# QUANTITATIVE CONVERSION OF CELLS AND PROTOPLASTS OF *PROTEUS MIRABILIS* AND *ESCHERICHIA COLI* TO THE L-FORM

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In the past few years considerable interest in the L-forms of bacteria has developed, and numerous descriptive papers have been published on their morphology, cytology, occurrence, and cultivation. These are well summarized in several reviews (Dienes and Weinberger, 1951; Tulasne, 1953; Minck, 1955; Sincovics, 1956). A thoroughgoing chemical and enzymatic study of Proteus L-forms has also been published recently (Kandler *et al.*, 1956*b*, *c*). In the extensive list of publications cited in these papers, the quantitative control of formation and growth of L-forms has received relatively little attention.

The present study of L-forms was initiated in order to gain a better understanding of the genetic or phenotypic changes associated with the various morphological changes of the L-cycle. Since many phases of such a study require enumeration and quantitative recovery of organisms, clarification of some of the unsolved methodological problems in L-form experimentation is required.

In this paper, attention is focused on the first step of the L-cycle—the change from bacillary form to large body to L-colony. Several authors have indicated the portion of viable cells which give rise to L-colonies. Tulasne (1953) stated that only 1 per $10^5$ organisms produced L-colonies in his system, while Medill and O'Kane (1954) reported that 1 per cent of the cells of a Proteus culture will form L-colonies. According to Dienes and Weinberger (1951), only 5 per cent of the large bodies of Proteus formed under the influence of penicillin are "viable."[1] These results leave open the question whether the ability to produce an L-colony is confined to certain variant cells in a population, or whether all cells are capable of giving rise to L-colonies under appropriate growth conditions.

[1] After this manuscript had been completed, a paper by Medill-Brown and Hutchinson (1957) appeared, reporting survival of up to 33 per cent of all organisms plated.

The present experimental approach to the problem of obtaining a high yield of L-colonies per bacillary plating unit is based on the idea that a protoplast stage (Weibull, 1953; Lederberg, 1956) is intermediate between bacillus and L-colony. One may picture the following sequence of events: The reaction leading to formation of cell walls is blocked in bacilli growing in the presence of penicillin. (Lederberg, 1956; Park and Strominger, 1957). In liquid media of high solute content, the organisms are preserved as intact protoplasts (Weibull, 1953). In this form they can increase in size but are usually unable to divide (McQuillen, 1956). Further developments might be postulated as follows: in solid medium, the original protoplast increases enormously in size and becomes a spherical "large body." The latter form gives rise to additional large bodies by a process superficially similar to budding and eventually a whole colony of large bodies results. This represents the 3B type L-colony of Dienes and Weinberger (1951). Withdrawal of penicillin permits the outgrowth of bacillary forms from individual large bodies.

If the above postulates regarding the relation between protoplasts and large bodies are correct, one may predict that media favorable to protoplast stabilization should also favor L-colony formation. Further, if appropriate stabilizing conditions could be found, every bacillus might be capable of giving rise to a 3B L-colony.

The results reported below verify these predictions. Under favorable conditions every cell and every protoplast in a *Proteus mirabilis* culture and in an *Escherichia coli* culture may give rise to an L-colony. The potential for L-colony formation is thus inherent in both cells and protoplasts and does not depend on special genetic or phenotypic characteristics.

The ratio of the number of viable cells plated to the number of L-colonies obtained—the cell:L ratio—is influenced by various factors which are mainly physical in nature. Much of this

paper is concerned with identification and evaluation of these factors.

## MATERIALS AND METHODS

*Strains.* Most experiments were performed with a strain of *P. mirabilis* freshly isolated from human feces, and a nonlysogenic prototrophic derivative of *E. coli* strain K 12. Both strains were maintained on nutrient agar slants. Additional strains were used to test the generality of some of the findings.

*Media.* Since this paper is concerned primarily with the effect of variations in medium composition on the cell:L ratio, the detailed data on this topic are presented in the Results section. Three media were developed in the course of this work: a serum-containing complex medium and a synthetic medium for *P. mirabilis* L-forms, and a semisynthetic medium for *E. coli* L-forms.

Complex medium:

| | |
|---|---|
| Brain-heart infusion (Difco) | 37.5 g/L |
| Enzymatic casein hydrolyzate | 10 g/L |
| Glucose | 6 g/L |
| Agar | 10 g/L |
| Sodium succinate | 0.5 M |
| Defibrinated horse serum | 10% v/v |
| Penicillin | $10^6$ units/L |

Final pH near pH 7.4

Synthetic medium:

| | |
|---|---|
| Sodium citrate | 0.0040 M |
| $K_2HPO_4$ | 0.0500 M |
| $KH_2PO_4$ | 0.0080 M |
| $(NH_4)_2SO_4$ | 0.0075 M |
| $MgSO_4$ | 0.0010 M |
| KCl | 0.2000 M |
| Agar | 1.5 per cent |
| Penicillin | 1000 units/ml |

Final pH near pH 7.4

Semisynthetic medium:

| | |
|---|---|
| Sucrose | 0.350 M |
| Glucose | 0.030 M |
| $KH_2PO_4$ | 0.035 M |
| NaCl | 0.060 M |
| $MgCl_2$ | 0.010 M |
| Penicillin | 1000 units/ml |
| Agar | 1.5 per cent |
| Enzymatic casein hydrolyzate | 2 per cent |

pH adjusted to 6.2 with KOH

*Inocula.* For experiments with complex medium, *P. mirabilis* cells were grown in Penassay broth (Difco) on a reciprocating shaker for 48 hr at 37 C to a density of about $2 \times 10^{10}$ cells per ml.

Cells for plating on synthetic medium were grown with agitation in Abrams' medium (1955) for 24 hr at 37 C to a count of approximately $2 \times 10^{10}$ cells per ml.

*E. coli* cells were grown overnight on the salts-glucose-amino acids medium of Fraser and Jerrel (1953) to maximum optical density and the next morning diluted approximately 20-fold in fresh medium. They were used after they had undergone a 3-fold increase in optical density and had attained a concentration of about $5 \times 10^8$ cells per ml.

*Dilutions and plating.* Dilutions were carried out in the growth media. All platings in synthetic media were made by the pour-plate method unless otherwise specified.

*Incubation.* Plates were incubated at 37 C and 55 per cent relative humidity. Plates of complex medium were counted after 7 days whereas plates of synthetic and semisynthetic medium could be read in 3 days. Bacillary colonies were counted after incubation overnight.

*Cell controls.* To obtain viable cell counts, penicillin was omitted from some of the plates in each experiment for calculation of the cell:L ratio. Bacillary colony counts did not vary substantially from one experiment to the next. From time to time, hemocytometer counts were made to compare viable and total counts. In every case, excellent agreement was obtained.

## RESULTS

Before turning to an account of the quantitative experimental data, a word is necessary concerning the morphology of the L-colonies which were encountered in this work. In early experiments with poorly stabilized spread-plates and large inocula, mixtures of L-colony types corresponding to the 3A and 3B types of Dienes and Weinberger (1951) were observed regularly, with both Proteus and *E. coli*. In the media which were finally developed, however, all Proteus cells gave rise to L-colonies which were unmistakably of the 3B type, recognizable as such even when imbedded in agar. *E. coli* colonies also contain large numbers of large, spherical bodies and thus present a predominantly 3B character. Since the *E. coli* colonies contain much fine debris reminiscent of 3A morphology, however, they might be regarded as intermediate between 3A and 3B. The factors that control the appearance of the 3A L-colony type are at present under investigation. All of the L-growth

considered in this publication is of the 3B or quasi-3B type.

Discussion of the variables which are significant to an efficient cell → L conversion can be systematized by considering the various components of the media in turn.

*Experiments with Proteus mirabilis.* (1) KCl and tonicity:—Experiments with protoplasts in many laboratories have shown that media of high solute content are essential to protoplast preservation (Weibull, 1953; Landman and Spiegelman, 1955; McQuillen, 1956). If protoplasts are an intermediate stage in L-colony formation, hypertonic stabilization effects should thus be reflected in the cell:L ratio. Determinations of this ratio at various solute concentrations were therefore carried out with several different stabilizing substances. Data obtained with graded KCl concentrations are shown in figure 1. For each point, the cell count at the same KCl concentration serves as the control; cell counts remain at the same level up to 0.6 M KCl and then decline. It is seen that L-colony formation is indeed strongly dependent upon a high solute level. Sucrose, sodium succinate, or sodium citrate present a similar picture and cell:L ratios of unity have been obtained with appropriate concentrations of all of these stabilizers.

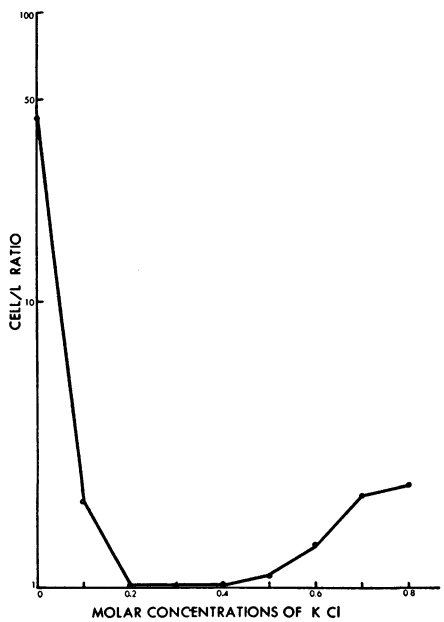


*Figure 1.* L-Colony formation in *Proteus mirabilis* as a function of KCl concentration (synthetic medium).

TABLE 1

*Effect of agar concentration and tonicity on the growth of L-colonies of Proteus mirabilis (Synthetic medium)*

| Agar | Cell:L Ratio at: | |
|---|---|---|
| | 0.2 M KCl | No KCl |
| % | | |
| 0.75 | 4.0 | >33000 |
| 1.0 | 2.1 | 32.4 |
| 1.5 | 2.1 | 83.5 |
| 2.0 | 9.0 | >33000 |
| 2.5 | >33000 | >33000 |

(2) Agar concentration:—Agar and solute have a mutually complementary effect on 3B L-colony stabilization. This is demonstrated in the experiment shown in table 1. It appears that low agar concentrations do not support L-colony growth in the absence of added stabilizer, but will allow fair L-colony development at 0.2 M KCl. Further, the deleterious effects of high agar concentrations are strongly modified at the higher solute level. The synergistic effect of agar and solute can be understood, if it is postulated that a physical stabilizing function of agar complements the osmotic stabilizing function of the solute.

The size of the imbedded L-colonies also varies quite strikingly with agar concentration. The colonies are medium-sized at low agar levels, largest (½ to 1 mm in diameter) at 1 and 1.5 per cent agar, and very small at 2 and 2.5 per cent agar.

The importance of agar to L-colony formation is also evident when L-colony counts from pour- and spread-plates are compared. In an experiment with synthetic medium, pour-plate counts gave cell:L ratios between 3.3 and 5.8 while the corresponding spread-plates showed no L-colonies at thousandfold lower dilutions. In this instance again, the agar effect is contingent upon other medium components; in complex medium, spread-plates regularly yield cell:L ratios of 3 while a cell:L ratio of unity is attained with the corresponding pour-plates.

(3) Effect of penicillin concentration:—In searching for an optimum medium for L-colony formation, several experiments were performed to determine the best concentration of penicillin.

TABLE 2

*Effect of penicillin concentration and tonicity on the growth of L-colonies of Proteus mirabilis (Synthetic medium)*

| Penicillin | 0.2 M KCl | | No KCl | |
|---|---|---|---|---|
| | Type of growth | Cell:L ratio | Type of growth | Cell:L ratio |
| *μ/ml* | | | | |
| 25 | None | — | Bacillary: survival 0.7 → 65% | — |
| 50 | L-forms | 305 | Bacillary: survival 0.1 → 4% | — |
| 100 | L-forms | 2 | L-forms | 4 |
| 200 | L-forms | 1 | L-forms | 3 |
| 400 | L-forms | 1 | L-forms | 7 |
| 600 | L-forms | 1 | L-forms | 10 |
| 800 | L-forms | 1 | L-forms | 10 |
| 1000 | L-forms | 1 | L-forms | 8 |
| 2000 | L-forms | 1 | L-forms | 8 |

Results which at first seemed inconsistent fell into a pattern when it was discovered that changes in penicillin concentration produced different L-colony growth patterns at different tonicities. This is shown in table 2.

Evidently, low penicillin concentrations are favorable to L-colony survival at low tonicities while high penicillin concentrations are required in well-stabilized synthetic medium. Further, there is a range of penicillin concentrations in which growth is poor or altogether absent; (Medill-Brown and Hutchinson, 1957) with 0.2 M KCl, this level is reached at 25 units of penicillin per ml. With no KCl and 25 or 50 units of penicillin per ml, survival varies from 0.1 to 65 per cent and is entirely in the forms of cells. Only L-forms appear at 100 units of penicillin per ml and above. These observations have not been investigated further.

(4) Effect of pH:—Synthetic medium was adjusted to pH values ranging from pH 5.5 to 8.0 and bacillary colony and L-colony counts were determined at each pH. Cell growth and L-colony formation were substantially the same throughout the range tested—the cell:L ratio was 1.0 between pH 6.5 and 7.5 and increased slightly to 1.2 at the high and low ends of the range tested.

(5) Carbon and nitrogen sources:—Maximal L-growth of *P. mirabilis* can also be obtained if the ammonium sulfate of synthetic medium is replaced by casein hydrolyzate or by aspartic acid. Further, the carbon source, sodium citrate, may be replaced by similar concentrations of glucose, lactate, or succinate. Experimental experience in this area indicates that some of the special effects produced by alterations in the carbon source which have been observed by other workers (Minck *et al.*, 1957) may have been due to the osmotic effect of these substances rather than to their chemical nature.

(6) Aerobiosis:—In both synthetic and complex medium, L-growth is equally good under aerobic or partially anaerobic conditions. In earlier experiments with Abrams' medium (1955), however, anaerobic incubation lowered the cell:L ratio in spread-plates from 286 to 6. Apparently, the effect of this variable as well as the improved growth in spread-plates observed in Abrams' medium is contingent upon differences in composition between Abrams' medium and synthetic medium.

(7) Age of inoculum:—Using different strains and media, Kandler and Kandler (1956) found that the age of the cells used as inoculum for L-colony formation had an important influence on the cell:L ratio. This factor proved to be of only minor significance in the system described

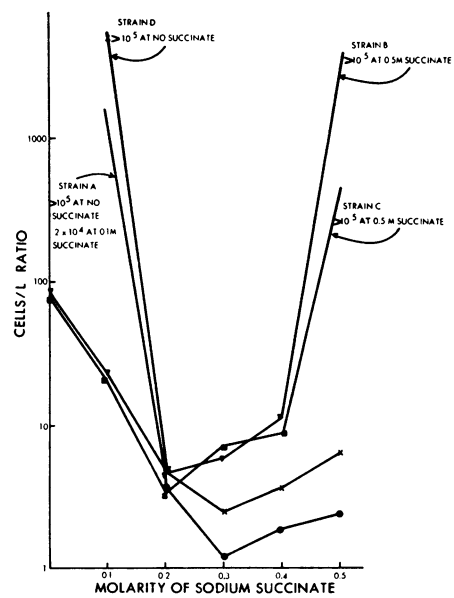


*Figure 2.* L-Colony formation as a function of tonicity in four strains of *Proteus mirabilis* (complex medium).

here. With complex medium, cell:L ratios of unity were obtained with 4-hr and 48-hr cells and no striking changes in cell:L ratio were observed between 24-, 48- and 72-hr cells. With synthetic medium, 24- or 48-hr cells were used routinely and there appeared to be no difference in their behavior.

(8) Strain differences:—According to the literature on L-forms, different strains and species of bacteria show marked differences in their ability to give rise to L-forms. Recent findings in this and other laboratories (Kandler and Kandler, 1956; Crawford *et al.*, 1957) which emphasize the importance of physical conditions in L-form survival prompted execution of a survey of L-colony forming ability of several strains of Proteus.

Four freshly isolated strains of *P. mirabilis* were inoculated into plates of complex medium containing varying concentrations of sodium succinate. Results of this experiment show (figure 2) that osmotic conditions which favor optimal L-colony development may differ for different strains. Obviously, several tests must be made before the L-forming potential of any bacterial strain can be adequately assessed.

(9) Relation between protoplasts and L-forms:—If a protoplast stage is intermediate between cell and L-colony, it should be possible to obtain L-colonies with an inoculum of protoplasts. To convert *P. mirabilis* cells into protoplasts, a 4-hr culture of Proteus in Penassay broth was transferred to Penassay broth containing 0.5 M sodium succinate, 1000 units penicillin per ml, and 0.01 M $MgSO_4$ and incubated on a shaker at 37 C. The change of the bacillary form to the protoplast form was followed both microscopically and by the watershock technique. In the latter procedure, 9 ml of distilled water were added suddenly to a 1 ml volume of suspended protoplasts or cells. Extensive killing due to osmotic rupture resulted in the protoplast population while cells remained unaffected.

Results from a protoplast plating experiment are shown in table 3. Clearly, each protoplast may give rise either to a bacillary colony or an L-colony in the absence or presence of penicillin, respectively. The conversion of Proteus cells to protoplasts is documented by the fact that after exposure to penicillin for 45, 105, and 165 min, a substantial fraction of the population is killed by watershock. Increased sensitivity to watershock presumably corresponds to increased protoplast fragility.

TABLE 3

*Ability of protoplasts of Proteus mirabilis to form L-colonies and bacillary colonies (Complex medium)*

| Time of Incubation in Succinate-Penicillin Broth | Loss by Shock | Bacillary Colony Count | L-Colony Count | Cell:L Ratio |
|---|---|---|---|---|
| *min* | % | $\times 10^{-7}$ | $\times 10^{-7}$ | |
| 0 | | 12.00 | 7.80 | 1.5 |
| 45 | | 12.00 | 6.90 | 1.7 |
| 45 Plus watershock | 75 | 4.50 | 3.70 | 1.2 |
| 105 | | 11.00 | 7.10 | 1.5 |
| 105 Plus watershock | 88 | 1.30 | 0.78 | 1.7 |
| 165 | | 11.00 | 4.00 | 2.7 |
| 165 Plus watershock | 95 | 0.49 | 0.18 | 2.7 |

While the results shown in table 3 show that protoplasts can give rise to L-colonies, they do not answer the question whether protoplasts constitute an intermediate stage between bacillary form and L-form; whether they reach the L-stage by a different pathway; or whether, perhaps, L-colonies are simply colonies of dividing protoplasts.

Some recent experiments have lent support to the latter hypothesis. It was shown that the survival of large bodies and of protoplasts in media of different agar concentrations follows a distinctive pattern which is very similar for both forms.

Protoplasts for these comparative experiments were prepared by transferring a 4-hr Proteus culture to succinate-Mg-penicillin-Penassay broth and incubating for 2 hr at 37 C. Large bodies were obtained by suspending the 3B surface growth from a plate of complex medium in 20 ml of liquid complex medium. Pour-plate counts of both suspensions were then made in penicillin-free and penicillin-containing media of graded agar content. Results are summarized in table 4.

It seems that protoplast and L-body suspensions give rise to a given number of bacillary colonies regardless of the agar concentration of the plating medium. Evidently, both forms are sufficiently stabilized by all of the media tested to allow them to change to the bacillary state. By contrast, the requirement for delimited agar concentrations for L-colony formation are clearly apparent and are quantitatively similar for both protoplast and L-body inocula.

These results show that 3B L-bodies and protoplasts have similar stability thresholds; they do not prove, of course, that these forms are identical.

TABLE 4

*Ability of Proteus protoplasts and L-body suspensions to form bacillary and L-colonies (Complex medium)*

| Agar in Plating Medium | 2-hr Protoplasts | | | 3B L-Bodies | | |
|---|---|---|---|---|---|---|
| | Bacillary colony count | L-Colony count | Cell:L ratio | Bacillary colony count | L-colony count | Cell:L ratio |
| % | $\times 10^{-7}$ | $\times 10^{-7}$ | | $\times 10^{-7}$ | $\times 10^{-7}$ | |
| 0.6 | 8.5 | <0.001 | >8500 | | | |
| 0.75 | | | | 1.1 | 0.0330 | 34 |
| 0.8 | 8.5 | 0.360 | 24 | | | |
| 1.0 | 8.2 | 1.900 | 4 | 1.1 | 0.3300 | 3 |
| 1.2 | 7.9 | 2.300 | 3 | | | |
| 1.5 | 6.4 | 0.140 | 45 | 1.1 | 0.0500 | 22 |
| 2.0 | 8.3 | <0.001 | >7600 | 1.1 | 0.0048 | 227 |

TABLE 5

*Effect of sucrose concentration on the growth of L-colonies in Escherichia coli*

| Molarity of Sucrose | Cell:L Ratio |
|---|---|
| 0 | 2390 |
| 0.05 | 510 |
| 0.10 | 23 |
| 0.20 | 2.9 |
| 0.30 | 2.4 |
| 0.35 | 2.0 |
| 0.40 | 2.1 |
| 0.50 | 1.8 |

Further data and considerations which bear on the question of the relationship between large bodies and protoplasts will be discussed in the section dealing with *E. coli* and in the Discussion of this paper.

*Experiments with Escherichia coli K 12 S.* (1) Tonic stabilization:—In initial attempts to obtain good L-colony growth with *E. coli*, sucrose was used as the stabilizing substance since this sugar has been the agent most widely used for protoplast stabilization with *E. coli* (Lederberg, 1956; Zinder and Arndt, 1956; Mahler and Fraser, 1956). Table 5 shows that the stabilization pattern of *E. coli* in sucrose is similar to the Proteus pattern exhibited in figure 1. By contrast, it was surprising to find that two other solutes tested, 0.05–0.5 M potassium succinate and 0.05–0.5 M potassium phosphate, allowed no L-growth at all. A fourth agent, KCl, gave erratic cell:L ratios, but regularly permitted some growth of L-colonies.

(2) Agar concentration:—Even with 0.35 M sucrose, L-growth becomes optimal only at agar concentrations of 1 per cent or above with K 12 S. The abrupt rise in cell:L ratio which is observed on lowering the agar concentration has not always occurred at the same point in our experiments—presumably, different lots of agar have slightly different L-colony-supporting properties. At 0.6 per cent agar, however, growth was always negligible and agar concentrations above 2 per cent were always highly inhibitory.

In *E. coli*, as in Proteus, agar and stabilizer complement each other in supporting L-growth. Thus, in 0.3 M sucrose, the cell:L ratio was raised only slightly when the agar concentration was dropped from 1.5 to 0.8 per cent. In the absence of sucrose, on the other hand, 1.5 per cent agar still allowed 1 cell in 50 to initiate an L-colony while the 0.8 per cent agar plates were completely devoid of growth even when inoculated with $10^5$ cells.

The importance of agar to L-colony growth of *E. coli* is further emphasized by the fact that inocula which produce $10^5$ L-colonies in semisynthetic agar by the pour-plate method produce no L-growth on a spread-plate.

Gelatin in concentrations from 10 to 20 per cent failed to support growth of *E. coli* L-colonies in both spread and poured plates.

(3) Effect of penicillin concentration:—The response of *E. coli* to varying penicillin concentrations partly parallels that of *Proteus mirabilis*. Again, a concentration range of penicillin is encountered where growth is largely suppressed. At 25 units of penicillin per ml of semisynthetic medium, almost every *E. coli* cell plated formed a colony, whereas at 50 units of penicillin per ml, only 1 cell in 14 initiated growth. In either case, the colonies consisted of bacilli but the cells growing at 50 units per ml were usually characteristically misshapen. They exhibited an optically transparent swelling—generally in their midsection. At 100 units of penicillin per ml, growth was of the L-colony type but the cell:L ratio was 22. At 200 units of penicillin, the cell:L ratio had dropped to 3. Depending on the particular isolate of strain K 12 S, the cell:L ratio remained low and constant between 300 and 5000 units of penicillin per ml or it rose again when the

penicillin concentration exceeded 500 units per ml. A shift in effective penicillin concentration due to tonicity alteration was also observed with *E. coli*: in the absence of sucrose or in 0.085 M sucrose, optimum growth was attained only with 800 or more units of penicillin per ml.

(4) Effect of pH:—L-colony development appears to be equally good at pH's ranging from 6.1 to 7.2. In several experiments, no L-growth was obtained at pH 7.5 and beyond. Below pH 6, the cell:L ratio gradually rises, but even at pH 5.2, 1 cell in 10 may give rise to an L-colony. Low agar concentrations are less efficient in supporting L-growth in the low pH range than they are at neutrality.

(5) Carbon and nitrogen sources:—0.03 M Xylose, 0.03 M fructose, and 0.06 M glycerol all served to replace glucose as carbon source in semisynthetic medium. Fructose 1,6-diphosphate and sodium succinate were also used, but gave higher cell:L ratios.

Among the nitrogen sources tested, only enzymatic casein hydrolyzate and vitamin-free acid-hydrolyzed casein supported L-growth. Even an amino acid mixture patterned after casein hydrolyzate and consisting of commercial DL- and L-amino acids gave negative results in several experiments, as did glutamate, glycine aspartic acid, asparagine, ammonium sulfate, and ammonium sulfate plus 0.2 per cent enzymatic casein hydrolyzate. Two and one per cent enzymatic casein hydrolyzate gave comparable cell:L ratios in most experiments, but lower concentrations proved less favorable.

At present, several interpretations for the superiority of hydrolyzed casein are still possible and experiments are in progress to determine the nature of this apparent nutritional difference between bacillary and L-growth of *E. coli*.

(6) Metal ion requirements:—Recently, experiments were performed to determine whether the requirement for 0.01 M $MgCl_2$ is specific or whether $Mg^{++}$ can be replaced by other divalent ions. It was found that $MnCl_2$ can replace $MgCl_2$ and that, in fact, $Mn^{++}$ is still completely effective at $10^{-3}$ M where $Mg^{++}$ no longer supports L-colony formation. $Ca^{++}$ showed slight activity only at the 0.001 M concentration level while $Co^{++}$ and $Fe^{++}$ were inactive.

(7) Strain differences:—A superficial survey of 20 coliform organisms—mostly *Salmonella* species —indicated considerable differences in L-forming propensity among these strains. Obviously, each strain must be investigated in detail to determine the conditions under which it can best form L-colonies.

TABLE 6

*Conversion of Escherichia coli cells and protoplasts into L-forms*

| Material Plated | Bacillary Colony Count | L-Colony Count | Cell:L Ratio |
|---|---|---|---|
| | $\times 10^{-7}$ | $\times 10^{-7}$ | |
| Original cells | 10.23 | 4.80 | 2.1 |
| Original cells, watershocked | 0.96 | 0.28 | 3.4 |
| Protoplasts | 5.01 | 4.46 | 1.1 |
| Protoplasts, watershocked | 0.0038 | 0.0018 | 2.1 |

Efficiency of cell → protoplast conversion:

$$\frac{5.0}{10.2} \approx 49\%$$

Efficiency of protoplast → L-colony development:

$$\frac{4.46}{5.01} \approx 89\%$$

Killing effect of 1:100 watershock on cells:

$$\frac{96}{1023} \text{ or } 94\% \text{ kill}$$

Killing effect of 1:100 watershock on protoplasts:

$$\frac{3.8}{5010} \text{ or } 99.92\% \text{ kill}$$

(8) Relation between Protoplasts and L-forms of *E. coli*:—If a population of young cells of *E. coli* is transferred to liquid semisynthetic medium containing 1000 units of penicillin per ml, protoplast formation is complete after 2 hr incubation. Protoplasts from such a suspension were diluted in 0.5 M sucrose semisynthetic medium and plated in penicillin-free and penicillin-containing semisynthetic agar. Another aliquot of protoplasts was watershocked and plated immediately in the two media.

In the experiment shown in table 6, half of the original cells survived to form protoplasts. Each intact protoplast, in turn, gave rise to a bacillary colony or an L-colony. If the protoplasts were watershocked, however, survival as either bacillary colony formers or L-colony formers was reduced by a factor of 1000. Evidently, the integrity of the protoplasts is as essential to L-colony formation as it is to bacillary survival.

## DISCUSSION

The experiments described in the preceding pages have demonstrated that every cell in strains of *P. mirabilis* and *E. coli* K 12 can give rise to an L-colony of the 3B type. The nutritional requirements of Proteus L-forms are extremely simple (Medill and O'Kane, 1954; Abrams, 1955) and appear to be the same as those of Proteus cells. *E. coli* L-forms are apparently more exacting than their parent form since they require magnesium or manganese and casein hydrolyzate. Whether these requirements can be considered as paralleling ordinary auxotrophy is doubtful, since they appear and disappear in every organism. It seems more plausible at present to attribute the need for these substances to a breakdown of the normal cellular transport or accumulation systems in the L-state. Similarly, the apparent requirement for a high concentration of penicillin for full L-form development in both Proteus and *E. coli* does not constitute evidence for a growth-factor-role for penicillin: it is well known that the L-state can be induced by many agents other than penicillin (Dienes and Weinberger, 1951) and that the metabolism and growth of a non-reverting L-strain of Proteus is completely unaffected by high penicillin concentrations (Kandler *et al.*, 1956*a*).

With the above exceptions of manganese, casein hydrolyzate, and penicillin, the special requirements for L-form growth in *E. coli* and Proteus are not nutritional in nature: in both organisms a high solute concentration is necessary for growth—a requirement which can be satisfied by succinate, sucrose, citrate, or KCl in Proteus, but only by sucrose in *E. coli*. Further, agar plays a crucial role in L-form development. This is evidenced by the partial (Proteus) or absolute (*E. coli*) superiority of the pour-plate method over the spread-plate method, the fact that gelatin will not replace agar, and by the striking response of L-form development to variations in agar concentration. Our presumption that the role of agar is physical rather than nutritional is supported by the finding of a synergistic interaction between solute and agar concentrations.

The concept of a generic relationship between protoplast and 3B L-body, which has been a major guideline in these experiments, has received so much support in our work and in recently published work of other experimenters (Liebermeister and Kellenberger, 1956; Bauman and Davis, 1957; Pease, 1957; Kandler and Zehender, 1957) that it may be considered as established. Further experimental work and semantic clarification are necessary, however, to circumscribe the nature of the relationship more precisely. Thus, it has been asserted that L-forms are nothing more than dividing protoplasts and that the recognition of this relation constitutes the essential solution of the "L-form problem." Alternatively, it may be argued that the protoplast stage is merely a transitional stage between cell and L-form. The answer to this question is partly terminological: in stabilizing media, the large bodies which make up the better part of the L-colonies are spherical in shape and to this extent are similar to protoplasts. On the other hand, L-spheres range in diameter from very small bodies to spheres of about 25 $\mu$ while a fresh protoplast population consists of very uniform spheres approximately 2 $\mu$ in size. Further, large bodies show a highly differentiated irregular internal structure as well as dispersed nuclear material (Liebermeister and Kellenberger, 1956) while unstained protoplasts show uniform optical density and presumably contain only as many nuclei as the parent cells from which they are derived. A substantial morphological distinction between large bodies and protoplasts can thus be made.

In the foregoing considerations, we have stressed the 3A-3B terminology. This emphasis stems from our belief that 3A L-forms represent a further departure from the intact cell, distinct from protoplasts and 3B L-forms. Our opinion is based in part on the filterability of 3A L-forms (Klieneberger-Nobel, 1956), on their characteristic microscopic morphology, and their altered chemical and enzymatic composition (Kandler *et al.*, 1956*b*, *c*), but most significantly on the observation that 3A L-forms often remain stable in the absence of penicillin and do not revert to the bacillary form (Tulasne, 1953, Kandler and Kandler, 1956). The interest of this phenomenon to the geneticist is heightened by the observation that the frequency of occurrence of the cell → 3A L-colony conversion (Kandler and Kandler, 1956; Altenbern, *unpublished observations*, 1957) seems to negate a mutational origin for the 3A L-form (In constrast to 3A L-forms, the diaminopimelic acid-less "C" type

L-colony forming strains of Kandler and Zehender (1957) and Bauman and Davis (1957) are clearly mutational in origin.)

Our current efforts are directed toward answering some of the questions implicit in the observed bacillus—3B-3A relationships.

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SUMMARY

Media and methods have been developed which allow the development of a 3B type L-colony from every cell in strains of *Proteus mirabilis* and *Escherichia coli* strain K12. One medium for Proteus consists entirely of mineral nutrients plus a carbon source and penicillin; the medium for *Escherichia coli* is also chemically defined, except for a requirement for a high concentration of vitamin-free acid-hydrolyzed casein. Success in the development of these media can be partly attributed to the working hypothesis that a protoplast stage is intermediate between bacillary form and L-colony: medium characteristics such as high solute and magnesium levels which are crucial to protoplast stabilization proved to be equally critical to L-colony development. In addition, penicillin concentration, agar concentration, and use of the pour-plate method were found to be of decisive importance to L-colony growth.

The relation between protoplasts and L-colonies was further supported in experiments which gave quantitative outgrowth of individual protoplasts into L-colonies.

The results obtained in this work are related to other recent advances in the study of protoplasts, 3B, and 3A L-forms.

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