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PROTOPLASTS AND L-TYPE GROWTH OF *ESCHERICHIA COLI*[1]

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A preceding article (Lederberg, 1956*a*) was devoted to the evolution of protoplasts from growing cells of *Escherichia coli* treated with penicillin. Further studies have strengthened the correspondence of these protoplasts with the "large bodies" and "L forms" described for many other bacteria. This paper will give an account of these studies, and an interpretation of others, in support of the hypothesis that L forms are outgrowths of protoplasts. Their cell walls may be impaired either by extrinsic inhibition, for example, with penicillin, or by intrinsic metabolic defects, consequences of genetic mutations.

The previous study was motivated mainly by the hope of furnishing protoplasts of genetically defined strains of *E. coli* for physiological and genetic analysis (Spiegelman, 1956; Spooner and Stocker, 1956). It was modeled on the experiments of Weibull (1953*a*) who forestalled the lysis of *Bacillus megaterium* exposed to lysozyme by maintaining the bacteria in a protective, hypertonic medium. It was found that the lysis of growing *E. coli* in the presence of penicillin could be forestalled in a medium containing M/3 sucrose and M/100 Mg^{++}; instead of lysing, the rods burgeoned out into osmotically fragile spheres, considered to be protoplasts.

Similar effects of penicillin on growing bacteria have been described extensively from an altogether different viewpoint as an aspect of the development of L forms. For various reasons, species other than *E. coli* have been preferred for such studies. In general, the wall defects have been recognized but not stressed as the essential feature of L forms, and their relationship to protoplasts obtained with lysozyme has been obliquely stated. The chief obstacle to a full coordination of protoplasts and the "large bodies" of the L form cycle has been the apparent inviability of lysozyme-produced protoplasts studied by one group of workers in contrast to the continued growth of L forms reported by others.

An adequate retrospect of the literature on L forms would be a herculean task. Fortunately, we may rely on a number of reviews for background documentation (Dienes and Weinberger, 1951; Liebermeister and Kellenberger, 1956; Klieneberger-Nobel, 1954; Tulasne, 1951; Kandler and Kandler, 1954). Studies on protoplasts and related aspects of bacterial morphology are included in a recent symposium (Spooner and Stocker, 1956).

As this paper is in part a restatement of previous knowledge, semantic questions loom large. *Protoplast* is borrowed from the botanical vocabulary where it serves to distinguish the living content of a plant cell from the lifeless cellulose wall. The walls of bacteria are not so well circumscribed, either chemically or morphologically, at the present time, and less direct measures help to define the protoplast. For the present, our operational criteria for the absence of a wall are (1) osmotic fragility and (2) loss of rigidity resulting in spherical or amoeboid form. To be sure, these criteria fail to distinguish between the total absence of a wall and its functional impairment. *L form* is a generic term which stems from the cultures labeled L_1, L_2, etc. (in honor of the Lister Institute) which Klieneberger had isolated from *Streptobacillus moniliformis*. These isolates were so bizarre in their morphology that their derivation from the bacteria could scarcely be credited, and Klieneberger felt instead that they were a symbiont. However, Dienes subsequently showed the direct, reversible conversion of bacillary into L type growths. Historically, L form refers to one of a specific series of strains. In this paper, however, "L" will be used more broadly to describe atypical growths resembling

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figure 3, in contrast to "B" for typical bacilliform. The time is perhaps nearly ripe for a notation that better reflects our concepts of these structures.

It will be convenient to distinguish the several ways in which protoplasts and L growth can be produced. Those achieved by the immediate presence of penicillin will be labeled with the prefix pc-; those obtained by withholding diaminopimelic acid (DAP) will be called dap-. "Stabilized" or "fixed" will refer to L forms which have an intrinsic hereditary defect, and therefore display this growth pattern on conventional media; they correspond to L forms in the strict sense advocated by Klieneberger-Nobel.

As this is an extensive rather than intensive report, we have appended much of the discussion to the findings as they are presented. For the same reason, we will stress, perhaps unduly, the hypothetical status of many inferences. Without question, many of the attendant details warrant thorough exploration as individual problems in their own right.

MATERIAL AND METHODS

Media. The media used were evolved by trial and error, starting from the formulations of pennassay broth and nutrient broth (Difco). The following medium containing 0.3 M sucrose, M/125 Mg^{++}, M/600 penicillin, plus a nutrient base, was found to be effective in the growth of L colonies of *E. coli*.

Sucrose broth (per L): casein digest (Sheffield Chem. Div., Norwich, N. Y.) 10 g; sucrose, 100 g; meat extract (Lemco or Difco), 10 g; NaCl, 3.5 g; glucose, 1 g; agar, if indicated, 10 g. After autoclaving, 10 ml of 20 per cent $MgSO_4 \cdot 7H_2O$ was added. If penicillin was indicated, we added 1000 u per ml unless otherwise stated.

"Bacterial hydrolyzate" was prepared as follows: 3 g of *E. coli* were suspended in 30 ml 2 N sulfuric acid, and autoclaved in a screwcap vial for 35 min; we then centrifuged, discarded the sediment, neutralized the supernatant with 10 N NaOH, and sterilized this by reautoclaving. Whenever indicated, the hydrolyzate was used at 1:10 dilution, giving a final concentration (in terms of the original cells) of about 10 mg per ml. The hydrolyzate gave an assay with a DAP auxotroph (see below) corresponding to 1 per cent of the dry weight of the original cells as DAP. Since possible stimulation by other factors has not been studied, the assay cannot be considered quantitatively reliable in absolute terms. However, as 10 μg of DAP will permit the growth of about 1 mg of bacteria, the correspondence is

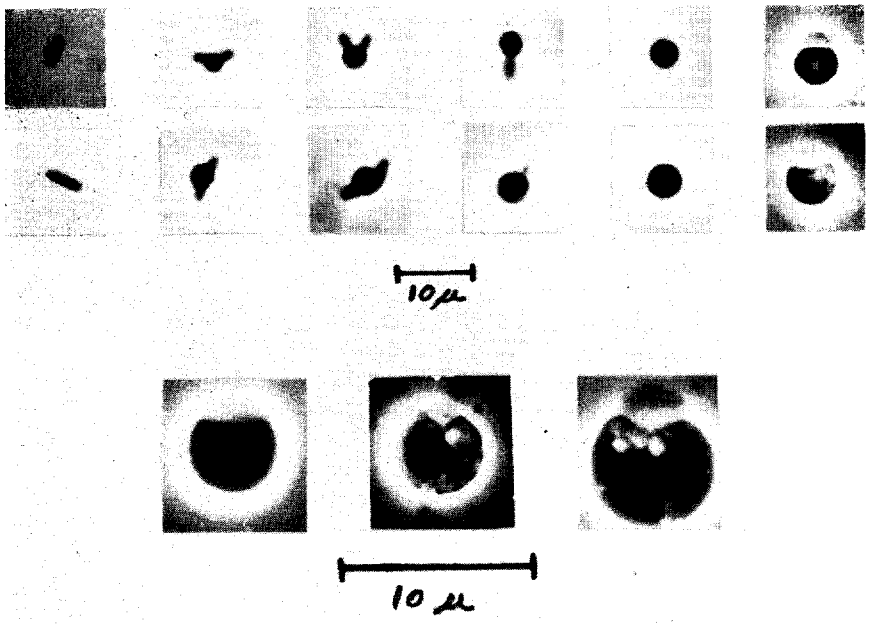

Figure 1. Escherichia coli Y-10 in penicillin sucrose broth. (*Above*) Various cells in successive stages. 0 to 4 hr. (*Below*) Late stage at higher magnification. Phase contrast.

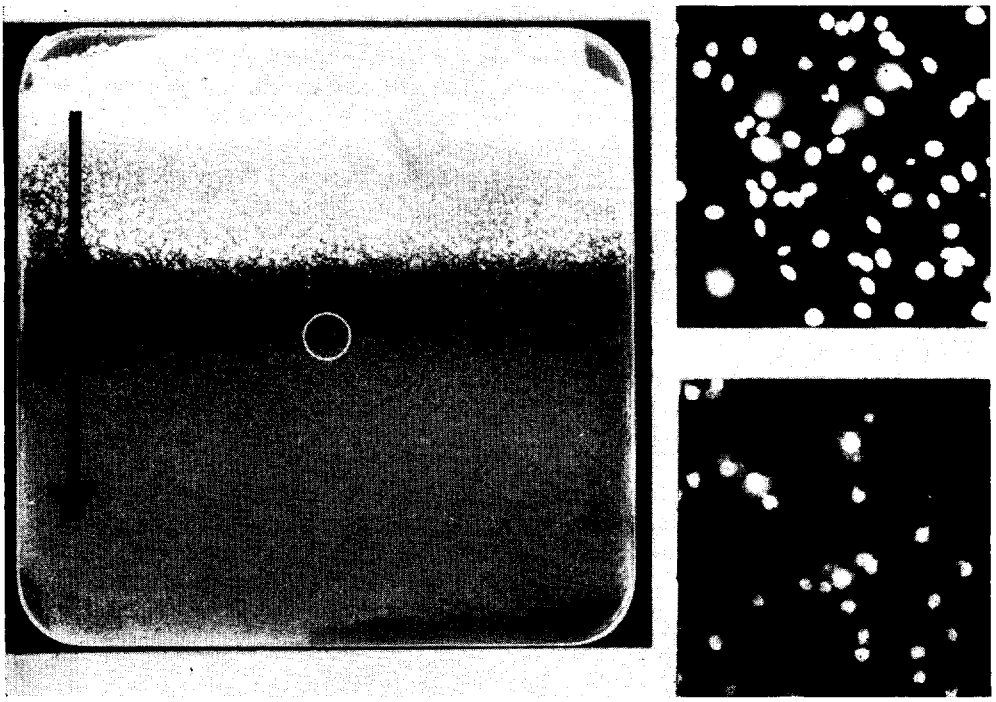

Figure 2. B and L colonies. (*Left*) A gradient plate of sucrose agar containing 0 to 700 units per ml of penicillin was seeded with *Escherichia coli* Y-10 and incubated 2 days. Note zones of B growth (above) and L growth (below) with intermediate zone of no growth. This print was obtained by placing the petri dish directly in an enlarger. (*Right*) B and L colonies at 48 hr, about 10×; dissecting microscope, oblique illumination.

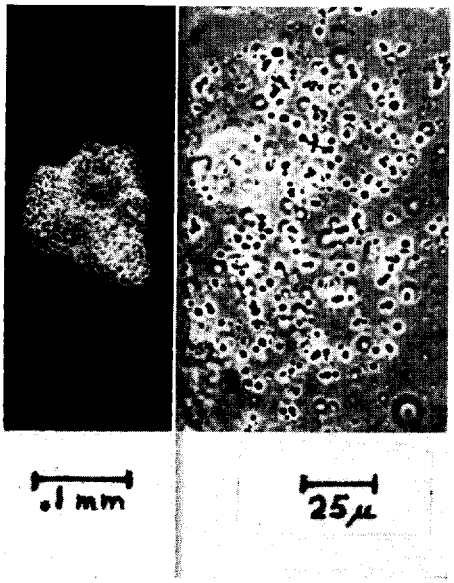

Figure 3. (*Left*) Young (20 hr) L colony, about 0.1 mm in diameter. Originally photographed at 100× phase contrast. (*Right*) Squash of L colony, phase contrast.

reasonable. The amount of hydrolyzate used as a supplement is a calculated tenfold excess.

After preliminary trials with other strains, most of the work reported here involved *E. coli* strain K-12 and a number of mutant substrains, such as W-6, Y-10, W-1895, and others, for the most part cited in earlier papers (Lederberg *et al.*, 1952). Strain K-12 and its derivatives will be considered collectively as *E. coli* line 1. Stock cultures were maintained on nutrient agar slants, or in stabs. Routine inocula were grown overnight in penassay broth and cultures aerated by holding the tubes on a rotator. All cultures were incubated at 37 C.

EXPERIMENTS AND CONCLUSIONS

Formation of protoplasts in penicillin sucrose broth. In the absence of sucrose or other stabilizer, cells of *E. coli* lysed after an initial swelling. However, as previously described, *E. coli* cells grown in sucrose-penicillin broth are converted one for one into protoplasts via the stages of figure 1. These protoplasts also lysed rapidly in

water, and more slowly in broth. In similar experiments, M/5 glucose and M/2000 clinical dextran (kindly furnished by Baxter Laboratories, Inc., Morton Grove, Illinois) had incomplete protective effects comparable to M/10 sucrose. Likewise, Weibull (1953*a*, *b*) found that M/50 carbowax protected the protoplasts of *Bacillus megaterium*. These findings suggest a more complex function for these solutes than simple osmolality, but the disruption by dilution in water will be referred to, for the present, as osmotic fragility.

Mg^{++}, replaceable by Ca^{++}, is essential for stabilization, perhaps by virtue of a reaction with lipid residues of the plasma membrane (Weibull, 1956). Citrate and Versene bind these cations and thus accentuate the requirement; the presence of citrate in some formulations of penicillin, and in the composition of some minimal media, should be kept in mind. The paradox that Versene is an essential part of one recipe for releasing protoplasts from *E. coli* (Repaske, 1956) has yet to be resolved.

It is well known that lysis by penicillin requires active growth of the susceptible bacteria, a principle that has a useful application in methods for the selective isolation of growth factor-dependent mutants (Davis, 1948; Lederberg and Zinder, 1948). Correspondingly, the evolution of protoplasts also requires growth, and will not occur in non-nutrient media, at low temperatures, or in the presence of inhibitory concentrations of streptomycin, chlortetracycline or chloramphenicol. For this reason penicillin is believed to act by inhibiting new wall synthesis, in contrast to the dissolution of the existing wall by lysozyme. The changes of figure 1 are therefore interpreted as the protrusion of an expanding protoplast against a progressively attenuated wall which finally collapses and releases the free protoplast. Owing to intercurrent growth, the penicillin protoplasts are perforce larger than those released by lysozyme, and even more so than the several protoplasts released from a single multiseptate rod of a *Bacillus* spp.

Some fifty and odd penicillin-sensitive strains of *E. coli*, of various serotypes, have been treated in penicillin sucrose broth with similar results. Protoplast formation has also been secured in a defined medium (Gray and Tatum, 1944) supplemented with sucrose, Mg^{++}, and penicillin, but was slower and incomplete, presumably because of the lower rate of protoplasmic increase in this medium as compared to broth.

The continued growth of protoplasts suspended in penicillin sucrose broth is reflected by increases in optical density and induced β-D-galactosidase as well as in the size of the individual protoplasts. The biosynthetic activity of these protoplasts is being more intensively studied in other laboratories (Spiegelman, 1957) where they have been found to be on a par with the intact bacteria. However, the increase in total mass is not matched by an increase in numbers, nor can any convincing division figures be seen under the microscope. On continued incubation, the protoplasts become very large and highly vacuolate, and they eventually lyse. No better evidence of proliferation was obtained with variations of sucrose broth medium, or by the addition of bovine or equine serum or of other proteins.

Freshly prepared protoplasts give a viable count, consisting of B colonies exclusively, amounting to 10 to 50 per cent of the input cells if diluted in sucrose broth (without penicillin) and plated in sucrose agar. This corresponds to the reversion of protoplasts to normal rods which takes place over the course of several hours in sucrose broth as has already been described (Lederberg, 1956*a*). The spheres develop protuberances, one or more of which elongate into filaments and segment terminally to give typical rods. These changes are taken to represent the resumption of wall-building when the inhibitor is removed. If the protoplasts are diluted in water instead of a protective medium, the viable count drops by a thousandfold or more. The residual viability may be accounted for by dormant "persisters" (Bigger, 1944). Resistant mutants might also be expected among the survivors, but were not found. Likewise, the B reversions from protoplasts maintained in sucrose broth and plated on sucrose agar have behaved like their parent B cultures in their formation of osmotically fragile protoplasts in response to penicillin. Mutants of *E. coli* line 1 resistant to 1000 u per ml of penicillin were never found in a single step in other experiments involving intense selection, and they are evidently much rarer than the phenotypically deviant persisters.

Many features of protoplast structure are still obscure. As the protoplasts enlarge, a lune-shaped vacuole appears at one side (figure 1). Older protoplasts often display a narrow crescent of

phase-dense material to the side of a large, nearly spherical, clear vacuole. The vacuole is bounded by a thin membrane, which presumably invests the entire protoplast, and may correspond to the wrinkled ghost which is seen after lysis. Nothing is known of the contents of this seeming vacuole.

India ink preparations show an additional envelope, a transparent capsule, even with strains (like *E. coli* line 1) which show no capsule in the B form. Since protoplasts of various serotypes are still agglutinable by homologous anti-O and anti-K serums, the capsule may well represent some disorganized elements of the cell wall. Further immunochemical studies may help to settle this point. When protoplasts are lysed in water, they appear to have dissolved altogether except for the residual ghost and some granular debris. In India ink, however, the lysed protoplast appears as an enlarged clear space in which the ghost is embedded.

Protoplasts are nonmotile, even when prepared from actively motile bacteria. However, they are still extensively flagellated when stained by Leifson's method (1951). This experience corresponds precisely to Weibull's (1953, *a*, *b*). As yet, we cannot say whether this paralysis represents a lesion of the flagella or reflects a role of the rigid wall in motility.

Preparations stained with Giemsa after HCl hydrolysis have exhibited a scattering of peripheral nuclear bodies as other workers have described for "large bodies" and L forms. Attempts to release discrete nuclei by controlled lysis with water or with lipase (Spiegelman, 1956) were unsuccessful.

Growth of L colonies of E. coli in penicillin sucrose agar. Many authors have stressed the importance of the physical texture of the medium in supporting the growth of L forms. In preliminary trials, the recipes given by Dienes (1949) were effective for *Proteus* strain 52 (kindly furnished by him). Good yields were also obtained in nutrient agar (Difco) with sucrose plus penicillin. It was necessary, as recommended, to limit the agar concentration to not more than 0.8 per cent. But comparable experiments with *E. coli* line 1 were much less successful at first. A long series of trial and error experiments finally uncovered the following prescriptions.

(1) Strain specificity:—Different lines and substrains within *E. coli* line 1 vary in their yield of L colonies, some cultures being completely unproductive in every medium tried. *E. coli* strain K-12 was only moderately productive, and was not readily maintained in serial passage of L colonies. Productivity for L colonies was uncorrelated with any other recognized genetic marker, isolated clones from the same stock culture sometimes showing wide variations, either in over-all yield, or in the conditions for optimal yield. Strain Y-10 of line 1 was one of the most productive, and has been used routinely in further experiments. It should be stressed that all strains produced protoplasts in penicillin sucrose broth, regardless of whether they went on to L type growth in agar.

(2) Agar concentration:—Unlike Proteus 52, *E. coli* required at least 0.8 per cent agar, the threshold varying with the gelling power of the lot. The most uniform results were obtained with 1 per cent Difco agar, which has been routinely adopted. Neither gelatin nor methocel replaced agar as a satisfactory gelling agent.

(3) Submerged versus surface growth:—We have not succeeded in securing L colonies of *E. coli* on agar surfaces; all observations in this paper are from pour platings. Agar shake cultures show no special aerobic requirements for L colonies; the largest colonies usually developed in a zone beginning just beneath the surface.

(4) Meat extract:—Stimulated the L growth of some strains. Yeast extract was, if anything, inhibitory.

(5) Mg^{++}:—Required just as for the stabilization of protoplasts in broth.

(6) Sucrose:—Indispensable for L colony formation in nutrient agar, but a small number of L colonies of *E. coli* developed in casein digest-meat extract agar without sucrose. So far as tested, these did not consist of mutants that would tolerate penicillin in the absence of sucrose. With Proteus, however, sucrose was dispensable in agar media (but improved the yield of protoplasts in broth).

(7) pH:—The range from pH 5.5 to 7.6 was imposed by the addition of M/10 phosphate buffer. The optimum was found at about pH 6.3 which approximates the pH of the unbuffered medium. Phosphate buffer has therefore been omitted from the recipe to minimize precipitation of magnesium phosphates.

(8) Growth factors:—In a defined medium (Gray and Tatum, 1944) supplemented with sucrose, penicillin and Mg^{++}, L growth was

perceptible but very sparse, each colony consisting of perhaps a hundred elements after 48 hr. This growth was accentuated by the addition of amino acid mixtures, but no single supplement was uniquely effective. A mixture of B vitamins, including riboflavin, did not stimulate L growth, contrary to the report of Tulasne *et al.* (1955) for Proteus.

(9) Penicillin:—One of the most critical factors for L growth (but not for protoplast formation) was found to be the concentration of penicillin. Although 100 u per ml suffices for the latter, most strains require 1000 u for high yields of L colonies, and may be stimulated even further by 10,000 u. A gradient plate thus shows three zones: B colonies at penicillin levels below about 50 u, a zone with virtually no growth, and L colonies at levels over 200 u (figure 2). The zone effect will be of some importance in later discussion.

Taking these considerations together, we have adopted the following regime, which gives yields of 10 to 50 per cent of the input cells as L colonies. Strain Y-10 is grown overnight in pennassay broth. The culture is diluted in broth, then mixed with molten penicillin sucrose agar for pour plates about 5 mm deep. (Small petri dishes, 6 cm in diameter, are convenient for many operations.) The plates are allowed to solidify and are incubated. L colonies grow more slowly than B, but can usually be counted after 24 hr, and may reach 1 to 2 mm diameter by 48 hr (figure 2). When viewed under a binocular microscope they can usually be distinguished by their translucent texture; they are also less compact than lens-shaped B colonies, and may show several leaf-like outgrowths. A plate is conveniently surveyed under darkfield illumination at 100 magnifications, at which the individual protoplasts can be made out; for certainty, the colony should be squashed out and examined under phase contrast at higher power. The unmistakable appearance of such squashes is shown in figure 3.

The evolution of L colonies from single bacteria has been followed in agar block preparations. A drop of seeded agar was spread into a thin film on a cover glass, then covered with mineral oil to prevent drying out. With a razor blade, slices were made at right angles to give rectangular blocks with a diameter of about 0.2 mm. Individual blocks were then spaced out in a regular pattern under the mineral oil. The cover glass was then inverted over an oil chamber. Since the individual blocks are readily centered under high power lenses, and can be re-located in the pattern, this furnished an efficient method of following several specimens in parallel for serial photography.

The formation of pc-protoplasts and their reversion to rods has been followed conveniently in microdroplets of broth preparations (De Fonbrune, 1949; Lederberg, 1954); but L growth did not develop in the liquid medium. Attempts to prepare microdroplets of agar were unsatisfactory, owing to the rapid congealing of the agar in the transfer pipette.

The development of L colonies from single rods is shown in figure 4. The initial stages are similar to the evolution of protoplasts in broth; however, the cell does not become perfectly spherical. Instead of generating a growing sphere, it forms a number of protuberances, some quite blunt, others very thin and difficult to photograph. These enlarge, and after a time, pinch off to give a "daughter protoplast." It has not been possible to follow the continuation of this cycle for very long by means of photomicrographs as the three dimensional aggregate soon becomes too confusing. While L colonies *in situ* contain more or less irregular elements, presumably due to constraint by the agar milieu, in squashes these are more uniformly spherical.

No L colonies have been found at the surface of the agar, either after surface inoculation or from pour platings. However, an L colony which starts beneath at a lower plane may break through the surface, resulting in a viscous drop or "colony" containing mostly debris and large ghosts. The appearance suggests that the L colony is under considerable pressure, and that its contents may swell, burst and spill out when it reaches a free surface.

The subculture of L colonies, especially for quantitative counts, therefore presents some technical difficulties familiar to other practitioners. It is expedient to cut out a block of agar, transfer it to a small shell vial with one or two volumes of sucrose broth, and to macerate the block with a short run of a VirTis Micro-Homogenizer. The mince can then be taken up in a pipette for dilution and plating. This treatment disperses most of the protoplasts into free suspension; some masses remain embedded in agar lumps.

The wide range of size of granules and spheres,

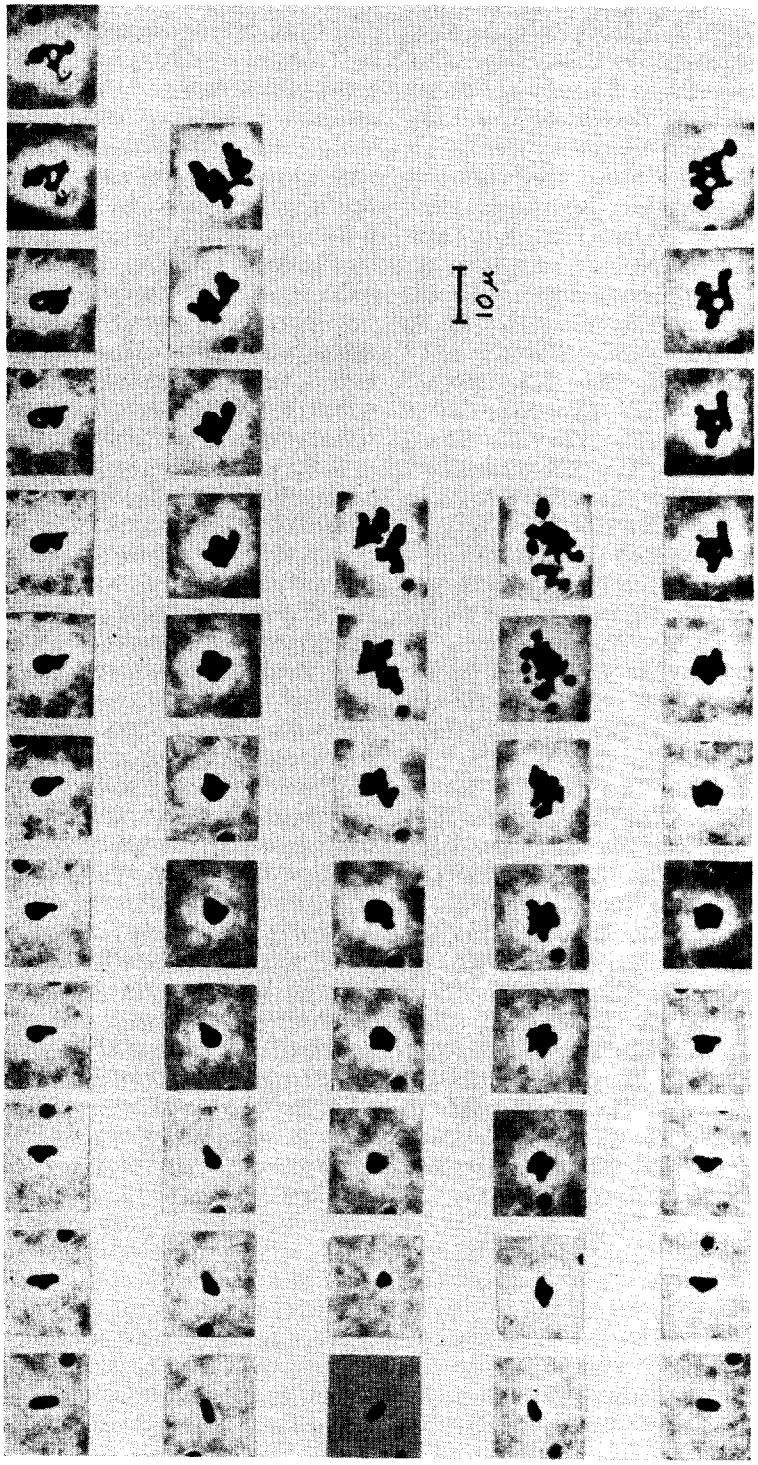

Figure 4. Evolution of L colonies from single bacteria in penicillin sucrose agar. Elapsed time up to 4 hr. Phase contrast

and the presence of agar fragments interfere with a total count. Single L colonies containing an estimated 10^3 to 10^4 spherical elements yielded from 10 to 50 L progeny when minced and replated. This low viability might be inherent in the elements of the colony, or might be ascribed to damage by release of external pressure or to injury from the maceration.

Several strains of *E. coli* have been subcultured by this method in the L phase for as many as 20 passages before the experiments were terminated. The dilution at each passage was about one tenth, so that the cumulative factor of increase was 10^{20}, leaving little doubt that the L colonies could have been propagated indefinitely in the presence of penicillin. Samples were also plated into sucrose agar, where they reverted to give approximately the same yield of B colonies as of L colonies in penicillin sucrose agar. These reversion cultures were indistinguishable from the original Y-10 in their growth habit, their sensitivity to penicillin, and their productivity of pc-protoplasts and L colonies.

Contrary to experience with other bacteria, the prolonged cultivation of *E. coli* in the L phase in the presence of penicillin gave no stabilized L forms.

A number of strains, e. g., K-12 and W-6, gave relatively low yields (10^{-3} or less) of L colonies. Reversion cultures were made of the obtainable L growth in the expectation of accumulating mutants better adapted to form L colonies in the adopted medium. This expectation was not fulfilled; the L colonies that do develop in these circumstances must be put down to phenotypic accidents.

Like pc-protoplasts, the L growth is osmotically fragile. Minces diluted in water gave less than 1 per cent of the viable count (B in sucrose agar, L in penicillin sucrose agar) shown by aliquots diluted in sucrose broth. Part of the residual survivorship may be ascribed to protected elements embedded in bits of agar.

These observations may be rationalized into the following conception of L growth as colonies of protoplasts. In broth, the uniformity of external pressure, the elasticity of the remaining envelope, and interfacial tension all tend to conserve the spherical shape of the protoplast. Without the normal division mechanism, which depends on the wall, the protoplast remains spherical as it grows. In agar the shape of the protoplast is imposed by local stresses in the medium, the network of agar fibrils taking the place of the missing rigid wall. At local points of weakness the growing protoplast herniates into an adjacent free space, expands, and pinches off a bleb. Many of the blebs may be expected to lack a full complement of vital internal structures, which might account in part for the low viability.

Diaminopimelic acid (DAP). The L growth so far discussed is an effect of external inhibition of wall formation. When the cells were placed in a sustaining medium without penicillin, they recovered their normal capacities and regenerated typical walls. However, many workers have reported on the occurrence of genetically stabilized L forms which remained defective in the same environment that sustained normal growth of the parental strains. Two questions concerning these mutants arise: (1) the biochemistry of the defect, and (2) the manner in which the L form arose and survived in place of the parental B form.

DAP is an amino acid originally discovered in hydrolyzates of *Corynebacterium diphtheriae* and subsequently identified as a component of the cell walls of numerous bacteria (Work, 1957; Cummins, 1956). Apart from the differential occurrence of the *meso-* and the LL-stereoisomers, DAP has been found in the walls of all bacteria examined except for gram-positive cocci and lactobacilli, and including actinomycetes and myxophyceae, but in no other biological source (except Chlorella). The preferential localization of DAP in the cell walls of the bacterial species in which it occurs suggests that it has no other structural significance, and the small amounts found in nonwall fractions may represent its role as a metabolic intermediate in wall formation and in the biosynthesis of lysine (Davis, 1952).

Davis has isolated an auxotrophic mutant of *E. coli* strain W that required DAP and lysine. Subsequently, Bauman and Davis (1957) and Meadow *et al.* (1957) observed that cultures grown on limited DAP underwent lysis when the DAP supply was exhausted. (We are indebted to Dr. Davis for initial samples of DAP, for a culture of his DAP-dependent mutant, strain 173–25, and for preliminary information on the osmotic fragility of DAP-starved cells.) DAP is now produced on a commercial scale as a metabolite accumulated by another mutant (Casida, 1956) and a generous sample has kindly been furnished by Chas. Pfizer and Co.

Strain 173-25 was grown in broth supplemented with bacterial hydrolyzate or with 10 μg per ml of DAP. The culture was then washed and inoculated into sucrose broth, which lacks DAP (having no constituents of bacterial origin). In contrast to the lysis observed in ordinary broth, these cells formed protoplasts by stages similar to figure 1. The same conversion was noted in minimal medium, supplemented with sucrose, Mg^{++}, and lysine, but was less complete. As a corollary, the sucrose medium gave a higher turbidity and more stable assay curve for DAP than the customary minimal medium without sucrose.

Strain 173-25 was also plated into sucrose agar, where it grew extensively and exclusively in the form of L colonies similar to, though somewhat smaller than, those shown in figure 3. Except for occasional (sometimes troublesome) reverse-mutants, no colonies at all were formed in the absence of sucrose. The dap-L colonies grew on serial passage in sucrose agar in the same fashion as already described for pc-L forms.

When DAP was restored, the passage strains promptly resumed B growth. Since DAP is absent from conventional bacteriological media, the DAP-dependent mutant would have been described as a fixed L form, had it been isolated prior to these studies.

DAP is not the only target whose impairment would block wall formation. Neither DAP nor hydrolyzate was found to reverse inhibition by penicillin, which may therefore antagonize some metabolite missing from the hydrolyzate, or interfere directly with a wall-building enzyme. Further, several stable L forms of Proteus were received from Dienes (strain Tulasne) and from Kandler (strains 6e, 6f, 5h, and 8y). The strains had been adapted to growth in liquid medium, and they grew quite well as L forms in the casein digest-meat extract agar without sucrose or serum. They showed no response to hydrolyzate or DAP, and the site of their block, like that of penicillin, remains unspecified. In addition, there was no effect when boiled Proteus cells were also added to furnish a possible primer of wall formation.

Selection of wall-defect mutants. The process of stabilization of L growths in the presence of penicillin has not been systematically studied, at least not to allow the discrimination of various genetic hypotheses. At least four come to mind: (1) that wall-synthesis is subject to spontaneous mutations which may block any of a number of steps and that such mutants have a selective advantage in the circumstances where they have been isolated; (2) that wall formation depends on a self-reproducing cytoplasmic particle whose reproduction is impaired by penicillin (Sharp *et al.*, 1957), by analogy with the effects of acriflavine on yeast (Ephrussi, 1953); (3) that wall-synthesis is itself a self-dependent process, with extension of the wall depending on the integrity of the pre-existing structure by analogy with the role of polysaccharides as primers for their own synthesis; (4) that penicillin has a specific mutagenic effect, so that the same compound produces both phenotypic and genotypic impairments of wall formation (Briggs *et al.*, 1957). These hypotheses are not mutally exclusive; however in the present material the last three are discouraged by the long-continued propagation of *E. coli* line 1 as pc-L growth without becoming a stabilized L form. Hypothesis (1) has been partially justified by the behavior of the DAP auxotroph, strain 173-25, which is an example of a wall-defect mutant. However, the circumstances of our experiments with penicillin evidently did not favor selective overgrowth of wall-less mutants, contrary to the experience of other workers. Under what conditions, then, would a wall-less mutant have an advantage, as would be necessary to complete the justification of the hypothesis (1)?

This question may have several answers; at least one clue comes from the zone effects of penicillin mentioned earlier (figure 2). In the presence of "zonal" levels, i. e., 75 to 125 u per ml, L growth of DAP-independent *E. coli* is quite sparse. On the other hand, in reconstruction experiments, the DAP-dependent strain 173-25 formed L colonies abundantly, regardless of penicillin concentration. But when DAP was added, L colony formation again failed in the intermediate zone. The component of pharmaceutical penicillin that is required at higher concentrations is believed to be the antibiotic itself, since the zone effect was also given by a highly purified sample (kindly furnished by Dr. J. Lein, Bristol Laboratories, Inc.) and was unaltered by the addition of an excess of heat-inactivated penicillin. We propose that incomplete inhibition accounts in some manner for the zone of poor L growth, a condition that can be relieved

either by the addition of more penicillin or by the superimposition of a genetic block. If this reasoning is correct, we should tend to find wall-defect mutants among those few L colonies which do form in the intermediate zone of penicillin.

A number of experimental designs to test this expectation have been tried, none extensively enough to be empirically preferred. The following procedure takes account of some general principles for the isolation of auxotrophic mutants which have been detailed elsewhere (Lederberg, 1950). Cells of Y-10 were harvested from penassay broth and resuspended in water, then irradiated with ultraviolet to a survivorship of 10^{-3} to 10^{-2}. The irradiated cells were then plated densely into penicillin sucrose agar and incubated for 24 hr. (This corresponds to an interval of intermediate cultivation during which the mutants can come to phenotypic expression in a neutral or perhaps advantageous environment.) A block of agar containing numerous L colonies was then minced and plated at various dilutions into sucrose agar containing zonal penicillin. The yield of L colonies was 10^{-3} or less than that obtained in the presence of optimal penicillin. These plates were incubated for 1 week to 10 days, during which time much of the penicillin has been inactivated, in part by the constitutive intracellular penicillinase, which is characteristic of *E. coli*. As a result, many non-mutant colonies revert to B form, and if recognized need not be picked in further tests. Suspicious colonies (figure 3) were replated in sucrose agar. If L colonies appeared on replatings, they were purified by one or two additional platings, and then tested for growth in hydrolyzate broth and DAP broth. To date, 197 colonies have been replated from sucrose agar with zonal penicillin to sucrose agar, and 4 have proved to be L forms stable enough for further characterization. All these have proven to be DAP auxotrophs, but a search for other classes of mutants is being extended with this and variant methods. Unless the compounds indicated have other functions important in over-all growth, mutants requiring muramic acid, hexosamines, D-amino acids and their conjugates with uridine-diphosphoglucose, and other metabolites are among those to be anticipated (Park and Strominger, 1957; Work, 1957). The DAP auxotrophs thus isolated from line 1 have resembled strain 173-25. One isolate may represent an incomplete block, as it grows substantially normally in broth and minimal liquid medium. However, it forms mainly protoplasts and intermediate forms in sucrose broth and L colonies in sucrose agar. As has already been suggested, hypertonic sucrose may play an active role in the herniation of the protoplasts, as well as in helping to maintain them (Zinder and Arndt, 1956). The addition of DAP restored normal morphology in broth or agar, with or without sucrose. The other strains required DAP for growth in media without sucrose.

Selection by phage and colicine. Since the cell walls play an important part in the adsorption and penetration of phages and colicines, these might also serve as selective agents for the isolation of wall-defective mutants. Pc-L colonies of *E. coli* line 1 were found to be relatively resistant to phages T1, T4, T5, and λ-2, but still formed plaques when seeded with T3 and T7, and faint plaques with T6, which speaks for differences among the receptors for the various phages. A number of colicines including Fredericq's (1948) type series was also tested for differential effect; most of them inhibited L growth to some degree, though usually less markedly than the corresponding B growth. Phages T1 and T5, and colicines E, K, and V have been tested further as aids in the selection of new mutants, analogous to the use of zonal penicillin. This approach has not been equally fruitful so far, although reconstruction experiments speak for its validity. The main difficulty has been interference background of transient L colonies which probably result from the wall-dissolving action of the phage itself (Zinder and Arndt, 1956). (The delayed lysis with concurrent L growth of phage-infected cells in sucrose agar warrants study in its own right.) None of the colicines tried has been completely innocuous to L colonies, and they, therefore, do not give perfect discrimination under the conditions used.

The only relevant mutant isolated with the help of phage was resistant to phage T1, grew nearly normally in broth and in minimal medium, but produced an abundance of protoplasts in sucrose broth. Unlike a partial DAP-auxotroph mentioned previously, this mutant (W3288) was unaffected in its growth pattern and its resistance to T1 by bacterial hydrolyzate.

Various chemicals have been used for the

selective isolation of pleuropneumonialike organisms (Morton and Lecce, 1953); Tulasne and Lavillaureix (1954) further state that thallium acetate differentially inhibits B versus L growth. In exploratory trials, we could find no differential resistance of L growth to graded concentrations of thallium acetate, sodium selenite, sodium tellurite, uranium nitrate, or crystal violet. These compounds were added in penicillin sucrose agar seeded with cells or protoplasts of *E. coli* Y-10.

The scarcity of L form mutants procured in *E. coli* contrasts with the reported regularity of their occurrence in other material treated with penicillin (e. g., Sharp *et al.*, 1957). We are unable to judge the relative importance of differences in technical details and in material, or whether other mechanisms (hypotheses (2), (3), and (4)) may be involved. However, wall-defect mutants may have a selective advantage in other environments besides zonal penicillin, phage, or colicine or in a genotype less well adapted to pc-L growth than that of Y-10. Further, most workers have used serum in their recipes, and low-titer antibodies against residual wall components might then play some part in the selection of spontaneous mutations. Even before a rationale for selection is perfected, its intensity can be estimated by means of reconstruction experiments.

Other observations. The unique role of DAP suggested that antagonistic analogues of this amino acid might have a therapeutic usefulness comparable to that of penicillin. A few compounds were tested briefly, against strain K-12 and against strain 173-25 with limiting DAP, but none was inhibitory. They included: D-glutamic acid; DL-α-aminopimelic acid, DL-α-aminobutyric acid (purchased from California Biochemical Foundation) and a sulfonic analogue of DAP, 1,5-diamino-1,5-pentanedisulfonic acid (kindly furnished by Dr. J. Lein, Bristol Laboratories, Syracuse, N. Y.).

The principal incentive for the study of pc-protoplasts in this laboratory was the hope that they might prove to be competent recipients for the transduction of genetically active deoxyribonucleic acid (DNA). This hope has so far not been realized in experiments with *E. coli*. The markers used in these experiments were Lac, M and S, for example $M^-S^r \times M^+S^s$, with selection for M^+S^r by platings on minimal medium supplemented with streptomycin. As a rule, the treated recipient protoplasts were allowed to revert to B forms before being plated, the penicillin being removed either by the addition of penicillinase, or by washing the suspension with fresh broth. Among the variables systematically tested were: DNA as crude lysates of donor protoplasts, or partially purified after detergent extraction of whole cells; substitution of Ca^{++} for Mg^{++}; addition of serum albumin; partial osmotic shock in the presence of DNA by dilution of protoplasts in water followed by reconstitution with concentrated sucrose. In addition, a large variety of strains was employed for recipients, including some 125 O-serotypes (kindly furnished by Dr. F. Ørskov). In some trials the recipients were treated as L colonies in penicillin agar.

Attempts to detect the fusion of protoplasts of sexually incompatible (F^-) genotypes were equally unrewarding. The design of the experiments was similar to that for DNA-transduction, mixtures of protoplasts being evoked and grown together in penicillin agar or broth. We also tried graded osmotic shocks, and spinning a mixed protoplast suspension in 10 per cent sucrose in an air turbine centrifuge at 80,000 × G for 20 min. Whereas the pellet showed evidence of considerable lysis, there was no indication of fusion of protoplasts either from microscopy or tests for recombinants. However, compatible strains ($F^+ \times F^-$) could recombine if either or both of the parents was introduced as pc-protoplasts. For example, protoplasts of Hfr_1M^- were mixed with protoplasts of $F^-Lac_1^-S^r$ in penicillin sucrose broth, and incubated for 1 hr. They were then agitated to break up residual complexes, diluted, and plated on EMB lactose streptomycin agar, supplemented with sucrose and Mg^{++}. On this medium, the Hfr parent is suppressed, and Lac^+S^r recombinants are distinguishable from the $F^-Lac^-S^r$ parent. In protoplast × protoplast tests, the ratio of recombinants to F^- parent varied from less than 1 to 5 per cent, compared to 5 per cent for the rod × rod controls. In view of the effect of streptomycin even on S^r pc-protoplasts (see below) this experimental design is not ideal, and further studies should be based on fuller knowledge of the reversion of protoplasts on various selective media. The mating of $HfrM^-S^s$ protoplasts × $F^-Lac^-S^r$ rods was consistently

fertile (perhaps because this difficulty is not in question), and a conjugal pair of these was readily observed as a rod with attached protoplast.

Hfr and F^- genotypes retained their characteristic compatibility behavior when reversions were tested after several passages as pc-L colonies. The wall defect imposed by penicillin thus has no influence on either the genetic continuity or phenotypic manifestation of this presumably surface-related property.

E. coli 204 is a pleiomorphic strain, briefly described by Klieneberger-Nobel (1949). The division mechanism of this strain seems to be intermediate between fission and budding, the units in a growing suspension consisting of deformed spheres with large protuberances. The culture is resistant to penicillin; hydrolyzate had no effect on its morphology. Its wall defect does not, however, extend to osmotic fragility, dilution from sucrose broth into water having had no effect on viability. No sign of the fragmentation mentioned by Klieneberger-Nobel was observed in living broth cultures seen under phase contrast, although many cells assumed irregular shapes and comprised an unusual range of sizes. It was undecided whether the discrepancy is due to differences of observational or cultural technique or to changes that may have occurred in the behavior of the strain.

In the course of other experiments, it was found that S^r (streptomycin-resistant) mutants of *E. coli* line 1, which grow well in 1 mg per ml of streptomycin, were inhibited by 20 μg per ml when grown as pc-L forms. This can be viewed either as the negation of the S^r effect when the wall is stripped, or a mutual reinforcement of penicillin and streptomycin in respect to another target. (Jawetz *et al.*, 1954; Linz and Lecocq, 1956). The former interpretation is favored by the sensitivity to streptomycin of dap-L colonies of an S^r strain.

Another hint of the relationship of mutation at the S locus to alterations of the cell wall was offered by the reported tendency of streptomycin-dependent mutants to grow as long filaments and occasional pleiomorphic colonies when deprived of streptomycin (Simon, 1955). An S^d mutant of *E. coli* line 1 was plated into sucrose agar and found to give a high yield of colonies consisting of very long filaments with occasional swellings (figure 5), rather than L colonies. On

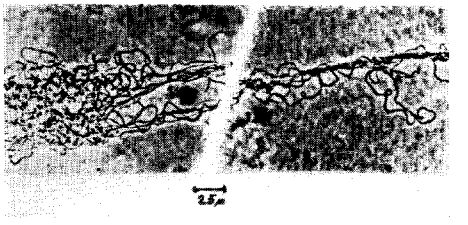

Figure 5. Escherichia coli streptomycin-dependent mutant; filamentous colony in sucrose agar without streptomycin. Phase contrast.

replating, the viability of these colonies was very low; the sucrose medium, therefore, could not compensate for the lesion imposed by lack of streptomycin.

Klieneberger-Nobel (1954) has listed some artifacts in sterile media containing lipoid material which can be mistaken for the globules of L growth. An equally rich source of artefacts is nutrient gelatin agar as used for the selection of motile bacteria. This medium tends to contain coacervates which give the appearance of germinating cysts.

GENERAL DISCUSSION AND RECAPITULATION

Our data support the conception that L growth is a result of a defect in wall synthesis. This defect may be imposed by external inhibition, as by penicillin, or by an internal genetic block. In some cases, the block has been defined biochemically, for example, as auxotrophy for diaminopimelic acid. In others (Proteus L forms) it has not been reparable even by hydrolyzates of the wild type bacteria. The irreparable mutants might be auxotrophs for metabolites lacking in the hydrolyzate as prepared, or might represent losses of the wall-building enzymes. Another hypothesis, that wall-building is self-priming, also warrants further consideration: the problem may be how to re-establish the primer at a suitable site.

L growth is an aspect of "unbalanced growth" (Cohen and Barner, 1954) in which one cell constituent, in this case the cell wall, is selectively inhibited. The prolonged subculture of pc- and dap-L forms furnished excellent evidence that penicillin and diaminopimelic acid play no essential part in the viability of the bacteria other than their influence on the cell wall, disregarding the role of DAP as a precursor of lysine which was provided independently. Since the over-all inhibition of cellular metabolism,

for example at low temperatures, is not lethal to bacteria, we should look to some form of unbalanced growth as a general mechanism of bactericidal action whenever a direct structural lesion is not evident.

The zonal effect of penicillin reported here is reminiscent of the paradoxical effect reported by Eagle (1951), namely, the greater bactericidal effect of lower than higher concentrations of penicillin. We can only speculate on the source of the protection afforded by higher penicillin levels; as far as L forms are concerned, it is probably still related to the wall, since deprivation for DAP has the same effect as augmenting the penicillin level.

A puzzling feature of previous researches, the appearance of genetically stabilized L forms, which persist as such when penicillin is removed from pc-L forms, also has a tentative explanation: that a superimposed genetic block to wall formation confers a selective advantage under the particular circumstances under which pc-L forms are growing. At least one such circumstance has been realized: zonal levels of penicillin. The development of fixed L forms in other material has not been recorded in sufficient detail to allow a judgment of the general applicability of this hypothesis.

The proposal that penicillin interferes with synthesis of the cell wall (Lederberg, 1957; Park and Strominger, 1957) does not imply that penicillin completely prevents the formation of each element of the normal wall. Nor does it specify the pathway of that inhibition. The functional tests—plasticity and osmotic fragility—speak only for the functional impairment of the wall, and there are strong hints that much residual wall material persists on pc-protoplasts. The diversity of wall components in gram-negative bacteria speaks for many potential sites of wall-defect. The reaction of pc-L forms with certain phages and colicines, and not others, and the retention of mating capacity, point to the specificity of the residual elements. It would be profitable to compare protoplasts made by different methods for these biological specificities.

The correlation of protoplasts with L forms is not novel and various aspects are supported by other workers (Bonifas, 1954; Vadasz and Juhasz, 1955; and other work already cited.) Apart from the morphological similarity between lysozyme- and penicillin-conditioned protoplasts, perhaps the most direct evidence is the analysis of streptococcal L and B forms (Sharp *et al.*, 1957), the stabilized L forms being found to lack the group A polysaccharide, both by immunological test and analysis for rhamnose. Similar analyses would be highly desirable for reversible, penicillin-conditioned L forms of the streptococcus and for the various types of L forms of *E. coli*.[2]

The mechanistic interpretation of L growth has been confounded by many cyclomorphic schemes of the greatest variety. L forms were, for example, invoked in speculations on mechanism of transduction in Salmonella. They were conceived of as "reduced cells" which might somehow persist with an incomplete genetic complement. The chief evidence for this scheme was the presence of what we would now call protoplasts in donor cultures treated with phage or with low levels of penicillin for the provocation of lysis. This fanciful speculation (Lederberg *et al.*, 1951) is mentioned now only to be condemned as baseless in the light of further studies (Zinder and Lederberg, 1952; Stocker *et al.*, 1953; Zinder, 1955) which have shown that bacteriophage particles are the vector in transduction.

Other roles that have been assigned to L forms are: an aberrant haplophase (Dienes, 1946); sexuality (Dienes and Smith, 1944; Klieneberger-Nobel, 1950); a resistant stage analogous to spores (Tulasne, 1955); a phase of regeneration or rejuvenation, possibly also correlated with sexuality (Klieneberger-Nobel, 1951). There is no indication that L growth is associated with deep-seated changes in genetic makeup; in any case, the vegetative phase of *E. coli* and probably other bacteria is normally already haploid.

When *E. coli* cells are exposed to penicillin, division is interrupted, even of cells which have started to constrict, and the protoplast almost always emerges from the middle of the cell at the point of incipient fission. This suggests that the septum and newly formed wall are the least rigid parts of the wall, or the most susceptible

[2] Kandler and Zehender (1957) report that stable L forms of *Proteus vulgaris* lack DAP while the B and pc-L forms contain it. The stable L forms include strains reported by them, and hereabove, not to be restored by the addition of DAP. Their metabolic defect is therefore presumed to be in the assembly of DAP into a wall component.

to inhibition by penicillin. At lower concentrations of penicillin, the cells fail to divide, but form long filaments, again as if the formation of the septum were more readily inhibited than that of the lateral wall. These features have caused some difficulty in the interpretation of fixed and stained preparations.

The fusion of protoplasts has been reported by several authors, e. g., Dienes and Smith (1944). Stained preparations can be misleading: for example, stages resembling those of figure 1 have been described as zygospores or fusion figures by Mellon (1925) and Klieneberger-Nobel (1950). Direct continuous observations on living material (Stähelin, 1954; Stempen and Hutchinson, 1951) leave no doubt that fusion can occur, but is it *de novo* or refusion? Published photographs generally show adjacent protoplasts that may have coalesced again before the completion of an earlier fission or budding. In our material, nearly all L colonies must arise from single cells (figure 4) and there is no warrant for the interposition of a fusion stage as an obligatory feature of L colony development. Further studies are needed to establish whether protoplasts stemming from different lines of cells can fuse with genetically interesting consequences, and whether this may occur with bacterial strains that do not also mate in their B phases.

It should be stressed that the normal conjugal mating process of *E. coli* involves typical bacillary forms (Lederberg, 1956*b*) and has nothing to do with L forms. However, the morphological details of the mating of protoplasts of compatible genotypes or of protoplasts with rods, though not yet studied, may prove of particular interest, as the two parents can be readily distinguished both by their appearance and their response to osmotic shock.

The "resistance" of protoplasts to penicillin is now understood as a corollary of the mechanism of action of this antibiotic; resistance to phages (and according to Dienes, also to complement) gives some credence to the adaptive value of protoplasts in special environments.

The spontaneous occurrence of protoplastic outgrowths in old cultures (Sinkovics, 1957) is understandable as an aspect of unbalanced growth whereby the growth of total mass outstrips the synthesis of new wall. The balance of these processes will of course depend on both environmental and genetic determinants. Perhaps the pleiomorphism of the pleuropneumonia organisms is a reflection of the nutritional inadequacy of the media. As much can be speculated for the propensity of *Streptobacillus moniliformis* to produce L forms.

Other cyclomorphic sequences, without prejudice to their biological interpretation, are apt subjects for possible correlations with wall synthesis. For example, the cycle of transformable competence of the pneumococcus (Fox and Hotchkiss, 1957) is patently due to some imbalance of growth of cellular constituents, for which the wall and other envelopes would be the first candidates. For another, the microcysts of *Spirillum lunatum*, as figured by Williams and Rittenberg (1956) show at least a superficial resemblance to the cycle of evolution and reversion of protoplasts. The spontaneous occurrence of large bodies and the osmotic fragility of marine luminous bacteria (Johnson and Gray, 1949) also have to be related to the rigidity of the cell wall.

While we have not undertaken a comprehensive comparative study of *Proteus* and other bacteria, our limited observations and the published record would indicate that *E. coli* is far more delicate and difficult to maintain in the L phase. For example, at suitable stages in the culture cycle, Proteus will give up to 10 per cent of pc-protoplasts in an unprotected medium (Liebermeister and Kellenberger, 1956), in which *E. coli* would lyse almost completely. Also, Proteus L forms have been successfully adapted to growth in liquid culture (Tulasne *et al.*, 1950; Dienes, 1953), and to some extent on the surface of agar, which we did not succeed in doing with *E. coli*. The easiest rationalization of such differences is that *Proteus* has a tougher residual membrane, a protection which may have made this species convenient for earlier investigations, but may also have helped to obscure the essential differences of B and L growth. A liquid-adapted culture of *Proteus* (strain Tulasne L), grows in massive clumps and the periphery consists of lysed protoplasts and debris. The pressure of the largely inviable mass of growth may take the place of agar in conditioning further outgrowth of the embedded protoplasts.

It was noticed that protoplasts subjected to osmotic shock, though leaving little to be seen under phase microscopy, still displaced a definite volume in an India ink suspension. This residual

structure should be looked for and taken into account in evaluating the biosynthetic capacities of disrupted protoplasts, and the association of specific enzymes, e. g., cytochromes (Weibull, 1953*b*) with membrane (ghost) fractions.

Another feature of L growth reported elsewhere has not been considered here: the occurrence of viable, filtrable granules (Klieneberger-Nobel, 1951; Kellenberger *et al.*, 1956; Sinkovics, 1957; Vadasz and Juhasz, 1955). The reported efficiency of filtration at low pore diameters is extremely low, which leads to some question as to the reliability of the minimum size estimates. Under these circumstances, there should be more concern for the homogeneity of pore size of filters and for the plasticity of a small protoplastic bud, which might be squeezed through pores smaller than its characteristic diameter. However, even if taken at face value, the filtrable granules of about 0.3 μ diameter are still ample to contain the material necessary for genetic continuity. Lacking L growths that could be propagated in liquid culture, we have made no filtration studies with *E. coli*.

On the same account, we have no basis to comment on other reported modes of growth of L forms, e. g., fragmentation of cysts, extrusion of granules, that have not yet been observed in the present material. The recent development of a technique for the production of protoplasts from yeast (Eddy and Williamson, 1957), suggests that further studies of wall-defective organisms will embrace a wide variety of organisms.

SUMMARY

Escherichia coli growing in the presence of penicillin forms localized swellings which enlarge to yield spherical protoplasts. The protoplasts lyse in dilute media, but may be maintained in protective media containing M/3 sucrose plus M/100 Mg^{++}. In penicillin sucrose broth, the protoplasts continue to enlarge but do not proliferate. In the absence of penicillin they revert to form normal rods.

An agar medium is described which permits the further growth of protoplasts in the presence of penicillin to produce L colonies. Growth depends on the formation of blebs which enlarge and pinch off. It occurs only in agar medium. Indefinite serial passages were made of certain such strains of *E. coli* K-12 as L growth in penicillin sucrose agar; these invariably reverted to normal bacillary form when replanted in the absence of penicillin.

Optimal L growth requires high concentrations of penicillin, neither L nor bacilliform colonies developing well at intermediate levels. This phenomenon has been applied to the selection of fixed L forms, which grow as L colonies in conventional media. So far, these isolates from *E. coli* have all proved to be auxotrophic mutants requiring diaminopimelic acid, a characteristic constituent of bacterial cell walls. These mutants, and another previously isolated by Davis, grow as L colonies in agar lacking diaminopimelic acid. They may be passed in series as such, and regenerate bacilliform growth when this metabolite is restored. However, other fixed L forms of Proteus responded neither to diaminopimelic acid nor to crude bacterial hydrolyzate.

Penicillin-produced L colonies of *E. coli* were found to have become resistant to certain phages and colicines, but not others; protoplasts of genetically compatible strains proved to retain their ability to undergo sexual recombination. Some but not all the surface receptors of the bacteria are therefore believed to have been lost. Protoplasts suspended in sucrose broth show a transparent capsule, visible in india ink preparations, which may represent remains of the cell wall. Lysed protoplasts, though expanded, are not fully dispersed through the solvent but occupy definite spaces, again as seen in India ink.

Attempts to convey genetic markers to protoplasts by means of extracts containing deoxyribonucleic acid were unsuccessful, as were efforts to promote the fusion of protoplasts of otherwise incompatible $F^- \times F^-$ genotypes.

These observations support the proposals that: (1) the mechanism of action of penicillin is to inhibit the synthesis of the bacterial cell wall; (2) that the L forms of Dienes and Klieneberger are colonies of protoplasts, whose aberrant mode of growth is conditioned by the loss of the wall and the failure of the normal division mechanism; (3) the partial or complete defect of the wall may be brought about either by external inhibition (penicillin) or by internal genetic blocks affecting any of various aspects of wall formation. So far, two types of wall-defect mutants are known: those repaired by diaminopimelic acid, and auxotrophic for it, and those not reparable even by crude bacterial hydrolyzates.

The evolution of L fixed growths in the pres-

ence of penicillin may be accounted for by a selective advantage under these conditions of spontaneous mutants with wall defects.

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