Penicillin, protoplasts and bacterial L-forms

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In the text-book classifications of the living world, the Bacteria are traditionally represented as minute organisms which reproduce exclusively by transverse fission. This conception is engrained in the very class-name of Schiso-mycetes, or fission-fungi. We now know that these organisms are more complex than our classification of them. For example, at least some species can participate in a process of sexual reproduction. Furthermore, even in asexual propagation, some bacteria under certain circumstances can assume very un-bacterial shapes and modes of growth, the L-forms which are the subject of this article.

'L' stands for the Lister Institute for Preventive Medicine, at London, where in the hands of Dr. Emma Klieneberger-Nobel the more recent chapters of our narrative began about 20 years ago. Many previous authors have described bizarre, atypcial forms of bacterial growth. For the most part, the earlier findings were ignored, or discounted, or relegated to footnotes in the textbooks. (It sometimes happens in science, when upsetting new knowledge intrudes on comfortable habits of thought, that the first reaction is "probably untrue", the second "well, not very important" and finally, "well, so-and-so discovered it years ago".)

Dr. Klieneberger was studying the life history of a bacterium called Streptobacillus moniliformis which is involved in a disease called Haverhill Fever. As the Latin name implies, this grows typically in the form of long chains of bacilli (rods) which remain attached for some time after they have increased in number by transverse fission. However, in some culture media,

she noticed a quite different growth form, which she designated "L_1" for this instance, and L_2, L_3 etc. for similar forms in other cultures. Unlike the typical bacilli, the L-form consisted of nondescript masses of granules and globules of all sizes, often distorted in shape. She made a concerted effort to separate the L-form from the bacillary growth, and succeeded to the extent of isolating true-breeding cultures of the L-forms. However, she did not succeed in isolating pure-breeding cultures of the bacilli free of the L-forms. She suggested two alternative hypotheses, a) that the L-forms were a stage in the life-cycle of the bacteria, and b) that they represented another organism, symbiotic with the Streptobacillus, and not readily separated from it. Another obscure group of organisms, the "pleuropneumia-like organisms", "PPLO", was a likely candidate for the affinities of such a symbiont, on account of certain morphological similarities. Her choice at the time was the symbiont hypothesis (b), partly because of her partial success in separating the bacillary from the L-forms, partly because this would put the least strain on traditional views of the life-history and morphology of an otherwise seemingly typical bacterium.

During the succeeding decade, her studies were complemented by those of Dr. L. Dienes, at the Massachusetts General Hospital in Boston. L-forms were isolated from a number of different bacteria, and in a few instances, he was able to show by direct microscopy the transformation of bacilli into L-forms and vice versa. Dr. Klieneberger-Nobel concurring in these observations, there was no longer any doubt of the life-cycle hypothesis, that bacteria were liable to another form of shape and growth. However, the biological significance of L-forms remained quite obscure; they have been assigned such functions as rejuvenation, adaptation to harmful environments, and sexuality (the last speculation being the initial source of my own interest in them).

A detailed analysis was held up for some time by the difficulty of reproducing L-form growth and quantitating it, in common bacteria, particularly in species like Escherichia coli which are best worked-over as materials of genetic experimentation.

One of the reasons for thinking that L-forms might be adaptations to noxious environments was the finding that they were quite resistant to penicillin, though the bacillary forms from which they were derived and to which they might revert were quite sensitive to this antibiotic. Penicillin has therefore been used systematically both to eliminate bacillary forms and to promote their transformation into the L-type of growth. By means of penicillin, Dienes and many other workers produced L-growth in a wide variety of organisms, though not with the genetically standardised strains of E. coli. Three aspects of the action of penicillin could be distinguished, at least in retrospect: 1) many bacteria were converted into fragile, globular bodies; in the same cultures the majority of cells would customarily be killed and dissolve. 2) In agar medium, containing penicillin the globular bodies could sometimes proliferate to give L-colonies like those pictured (figure). 3) While the L-colonies would ordinarily revert to the bacillary form in the absence of penicillin, occasionally a stable L-form could be isolated which would retain its peculiarities on standardised medium, the same medium that would support bacillary growth of the parent culture. Any comprehensive theory of L-forms must therefore account both for penicillin-induced and genetically stabilised L-type growth. We must leave this thread of our narrative, which now dates about 1953, with the anticipatory comment that most of the clues needed for the explicit statement of such a theory had already been collected, though they were scattered through a large number of observations on many species. It is equally remarkable that while the problem of the mechanism of

action of penicillin had been vigorously, but unconvincingly, attacked from a biochemical point of view, the morphological effects of penicillin were either ignored or put down to non-specific reactions to injury.

The impact of Sir Alexander Fleming, the renowned discoverer of penicillin, hangs over this story, for (in 1922) he had also discovered lysozyme, an ensyme found in egg-white, and secretions such as salive and tears. The ensyme dramatically lyses certain bacteria, evidently by attacking their cell walls. This conception of its action was extended by Weibull and by Tomscik in 1953, with studies on another bacterium, Bacillus megaterium, a species whose very large cells (a property again coded in the name) make it apt for cytological studies. Whereas lysozyme rapidly dissolves these cells in ordinary media, the addition of large amounts of sucrose, to give a concentrated medium of high osmotic pressure, protects the cells from complete lysis. Instead, the dis-solution of the cell walls releases globular protoplasts, which are the soft living unit contained within the rigid cage. The term protoplast is borrowed directly from botanical usage, where it means precisely the same thing. However, unlike the protoplasts of plant cells, bacterial protoplasts can survive only in concentrated media. Dilution into ordinary media constitutes an 'osmotic shock' which causes the protoplasts to burst. The very ease with which the protoplasts can be broken has made them excellent sources of intracellular constituents for biochemical studies, and this alone has motivated studies on the development of similar procedures for other bacteria, e.g. again E. coli.

The spherical form and the fragility of these protoplasts at once suggests a possible relationship with L-forms. This was at first obscure, as the lysozyme-induced protoplasts of B. megaterium have not yet been made to proliferate into L-forms.

The lytic action of penicillin on growing bacteria had been used in this laboratory for several years for the isolation of growth-factor-requiring mutants. Since non-growing bacteria are left intact (an earlier empirical observation), penicillin is used to kill the preponderance of cells that can grow in a defined medium, and leave behind those that are starved for specific components in which the medium is lacking.

Since sucrose would forfend the lysis of Bacillus megaterium by lysozyme, would it have the same effect on growing E. coli, and thereby furnish a method for producing protoplasts of this species?

The experimental answer to this question was indeed yes. In the presence of penicillin, growing cells, instead of dividing by fission, enlarged and in two hours or so popped out of their skins to release rather large (that is to say, about 5 μ or 1/5000ths of an inch across) spherical bodies. On account of their shape, and their easily verified fragility to osmotic shock, these are also considered protoplasts. It would, however, be difficult to say that either these protoplasts or those obtained by lysozyme lacked every element of the normal cell walls, a question that needs further study.

But are not these protoplasts in fact the same elements as have been described earlier, as the initial stages of the L-form cycle? The chief novelty in these experiments was the viewpoint that motivated them, and the fact that the use of a protective medium allowed the transformation from rod to globule to be quantitatively described. In addition, E. coli had not previously been amenable to this shift. The very reason that this species displays sensitivity to osmotic shock in the most dramatic form, has also helped clarify the significance of protoplasts.

The facts are now ready for a systematic interpretation, some of the conjectural elements of which have since been confirmed. The mechanism of

action of penicillin is to inhibit the synthesis of cell wall substance. Unbalanced growth, namely of protoplasmic mass versus the wall, leads to the rupture of the cells. The naked protoplasts will promptly lyse in ordinary media, but can be protected in concentrated media. The fate of these protoplasts depends on their environment. If penicillin is removed, cell wall will be resynthesised and the normal shape and osmotic tenacity will be restored. However, if the protoplasts are subjected to osmotic shock, or allowed to grow too long in the presence of penicillin, they will burst, and of course lose their viability. This contrast shows that no other action of penicillin has to be invoked to explain its lethal action. The unique chemical makeup of bacterial cell walls accounts for the chemotherapeutic specificity of penicillin; the animal body lacks any targets which are chemically similar to these walls. This hypothesis is not yet complete; the second and third aspects of penicillin L-form relationships will be considered shortly.

This view of the biochemical target of penicillin as the synthesis of the bacterial wall was not only compatible with existing data on its influence on cellular permeability, but was soon corroborated by a completely different line of evidence.

At the Department of Biochemistry (which is next door to Genetics at Wisconsin) T. S. Park and his professor, M. Johnson, had found that staphylococci exposed to penicillin accumulated a series of compounds related to nucleic acids, but of a formerly undescribed structure. Later work by Park established that these compounds were conjugates of Uridine-Diphosphate coupled with amino compounds: an amino sugar (muramic acid) and certain amino acids. The significance of these compounds was quite obscure at the time, and they were related to the synthesis of <u>nucleic acids</u>, which for various other reasons, not now convincing, were thought to be the targets of penicillin action.

Meanwhile, other derivatives of Uridine diphosphate have been found to be important, not so much in nucleic acid metabolism, as in the metabolism and coupling of various sugars. That is, it is now more significant to regard these compounds as activated forms of amino-sugars, rather than nucleic acid derivatives. (It is quite possible that both points of view will be justified, when we learn more of the role of nucleic acids in controlling the synthesis of proteins and other specific macromolecules.) Simultaneously with the morphological studies just reviewed, Park and Strominger found that the amino-residues were equivalent to those found in the cell walls of untreated staphylococci. They could therefore propose, quite independently, that penicillin blocked the further metabolism of these activated amino-residues, and furthermore that their normal metabolic fate is incorporation into wall substance. However until the enzyme systems which are responsible have been isolated, it is impossible to judge whether the primary inhibition by penicillin is of a polymerizing enzyme, on the synthesis of other building blocks, or even on some more organized structure which is involved in wall-building.

What of the further growth and viability of the protoplasts? In liquid medium, they grow rapidly in the sense that proteins, including new enzymes, and nucleic acids are synthesized. (In fact, they have been used, together with partially disrupted fragments of protoplasts, for exciting studies on the biosynthesis of these compounds by Dr. S. Spiegelman at the University of Illinois). But when followed microscopically, the protoplasts actually do not divide in liquid cultures. They enlarge enormously and eventually burst, even in a protective medium. However, most students of L-forms have emphasized the role of agar in supporting this type of growth.

Initial attempts to grow the protoplasts of E. coli in penicillin agar gave erratic results, which were finally traced to still mysterious strain

specificities, to the concentration of penicillin used, and to the enrichment of the medium. Some cultures have simply given no L-type growth under any conditions tried, although they are indistinguishable from the others in forming the initial protoplasts. It was also found that levels of penicillin which were quite sufficient to inhibit bacillary growth still were not enough to support optimal L-type growth. Since there is then a zone of intermediate penicillin concentration that permits neither, this will be referred to as the zone effect. Finally, the addition of enrichments such as meat extract, in combination with sucrose, gave quite high 'efficiencies of plating' so that, e.g., half the cells plated into an optimal medium might give L-colonies, as might be counted when they developed fully. These colonies have a transparency, slow growth and texture that distinguishes them from ordinary bacillary colonies even at low magnifications. When they are studied at high magnifications, they give the bizarre appearances shown in figure .

These colonies could be passaged in subculture by cutting out the blocks of agar in which they were embedded, grinding them cautiously, and replating the dispersion into fresh plates containing the same penicillin agar medium. However, despite the thousands of globular elements that can be seen in such an L-type colony, the yield of subculture colonies will be only from ten to fifty; most of the visible elements are therefore inviable or extremely fragile. Even after twenty passages under optimal conditions, all viable elements promptly reverted to typical bacteria in the absence of penicillin. These experiments had therefore not yet reached the third stage: genetic fixation. The described effects could all be referred to the inhibition of wall synthesis in the uninterrupted presence of penicillin, and its resumption when the penicillin was withheld.

The manner in which protoplasts proliferate requires more study by refined techniques, since the elements are so small as to be at the useful limit of magnification of the phase contrast microscope. For this reason, the picture to be described may be incomplete. It appears, however, that the protoplast is a semifluid droplet, which changes its shape in response to its own surface tension. In agar, however, it will be compressed by the matrix in which it is embedded, and especially as it enlarges, it extends into the spaces in this matrix. These extensions or blebs, may remain minute granules, become long or fingerlike, or may round up immediately, but round up they will until they actually pinch off from the mother protoplast. Some of the daughter forms, perhaps those which have received a nucleus along with a blob of protoplasm, will continue to grow and generate their own progeny. At a glass surface, a developing L-colony has been seen to spread along the glass and break up into droplets of varying size, exactly as expected on this picture. The semi-fluid protoplast thus has no regular mechanism of division, which normally depends on the wall, but is at the mercy of external pressure and surface tension which can cause it to fragment as it grows in a confining milieu. As another aspect of L-type growth some authors have stressed the viability of minute granules, some perhaps half the size of the normal bacterium. It has been very difficult to get direct evidence of the viability of such a particle, which would be nearly submicroscopic; indirect measurements based on the occasional passage of viable elements through filters of graded size may be influenced by the plasticity of the protoplasts, which might enable them to be squeesed through pores that would restrain a more rigid particle. However, the possible 'filtrability' of L-form granules is more a technical question than anything else, so long as the limiting dimensions still leave room for packaging the minimum apparatus for genetic continuity of the bacterial cell.

Perhaps the most puzzling feature of L-forms, is the third aspect, the genetic fixation of L-type growth after prolonged passage in the presence of penicillin. The leading clues for this came from still another source.

By and large, the chemical makeup of all cells is startlingly uniform. The same array of amino acids is basic to the structure of proteins in bacteria as in man. But there are exceptions, and one of the clearest of these was the discovery by Dr. Elizabeth Work, in London, of a new amino acid, DAP or di-aminopimelic acid, which has been proved to occur in a variety of bacteria, and nowhere else in nature. Further studies have localised DAP entirely or nearly entirely in the cell walls of the various bacteria. Dr. B. D. Davis of New York isolated a mutant of E. coli which required DAP for growth, and this mutant has been very useful both as a biological assay for DAP and for the further elucidation of its function. In a striking corroboration of the hypothesis for penicillin action, when cells of the DAP-less mutant are deprived of DAP in an otherwise rich medium, they undergo the same transformation into protoplasts as the wild type strain does in the presence of penicillin. The DAP-less protoplasts are osmotically fragile, and will revert to normal bacillary forms if they are furnished with DAP. It is therefore inferred that unbalanced growth, in which wall-synthesis is now impeded by the lack of this essential building block, while other aspects of protoplasmic synthesis continue. All this is exactly analogous to the effect of penicillin, which however affects another aspect of wall formation.

In addition, when DAP-less cells are plated into enriched agar, whether penicillin is present or not, they proceed through the same cycle of L-type growth. Since ordinary media are deficient in DAP, this mutant would be recognised as a stabilized L-form by the criteria applied to those isolated by other workers. We therefore are led to the conclusion that stabilised

L-forms are mutant strains which have suffered a genetic block to wall formation. This block need not be in DAP-metabolism, nor at the still unknown target of penicillin action. Any mutational block to an essential part of wall synthesis should give the same result. In fact, the stabilized L-forms isolated by other workers have not responded to DAP, and must therefore have another defect. However, the defect would have to be for a function unique to wall formation, since one that influenced overall growth would inhibit bacillary or L-type growth alike.

There remains the question of why such wall-defect mutants should tend to occur in the presence of penicillin. Is penicillin itself mutagenic, or does the cell-wall contain or depend on a self-perpetuating structure? These notions are not readily ruled out, and in certain contexts they might be plausible, but an even simpler explanation is possible.

The zone effect of penicillin was mentioned previously. That is, certain levels of penicillin discourage both bacillary and L-growth. In fact, even to very high levels, penicillin continues to stimulate L-type growth. On the other hand, the DAP-less mutant produces good L-type growth at any level of penicillin. This suggests that L-type growth is best when wall formation is completely inhibited, either by saturation with penicillin or by the superimposition of a genetic block. Therefore during the passage of L-type colonies in the presence of penicillin, any random mutation imposing a genetic defect on wall-formation, will have a selective advantage and will tend to overgrow its neighbors. Since quite large populations of cells are always involved in these experiments, even mutations occurring at quite ordinary, that is low, rates, can lead to the results found. It is quite possible, however, that some bacterial species have much higher rates of mutation than others for the occurrence of such defects.

By the application of these principles, it has been possible to isolate new, 'stable' L-types from E. coli; these have so far proved to be mutants requiring DAP, but the same rationale is expected to lead to the detection of other metabolic defects, e.g. for the amino-sugars mentioned previously which seem also to be unique to bacterial walls.

What then of the functional significance of the bizarre L-type or, as may now be more appropriate to call it simply, protoplastic growth? This has been tested by genetic means, and found to be irrelevant to sexual reproduction (which other experiments have related to the pairwise conjugation of intact, full-walled bacteria). It now appears to be nothing more than a sign of the nakedness imposed either by external inhibition or internal defect. However, the bacterial wall has many functions, of concern both to the microbe and the microbiologist. For example, it is the site of attachment of certain viruses, so that the protoplast is essentially resistant to their action. As these viruses also carry a wall-dissolving enzyme similar to lysozyme, they may play some part in the occurrence of protoplastic forms in nature. But at this instant, the naked forms are perhaps of most interest as laboratory tools, both to study the function of walls, and for hopeful attempts to elude them. Insofar as the wall may be a barrier to the movement of substances we may wish to import and export, its removal from a still viable unit is a hopeful stategem in the scientific siege of the Cell.

In this account, I have indulged in name-dropping to good purpose. It is perhaps more obvious, but no truer of this subject that its development has depended on the discoveries and insights of unnumbered researchers working throughout the globe. It is no accident that a Swede working in California, an Austrian in Massachusetts, a German in London and a Hungarian in Switzerland are among the leading characters in this memoir.

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