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On the Mechanism of the Irreversible Antimicrobial Effects of <latex>β</latex>-Lactams

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On the mechanism of the irreversible antimicrobial effects of β-lactams

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[Plate 1]

A full understanding of the mechanism of action of an antibiotic involves not only the identification of the drug-sensitive biochemical targets, but also the pathway by which the inhibition of the target reaction(s) leads to the eventual physiological consequences, i.e. inhibition of the reproductive capacity of the whole cell. The complexity of such a pathway may be illustrated by the case of the penicillin response of pneumococcal mutants defective in the activity of murein hydrolases, i.e. enzymes that do not seem to react directly with the penicillin molecule. These hydrolase-defective mutants seem to contain all the 'normal' penicillin-binding proteins; penicillin treatment causes typical morphological and biochemical effects (interference with cell wall metabolism) and the minimal inhibitory concentration as well as dose response to penicillin are identical in the hydrolase-defective mutant cells and in the wild-type bacteria. In spite of all these similarities, the eventual physiological response of mutants is strikingly different from that of the wild-type cells: in contrast to the wild-type cells, mutants do not lyse during penicillin treatment and their rate of loss of viability is greatly suppressed. These observations suggest that inhibition of the primary biochemical targets of penicillin (penicillin-binding proteins) is a necessary but not sufficient condition for the induction of the pharmacologically most useful irreversible effects of β-lactams.

During the past 4–5 years, our laboratory has been engaged in an intensive effort to unravel the mechanism of penicillin-induced death and lysis in several species of bacteria and I shall summarize briefly some of our conclusions here, using mainly observations obtained in two experimental systems: the Gram-positive pneumococci and the Gram-negative *Escherichia coli*. Our approach to the mode of action of β-lactams involves an attempt to identify cellular factors that are directly responsible for the events of cell death and lysis. In my mind, such studies complement the other approaches (biochemical, enzymological and genetic analysis of penicillin sensitive enzymes and binding proteins), summarized in the talks of Professor Strominger, Professor Ghuysen and Dr Spratt at this meeting.

Growth inhibitory, bactericidal and lytic effects of β-lactams

Recent studies have directed attention to two puzzling features of β-lactam action. First, observations summarized by Dr Spratt indicate that structurally different β-lactams may cause very different biochemical and physiological effects when added to the same bacterium. Conversely, the same β-lactam added to different bacteria may also produce strikingly different physiological effects. This second type of puzzle is illustrated in figure 1 by the response of three different species of streptococci to benzylpenicillin (figure 1). *Str. sanguis*, *Str. pyogenes* (group A) and *Str. pneumoniae* each have about the same minimal inhibitory concentrations (m.i.cs) to

benzylpenicillin and in each species the antibiotic interferes with cell wall biosynthesis and also causes typical morphological effects. Yet the eventual, physiological consequences of drug treatment are strikingly different in the three species: in *Str. sanguis* benzylpenicillin acts primarily as a reversible growth inhibitor; group A streptococci treated with penicillin rapidly lose viability without any sign of lysis or structural damage to the cells, while pneumococci lose viability and lyse during penicillin treatment (Horne & Tomasz 1977).

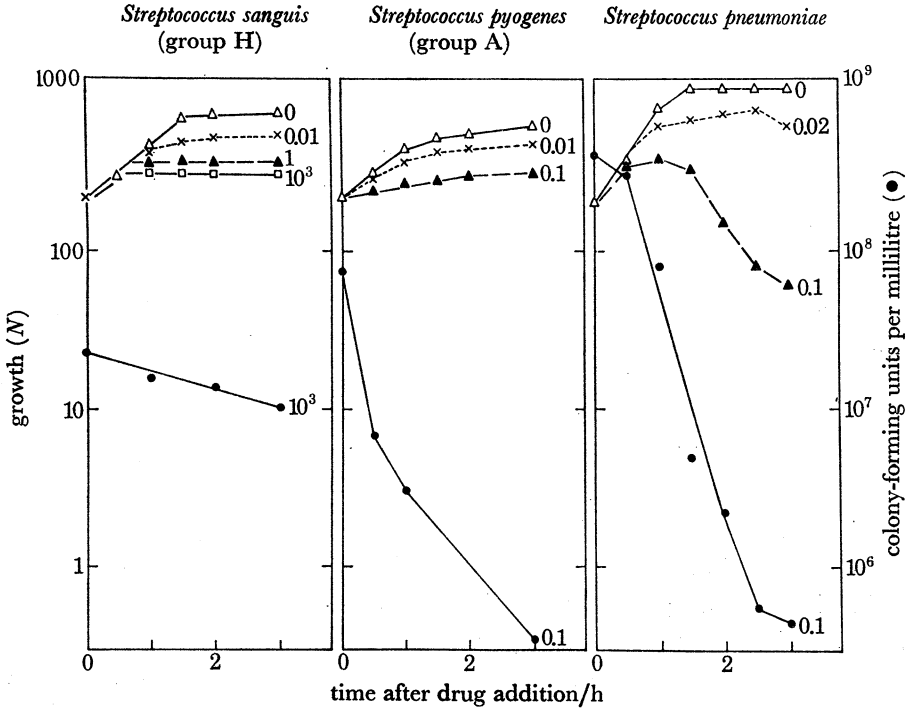

FIGURE 1. Effects of benzylpenicillin on three species of streptococci. Numbers by each curve indicate concentration of drug (micrograms per millilitre). Closed circles indicate viability of bacteria (units per millilitre). All other symbols indicate turbidity. Dashed lines indicate m.i.c. of drug. Reprinted with permission from *Antimicrobial Agents and Chemotherapy*.

These findings allow several conclusions. (i) They clearly show that loss of viability and lysis are *not* always synonymous, a fact often overlooked in the literature. It seems, indeed, that even in *E. coli*, there may exist several different mechanisms for loss of viability depending on the nature of the β-lactam used to inhibit the cells. (ii) The different response of the three streptococci may mean that in *Str. sanguis* and in group A streptococci certain penicillin-binding proteins (PBPs) needed for the bactericidal and lytic effects, respectively, are not available to the drug molecules. As an alternative mechanism, we suggested that the irreversible effects of penicillins (killing and lysis) may always be indirect and that they are caused by cellular factors (e.g. autolysins), the activity of which becomes triggered in the penicillin-treated bacteria (Tomasz & Holtje 1977). It should be noted that of the three streptococci studied, only pneumococci have demonstrable autolysin activity. Loss of viability in group A streptococci could conceivably be caused by a triggered phospholipase activity or by a combination of irreversible PBP inhibition and inhibition of protein synthesis (Tomasz 1979).

In this model the *direct* physiological consequences of the inhibition of PBPs may be only inhibition of growth; killing and lysis follow this primary (bacteriostatic) response as a result

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of the triggering of secondary mechanisms (e.g. autolysin activity). This model is based on findings obtained in the analysis of autolysin-defective pneumococcal mutants, to be discussed briefly below.

The role of murein hydrolases (autolysins) in the penicillin-induced lysis of bacteria

Suggestions concerning a possible role of autolysins (murein hydrolases) in the penicillin-induced lysis of bacteria appeared in the literature soon after the identification of the bacterial cell wall synthesis as the biochemical target of penicillin action (Weidel & Pelzer 1964; Rogers 1967). These suggestions were confirmed in 1970 by the observation that pneumococcal mutants or physiological variants defective in the autolytic system do not lyse when treated with penicillin (Tomasz *et al.* 1970). The rate of viability loss was also found to be greatly decreased in these bacteria. Interestingly, the lysis-defective mutants have remained sensitive to the growth inhibitory effect of penicillins and exhibited m.i.c. values characteristic of the lysis-prone (parental) pneumococci. The term 'antibiotic tolerance' was coined to refer to this novel, and at first sight paradoxical, phenomenon. A further interesting feature of the pneumococcal tolerant mutants was that they could be made sensitive to exogenous wild-type autolysin (added to the culture medium) by pretreatment of the bacteria with β-lactams and other cell wall synthesis inhibitors. The inhibitors added alone only caused inhibition of growth, and autolysin added alone had no effect on the growth of the bacteria. However, antibiotic plus enzyme in combination caused a great stimulation in the rate of loss of viability and also induced culture lysis.

Analysis of the tolerant pneumococci has clearly indicated that the peculiar penicillin response was caused by the suppression of the autolytic system, which could be achieved by a variety of means including a mutational defect in the autolytic enzyme (an *N*-acetylmuramic acid-L-alanine amidase). Particularly interesting was the observation that the addition of high concentrations of the pneumococcal Forssman antigen to the medium protected the wild-type pneumococci against penicillin-induced lysis, i.e. in the presence of this compound, pneumococci were only inhibited in their growth by penicillin (figure 2, plate 1). The Forssman antigen, a choline- and lipid-containing complex teichoic acid, was earlier shown to be a specific and powerful inhibitor of the pneumococcal autolysin (Holtje & Tomasz 1975). Interestingly, the pneumococci were not only protected from penicillin-induced lysis but formed chains when grown in the presence of Forssman antigen (see figure 2*c*). Both of these observations suggest that the exogenous lipid–teichoic acid was readsorbed onto the bacterial surface in such a manner that it could achieve at least a partial suppression of autolysin activity.

Penicillin tolerance in other bacteria

The resistance of autolysin-defective mutants against penicillin-induced lysis was confirmed in a number of Gram-positive bacteria such as *B. licheniformis* (Rogers & Forsberg 1971) and *B. subtilis* (Ayusawa *et al.* 1975). In the paper describing the penicillin tolerance of mutants, it was predicted that tolerant bacterial variants (resisting the irreversible effects of penicillin) may be selected in persistent bacterial infections (Tomasz *et al.* 1970). Recently, the isolation of penicillin-tolerant staphylococci was described from clinical specimens originating from

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patients with relapsing infections. A characteristic feature of these isolates was a wide dissociation of m.i.c. and minimal bactericidal concentration (m.b.c.) values for certain β-lactams and other cell wall synthesis inhibitors. At least some of the isolates were reported to have a defective autolytic system (Best *et al.* 1974; Mayhall *et al.* 1976; Sabath *et al.* 1977). Tolerance to the irreversible effects of cell wall inhibitors was also described in several laboratory strains and clinical isolates of *Streptococcus sanguis*. These strains also appear to be defective in autolysin activity (Horne & Tomasz 1977).

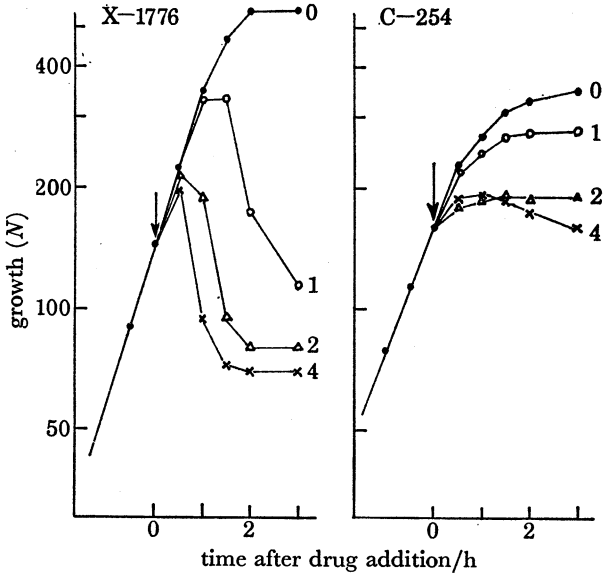

FIGURE 3. The response of parental and mutant (penicillin-tolerant) *E. coli* cultures to treatment with cephaloridine. Drug was added (arrow) at concentrations representing multiples of the m.i.c. Growth and/or lysis were measured by monitoring the light scattering of cultures.

Tolerant mutants of *E. coli* have recently been isolated by K. Kitano in our laboratory (Kitano & Tomasz 1979). In contrast to the parental bacteria, the mutants were only inhibited in growth by penicillin concentrations ranging from the m.i.c. (30 μg/ml) to about eight times the m.i.c. (figure 3). Both lysis and viability loss were inhibited and the tolerant bacteria had a tendency to grow in chains. A comparison of the crude autolysin extracts of parental and mutant cells has revealed a lowered activity in the mutant bacteria. In all these properties, the *E. coli* tolerant mutants resemble the autolysis defective mutants of pneumococci.

POSSIBLE CAUSES OF AUTOLYTIC CELL WALL DEGRADATION IN PENICILLIN-TREATED BACTERIA

Two different types of general mechanisms have been proposed to explain the degradation of murein in penicillin-treated bacteria. The first model, which I shall refer to as the 'constitutive' model (Weidel & Pelzer 1964), assumes that murein hydrolases acting in concert with wall synthetic enzymes are part of the mechanism of cell wall enlargement, the 'nicks' in the cell wall (created by a hydrolase) serving as growing points at which new cell wall elements are added to the pre-existing wall by synthetic enzymes. Inhibition of the synthetic reactions (by wall inhibitors), without a parallel inhibition of the hydrolases, would then

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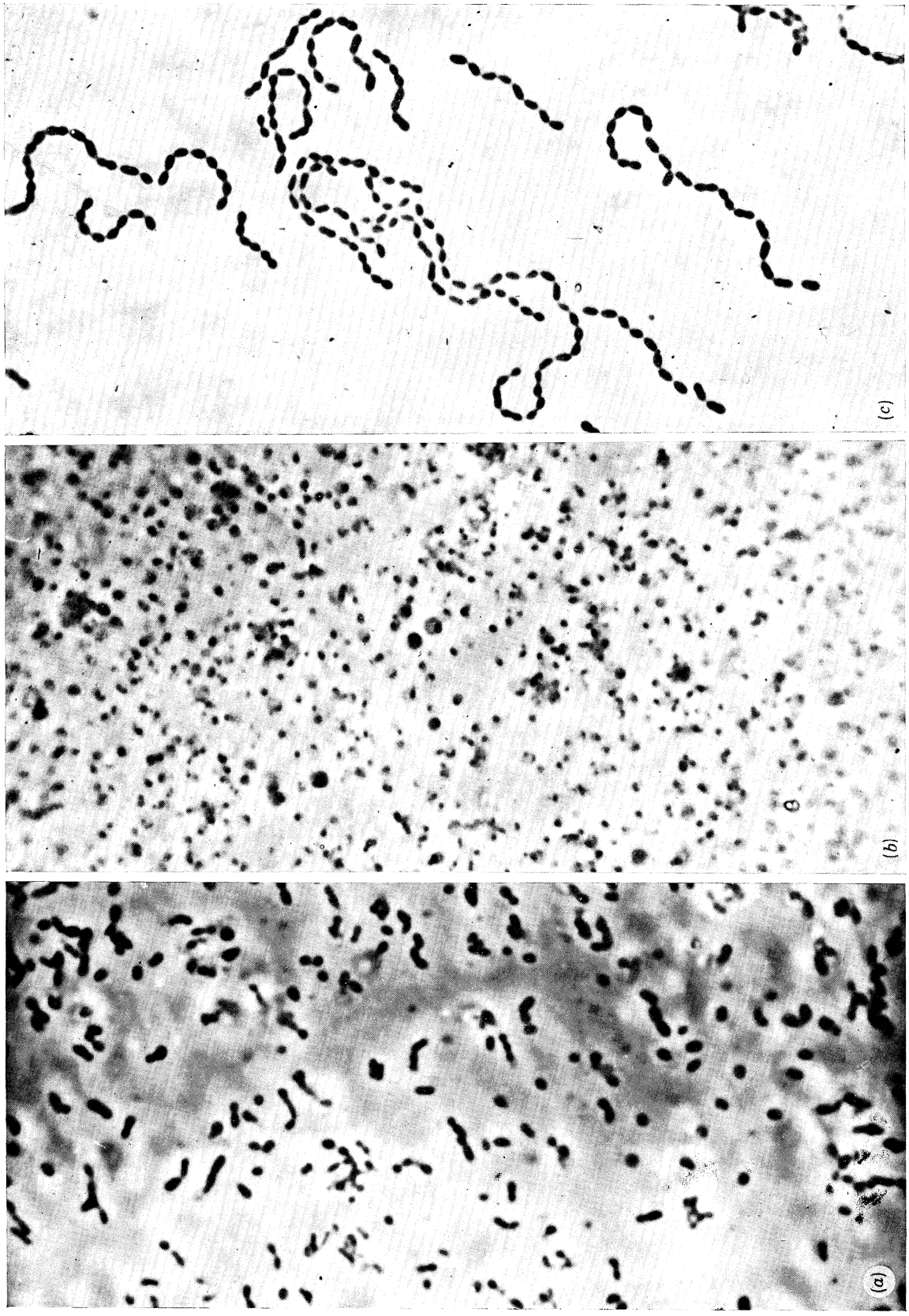

FIGURE 2. Protection of wild-type (lysis-prone) pneumococci against penicillin-induced lysis by the addition of Forssman antigen to the growth medium. (*a*) Pneumococci in normal growth medium before the addition of penicillin. (*b*) Pneumococci after 3 h of penicillin treatment. (*c*) Pneumococci grown in medium containing Forssman antigen (3 mg/ml) after 3 h of penicillin treatment.

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automatically lead to cell wall rupture and lysis. Indeed, the penicillin tolerance of autolysin-defective pneumococci has first been interpreted as an experimental verification of this model (Tomasz *et al.* 1970).

It is conceivable that in some bacteria a murein hydrolase activity coupled to wall synthesis is responsible for cellular lysis during inhibition of wall synthesis, as predicted by this model. However, in pneumococci, subsequent studies have encountered difficulties with this interpretation. In short, while the lytic agent 'provoked' during penicillin treatment was clearly the amidase, it was difficult to see how this type of activity could be coupled to a synthetic reaction. Also, if this enzyme activity were needed for wall expansion, constitutive amidase-defective mutations should be lethal. Furthermore, indirect evidence indicated that the pneumococcal amidase may only be active at the end of the cell cycle catalysing the separation of daughter cells.

In view of these findings, it has become a mystery again why arrest of cell wall synthesis should provoke the suicidal activity of the pneumococcal murein hydrolases.

Natural inhibitors of murein hydrolases

In 1975, J. Holtje, in our laboratory, demonstrated the powerful and specific autolysin-inhibitory effect of the pneumococcal Forssman antigen and suggested that regulation of autolysin activity may be one of the physiological functions of lipoteichoic acids (Holtje & Tomasz 1975). A collaborative study has extended the validity of the pneumococcal findings by demonstrating the autolysin inhibitory properties of polyglycerophosphate-type lipoteichoic acids in several other bacterial systems (Cleveland *et al.* 1975). Subsequently, certain phospholipids were also shown to inhibit the autolysis of *Streptococcus faecalis* (Cleveland *et al.* 1976).

The existence of such potential autolysin regulatory substances in bacteria has turned our attention to the metabolism of Forssman antigen during penicillin treatment of pneumococci. It was found that autolysin-defective mutants (and wild-type cells as well) have rapidly released an autolysin inhibitor into the growth medium upon addition of penicillin and other cell wall inhibitors to the cultures (Tomasz & Waks 1975). The inhibitor was subsequently identified as a complex of the Forssman antigen and some as yet unidentified macromolecules (Hakenbeck *et al.* 1978). Rapid and massive release of acylated polyglycerophosphate-type lipoteichoic acids as well as cellular lipids (Horne *et al.* 1977; Horne & Tomasz 1979) was also documented during treatment of *Str. sanguis* and other bacteria with cell wall inhibitors.

On the basis of these and other observations, another model ('autolysin triggering') was suggested to explain the mechanism by which a hydrolase-catalysed cell wall degradation may be provoked during penicillin treatment. In essence, this model proposes that the pneumococcal amidase is inhibited during most of the cell cycle by inhibitor(s) containing the Forssman antigen. Penicillin treatment causes a dissociation of the enzyme–inhibitor complex and the inhibitor is actually lost to the medium by a mechanism that is not fully understood at present. On the other hand, inhibitors of protein, RNA and lipid synthesis (i.e. agents that are known to antagonize the irreversible effects of penicillin) were all found to suppress the penicillin-induced release of lipids and lipid–teichoic acid complexes (Tomasz & Waks 1975). At the present time, the only known physiological function of the pneumococcal autolysin has to do with the separation of daughter cells. It is conceivable that the physiological activity of this enzyme is also triggered at the end of the cell cycle by a transient, genetically programmed halt or

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perturbation of murein synthesis by a mechanism similar to the premature antibiotic-induced triggering, except that the physiological activity would be localized, transient and properly timed. The essence of the penicillin-induced cell lysis (and, at least in part, cell death as well) would then be the disturbance of the cellular control of this enzyme, the activity of which would be provoked by the antibiotic treatment on the wrong scale, at the wrong place and at the wrong time (Holtje & Tomasz 1977).

Clearly, disturbance of autolysin regulation must be the ultimate consequence of the inhibition of PBPs, which represent the primary targets of β-lactams. This inhibition can initiate a series of events along the pathway that eventually leads to what I referred to as 'triggering' of self-destructive autolysin activity. The nature of triggering is not well understood at present. In our model for pneumococci, we envision triggering as the dissociation of the autolysin–inhibitor complex which may be caused either by some cell wall precursor (e.g. an anomalous form of the bactoprenol carrier lipid, or change in the intracellular concentration of a normal precursor) accumulating in the drug-treated bacteria or by some other aspect of the penicillin-treated cell (e.g. a localized abnormality of wall structure). A more detailed discussion of observations and ideas that bear upon the mechanism of β-lactam-induced cell death and lysis may be found in a recent review (Tomasz 1979).

The observations described in these comments further illustrate the extreme complexity of the mode of action of penicillins. In fact, it is fair to state that studies during the last 5 years have produced more questions than answers on every level of the interactions of β-lactams and bacteria, and at present we do not understand exactly how and why the inhibition of the primary biochemical targets of β-lactams leads to the various types of growth inhibitory effects. It is safe to predict that 50 years after the discovery of these remarkable antibiotics, the mode of action of β-lactams will remain an exciting and rewarding area of studies.

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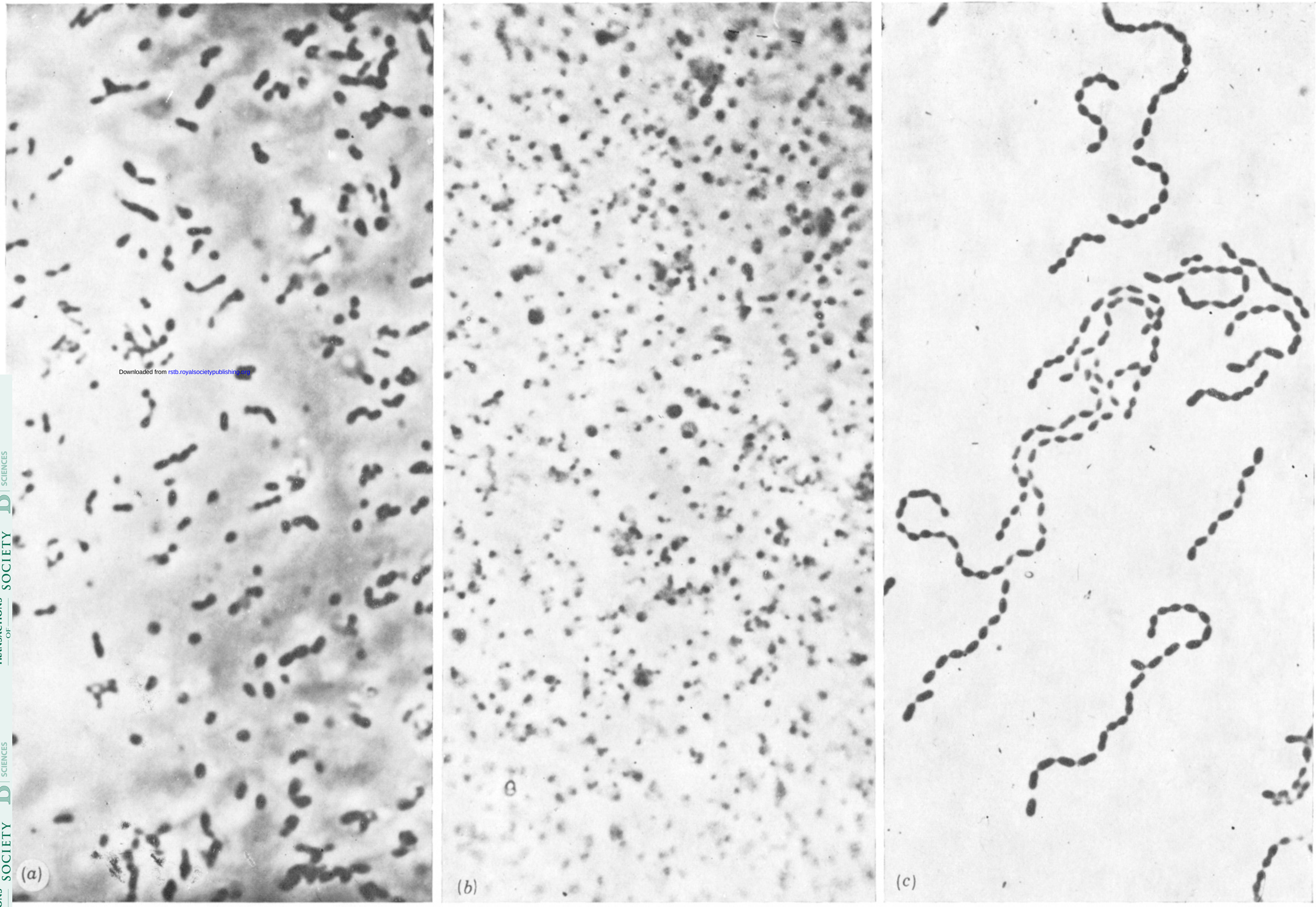

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