume as compared with the uninfected heart cell controls (Fig. 6). Refeeding of

![Fig. 2. Isoelectric focusing of the cytoplasm of the established L-form in extracted medium with (A) 0.5% (wt/vol) sodium chloride plus oleic acid (2 μg/ml of medium), (B) 0.85% (wt/vol) sodium chloride, and (C) 3.5% (wt/vol) sodium chloride (control).](image)

![Fig. 3. Comparative osmotic fragility (survival) in tissue culture medium of the established streptococcal L-form growing at lower concentrations of sodium chloride. L-form inoculum from extracted growth medium with 3.5% (wt/vol) sodium chloride () , 0.85% (wt/vol) sodium chloride ( ), and 0.5% (wt/vol) sodium chloride plus oleic acid (2 μg/ml of medium) ( ).](image)

![Fig. 4. Uptake and release of the established physiological isotonic L-form by Girardi human heart cells.](image)
such cultures 3, 5, and 7 days after inoculation did not lead to renewed growth as deduced by microscopy and lack of change of the medium pH indicator. However, if these inoculated tissue cultures were trypsinized, even 9 days after L-form inoculation, and the few remaining viable heart cells were allowed to establish themselves before being refeed and trypsinized again 3 days later, complete recovery eventually occurs. Thus, at least two trypsinizations plus multiple refedings were necessary for infected heart cells to recover and exhibit their

![Image](http://iai.asm.org/images/article/Fig5.png)

**Fig. 5.** Uninfected Girardi human heart cells 5 days after splitting with trypsin (control). May-Grunwald-Giemsa stain. ×300.

![Image](http://iai.asm.org/images/article/Fig6.png)

**Fig. 6.** Girardi human heart cells 4 days after infection with the established physiological isotonic L-form and 5 days after trypsinization. ×300. Stain as in Fig. 5.
typical growth rate and morphology. These same results were noted when infected heart cells were removed from flasks by scrapping with a rubber policeman in lieu of trypsin. In contrast to the above, no apparent deleterious effects occurred when the host cells were inoculated with the L-form 48 h after trypsinization.

Tissue cultures inoculated with heat-killed (1 h at 70°C) L-form cells, either washed with saline or suspended in their own growth medium, showed some morphological damage that was completely reversible after only one trypsinization, two to five refeedings alone being ineffective for restoration of a normal growth rate and cellular morphology. Supernatant culture medium and saline washes from live and heat-killed L-form cells did not alter the microscopic morphology or otherwise affect these infected heart cells.

The effect of trypsin on the viability of the L-form was determined. It was found that subjecting an overnight culture of the L-form suspended in tissue culture medium to a 0.25% concentration of trypsin for 20 min resulted in an 18.5% decrease in viable count. The time exposure and concentration of enzyme used is equivalent to that for the perpetuation of the uninfected heart cell line.

To answer the question "Does the L-form penetrate or only attach itself to the surface of the heart cells?", the L-form was labeled with potassium tellurite. Viable counts taken of the physiological isotonic L-form growing in the presence of 0.001% potassium tellurite after 24 h of incubation were 55% of the control in the absence of this reagent. This concentration of potassium tellurite permitted maximum labeling of the remaining viable cells for ease of differentiation from the internal structures of the heart cells by electron microscopy. Figures 7 and 8 are controls of uninoculated heart cells, with their intracellular detail clearly evident, and the L-form with its heavy deposits of metallic tellurium or tellurium oxide on and near the inside of the cytoplasmic membrane, respectively. Figures 9 and 10 show the L-form within the heart cell.

**DISCUSSION**

A result of this investigation was the rapidity with which an osmotically fragile L-form of *S. pyogenes*, type 12, was adapted to survival and growth at or below physiological isotonicity. Approximately 20 transfers were required, at times with oleic acid, for growth to occur in medium with 0.7% sodium chloride. Previously, others had shown that over 200 transfers during a stepwise reduction in the salt content of the

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**Fig. 7.** Electron micrographs of thin sections of uninfected Girardi human heart cells (control). Bar = 1 μm.
Fig. 8. Electron micrograph of thin section of the established physiological isotonic L-form 1 or 2 days after growth with potassium tellurite. Arrows exemplify intracellular tellurite deposits. Bar = 1 μm.

Fig. 9. Electron micrograph of thin section of the tellurite-labeled physiological isotonic L-form within the heart cell. Note loss of intracellular detail of the infected heart cell. Arrow indicates intracellular L-form. Bar = 1 μm.
growth medium was necessary for an L-form from S. pyogenes, strain S8, to finally grow at near physiological isotonicity (13). Also, it had been reported that, once adapted to a particular concentration of sodium chloride, growth of an L-form could not be sustained in broth containing either higher or lower concentrations of this electrolyte (13). This was not the case here. The physiological isotonic L-form grew readily when returned to the hypertonic medium. However, its inability to continue to grow in the isotonic medium, once transferred several times in the hypertonic medium, indicates that adaptation to growth in an isotonic environment is not analogous to that of an L-form made to grow in liquid medium. As is known, once achieved, the ability to grow in broth from agar is never lost.

Osmotically fragile (salt-requiring) L-forms have a lower phospholipid content than salt-nonrequiring L-forms (21). Because of this, it has been hypothesized that a high sodium chloride content in the medium for growth of osmotically fragile L-forms may reflect a need for the condensation and solidification of their membrane phospholipids by metal ions (21). While being adapted to lower sodium chloride concentrations with oleic acid, the membrane is highly elastic, as evidenced by the total amount of this acid present (over 30%) in the membrane and from concomitant electron spin resonance spectroscopy (M. Chevion and C. Panos, Biochim. Biophys. Acta, in press). However, once established, the saturated/unsaturated fatty acid ratio of the L-form showed a decrease in its unsaturated fatty acid content from the hypertonic medium to osmolarities at or below physiological isotonicity. This may reflect the ability of the L-form to slowly strengthen its membrane by increasing the packing mode of its fatty acid content through increased saturated fatty acid formation (12), thereby negating or minimizing a solidification and condensation effect by sodium chloride. This may explain the inability of the physiological isotonic L-form to continue to grow in the isotonic medium after having been returned to the hypertonic medium for several transfers, as already mentioned above. Our electron spin resonance spectroscopy findings have shown that the physical nature of the membrane of the established physiological isotonic L-form has changed. The resulting lipid chain rigidity of the membrane is in the order physiological isotonic L-form > osmotically fragile L-form > parental streptococcus (Chevion and Panos, in press), which agrees with the changes in the saturated/unsaturated fatty acid ratios already
VOL.
and
subtilis
protein differences
Acholeplasmas, organisms unable
distribution
tering
osmotic
in
be
in
this coccal L-form
osmotic
mum
and possibly
in rearranging or redistributing its
membrane lipid protein components for
maximum osmotic stability. Earlier findings with the
Acholeplasmas, organisms unable to synthesize
only long-chain enoic fatty acids, had shown
that addition of long-chain unsaturated fatty
acids to the growth medium resulted in greater
membrane elasticity and decreasing osmotic
fragility (16; Razin, in press). However, with
this coccal L-form one must consider the de
novo synthesis of both long-chain saturated and
unsaturated fatty acids, and the simultaneous
effect on their synthesis by preformed fatty
acids when added to the growth medium, on the
physical state of its membrane.

An optimum ratio of saturated to unsatu-
rated fatty acids is believed necessary for both
growth and osmotic stability of the mycoplasma
membrane. Although both the physiological
isotonic L-form and the L-form growing at 0.5%
sodium chloride plus oleic acid are osmotically
stable, their growth and viability characteristics
were markedly different when suspended in
the isotonic but non-nutritive tissue culture
medium. Therefore, the saturated to unsatu-
rated fatty acid ratio may reflect a versatility
in membrane stability to different environ-
ments for the adapted streptococcal L-form
culture, from maximum flexibility at lowest
sodium chloride concentrations and an avail-
able enoic acid to maximum rigidity in isotonic
media without such acids.

It is known that abnormally high levels of
free fatty acids occur in the blood of patients
with bacteremias from gram-negative infections
(6). Therefore, our adaptation scheme with the
temporary use of oleic acid makes the presence
of unsaturated fatty acids, in such pathologic
conditions or during mixed gram-positive and
-negative bacterial infections, for use by any
osmotically fragile L-form for rapid adaptation
to survive in less hypertonic environments seem
quite reasonable.

Earlier, we had demonstrated quantitative
protein differences in the membrane of S.
pyogenes and this stabilized L-form (15). Later,
Gilpin et al. (7) showed that conversion of B.
subtilis to an L-form also resulted in the re-
distribution of membrane proteins. The changes
in the protein profile of the cytoplasm noted
with the adaptation to growth at lower and
lower salt concentrations with this coccal L-
form may represent shifts in the soluble protein
precursors for membrane synthesis. Therefore,
these collective changes may be analogous to
those observed with several mycoplasmas, sug-
gesting that membrane proteins, including
those bound electrostatically to membrane
lipids, may influence the physical state of mem-
brane lipids (Razin, in press). Finally, electron
micrograph measurements of thin sections of
the osmotically fragile and physiological iso-
tonic L-forms and of the parental coccus failed
to show any correlation between membrane
thickness and increased osmotic stability
(Chevion and Panos, unpublished data).

These studies have demonstrated unequivoco-
ally that an L-form surviving in a physiological
isotonic environment can destroy human heart
cells in tissue culture rapidly. Therefore, we are
dealing with a cytopathic effect(s) rather than a
latent infection as has already been described
(17–19) with other L-forms in tissue culture.
This destructive effect probably occurs before
and after penetration of the viable L-form since
multiple refeddings alone fail to result in re-
covery of the infected heart cells; complete
recovery only occurred after multiple tryp-
sinizations and refeddings. Splitting of infected
heart cells by scraping, in lieu of trypsin treat-
ment, and the use of multiple refeddings did not
result in their recovery. However, human
embryonic kidney cells infected with a stable
L-form of S. faecalis have been maintained for
up to 60 days by splitting cells by scraping with
a rubber policeman (8). Also, use of heat-killed
L-forms caused some tissue destruction that
was reversible only after trypsin treatment.
Therefore, the necessity of trypsin for complete
recovery may be due to its digestive effect on
viable and nonviable L-forms occluded by the
host cells. It had been established that this
L-form, when lyophilized and injected into
rabbit hearts, can cause granulomas at the site
of injection that differ from those induced by
cell walls or by sonic extracts (2). In these
tissue culture studies, infection by the L-form
always resulted in almost complete loss of intra-
cellular detail of the host cell and resembles,
microscopically, the cytopathic effect of tissue
culture cells when infected with certain viruses.

Osmotic lability is not the only reason for
the inability of an L-form to persist in vivo.
Clasener et al. (4), using three different strep-
tococcal L-forms adapted to physiologic osmotic
conditions, were unable to demonstrate their
persistency in mice and concluded, "... (this
adaptation) did not make their capability to
survive in vivo comparable to that of the bac-
terial phase." The variability of L-forms of the
group A cocci in induction and persistence in
tissue culture, adaptation to low salt, and hemolysin production is known. This is in concert with their morphological homogeneity but biochemical heterogeneity, even when from the same parental bacterium. This has made the study of L-form pathogenesis in animal systems difficult. It is probable that adapted, osmotically stable L-forms from gram-positive bacteria may also have to be adapted to growth in tissue culture cells before their full pathogenic potential in an acceptable host is successfully realized.

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LITERATURE CITED