CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

IV. Comparative Responses of Gram-positive and Gram-negative Bacteria to Penicillin[1]

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It is well established that in general large differences exist in the sensitivity of gram-positive and of gram-negative organisms to penicillin; the differences are so large, in fact, that most infections due to gram-negative organisms are classed as refractory to penicillin treatment, although it is known that at sufficiently high concentrations of the antibiotic *in vitro* many such organisms are inhibited. It is also well established that there are relatively wide differences in sensitivity to penicillin among different species of susceptible bacteria and even among different strains or races of a single susceptible species (Herrell, 1945). It seems of interest, therefore, to ascertain whether the same mechanism of penicillin action operates in gram-negative organisms as in gram-positive, but perhaps at a higher threshold level, or whether an entirely different mechanism of action must be sought. This problem is of practical as well as theoretical importance, since, if the same mechanism is operative in both types of organisms, but merely at different threshold levels, it may be possible to find a practical means of lowering the threshold of sensitivity in the more resistant organisms and thus to bring them within the scope of effective action of penicillin in practical clinical doses. The advantages to be anticipated from such a procedure are obvious, since the superiority of penicillin over other currently available antibiotics on the basis of toxicity, untoward reactions, development of fastness in the organisms under treatment, etc., is generally recognized.

It is difficult, if not impossible, in the present state of knowledge to define with certainty the precise biochemical and biophysical mechanisms through which penicillin exerts its effect on susceptible organisms. The fact that it exerts a strong selective action in relatively low concentrations against many types of bacterial cells without manifesting any appreciable toxicity toward other living structures indicates that it does not owe its action to a drastic, general protoplasmic poisoning as do many other antibacterials (halogens, salts of heavy metals, phenols, etc.), but it is reminiscent rather of the "receptor" hypothesis of Ehrlich (1908, 1914), who believed that antiseptic agents act against susceptible cells by linking chemically with reactive groups contained therein. Specific tests with different stains and reagents may be visualized, in a way, inasmuch as they help to reveal chemical changes that occur in cells and colonies under the

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influence of penicillin, as tending to define the "receptor" systems chemically. No one of the tests alone can be taken as conclusive evidence of the chemical groups or systems involved in inactivation of bacterial cells by penicillin, but the results of a number of such tests with different reagents may be interpreted as convergent lines of evidence that tend to suggest mechanisms of action. The results of such investigations on *Staphylococcus aureus* have provided evidence that a threshold effect obtains on penicillin assay plates and that that effect depends, in part at least, upon an oxidation-reduction threshold. The sharp boundaries at the margins of inhibition zones may be taken as representing visible expressions of an irreversible shift of -SH to S-S in the cytoplasm of the cells (Dufrenoy and Pratt, 1947*a*). The outlines of the zones of inhibition, where little or no growth occurs, are accentuated by the fact that the bounding rims of the zones are composed of narrow bands in which the growth of the test organisms is enhanced over that of the cells further removed from the central cylinder containing penicillin. These bands occupy a region of the plate within the range of diffusion of appreciable concentrations of penicillin but slightly beyond the range of diffusion of bacteriostatic concentrations. This observation is consistent with the observation that at sufficiently low concentrations *in vitro* penicillin exerts a stimulating effect on microorganisms (Miller, Green, and Kitchen, 1945; Eriksen, 1946; Curran and Evans, 1947).

Techniques similar to those previously employed in this laboratory were used in the present work to study the reactions on penicillin assay plates seeded with the gram-negative organisms *Escherichia coli* and *Proteus vulgaris*, as well as on plates seeded with the gram-positive *Staphylococcus aureus* and *Bacillus subtilis*.

MATERIALS AND METHODS

Assay plates were prepared and incubated, except where noted to the contrary under the individual experiments, according to the procedure prescribed for the modified cylinder plate method involving physical development (Goyan, Dufrenoy, Strait, and Pratt, 1947). After being seeded and subjected to suitable periods of preincubation (without penicillin) and of secondary incubation (with penicillin), they were treated according to the techniques previously described (Dufrenoy and Pratt, 1947*a*,*b*; Pratt and Dufrenoy, 1947*a*,*b*) with appropriate reagents intended to reveal specific reactive compounds or groups, or classes of reactive compounds or groups. Critical examination and study of different parts of plates so treated reveal much information concerning chemical changes that occur in bacterial colonies exposed to lytic, bactericidal, bacteriostatic, and subbacteriostatic concentrations of different agents.

A crystalline preparation of sodium penicillin G that assayed 1,549 units per milligram was used for the experiments. The organisms that were studied were *Staphylococcus aureus* NRRL-313 (same as FDA strain 209P), *Bacillus subtilis* NRRL-B-558, *Escherichia coli*, and *Proteus vulgaris* HX19. It was necessary to use different schedules of incubation for each organism because of their different inherent rates of growth. The purpose of the preliminary incubation is to permit the organisms to reach the logarithmic phase of their growth curve in which

they are most sensitive to penicillin before coming in contact with the antibiotic. In the 3-hour cylinder plate assay for penicillin (Goyan, Dufrenoy, Strait, and Pratt, 1947) it is desirable to choose the length of the second period of incubation, the period during which penicillin is permitted to diffuse on the plates, so as to provide a balance between growth of the organisms (an exponential function of time) and diffusion of antibiotic (a linear function of time) such that the curve relating log diameter of zone of inhibition to log concentration of antibiotic is linear. If too short a period of diffusion is permitted, obviously no zone of inhibition develops. If too long a period of secondary incubation is provided, the biological factor (growth) overrides the physical factor (diffusion) and a nonlinear calibration curve results. Proper control of the secondary period of incubation was important in the present work also, because, if too long a period of contact with inhibiting concentrations of the antibiotic was permitted, extensive lysis occurred in the inhibition zones, and colonies in intermediate stages of inactivation, such as are desirable for cytochemical studies, became very scarce. The most useful schedule of incubation was found to be as follows:

ORGANISM	DURATION OF INCUBATION AT 37 C	
	Primary period (without antibiotic)	Secondary period (with antibiotic)
S. aureus	3 hours	3 hours
B. subtilis	2 hours	2.5 hours
E. coli	3 hours	5 hours
P. vulgaris	3 hours	3 to 6 hours

EXPERIMENTS AND RESULTS

Results observed following tests with various dyes and reagents on penicillin assay plates seeded with gram-positive and with gram-negative organisms are summarized in tables 1 and 2, respectively.

The experiments have shown that, in general, essentially the same pattern appears on penicillin assay plates subjected to the various stains and reagents after incubation whether they are seeded with the penicillin-resistant, gram-negative organisms *Escherichia coli* and *Proteus vulgaris* or with the sensitive gram-positive organisms *Staphylococcus aureus* and *Bacillus subtilis*. This suggests that penicillin elicits essentially the same responses in the two classes of organisms if appropriate concentrations are employed (see below). It is interesting to note in this connection that, using a different technique, Schuler (1947) concluded that penicillin acts on gram-positive and on gram-negative organisms through the same mechanism.

Each zone of inhibition on the assay plates is bounded by a clearly discernible narrow ring formed by a very abundant growth of the test organisms that markedly exceeds the growth on other portions of the plate. Since a diffusion gradient must exist around the cylinders containing the penicillin solutions, these regions of enhanced growth immediately outside the zones of inhibition may be interpreted as representing areas in which the concentration of penicillin, although

TABLE 1

Response to different dyes and reagents on penicillin assay plates seeded with gram-positive organisms

REAGENT	GROUP ASSUMED TO BE ACTIVE	AUTHORITY* AND DESCRIPTION OF TEST	REACTION ON ASSAY PLATES					
			Staphylococcus aureus			*Bacillus subtilis*		
			Color		Definition of boundary	Color		Definition of boundary
			Inside of zone	Outside of zone		Inside of zone	Outside of zone	
K-ferricyanide, ferric sulfate	—SH	Mason, H. L., 1930	Faintly bluish	Deep blue	Very sharp	Faintly bluish	Deep blue	Very sharp
Schiff's	Free aldehydes	Oster, K. A., 1946	Clear, faintly pink	Red	Very sharp deep red ring of enhanced growth	Clear, faintly pink	Deep red	Sharp
Schiff's (after pretreatment of plate with $HgCl_2$)	Aldehydes bound in cells	Oster, K. A., 1946	Faintly pink	Deep red	Very sharp	Test not performed		
Osmic acid	Dienol (*o*-polyphenols)	Dufrenoy, J., 1945	Clear	Dark	Very black ring	Clear	Dark	Sharp black ring
Azo-reaction (in alkaline solution)	Dienol (*o*-polyphenols)	Lison, L., 1936	Faintly pink	Red	Very sharp	Orange	Red	Very sharp, brilliant orange-red ring of enhanced growth
Sakaguchi†	Substituted guanido	Sakaguchi, S., 1925; Vincent, D., & Brygoo, P., 1946	Clear	Pink	Very sharp	Faintly pink	Pink	Poor
Molybdate	$PO_4^{\equiv}$	MacDougal, D. T., & Dufrenoy, J., 1944	Clear gray-blue	Blue	Very sharp deep blue ring of enhanced growth	Golden	Blue	Sharp by transmitted light, poor by reflected light
Hematoxylin after molybdate	Lipidic complex	MacDougal, D. T., & Dufrenoy, J., 1944	Light blue	Deep purple	Moderately sharp. Black	Faint violet	Deep violet	Poor
Toluidine blue	Ribonucleic acid	Jeener, R., & Brachet, J., 1943	Lavender	Purple	Very sharp	Faintly bluish	Deep blue	Sharp
$FeCl_3$	*o*-Diphenols	Lemoigne, M., 1928	Light gray-green	Greenish	Poor	Faintly bluish	Dark blue-green	Sharp
Methyl green (approximately 0.02% solution)	Polynucleotides dehydrogenase systems	Brachet, G., 1942	Green	Pink	Sharp	Faintly greenish	Pink	Poor

* For complete citation see list of references at the end of the paper.
† Plates must be iced before reagents are applied.

TABLE 2

Response to different dyes and reagents on penicillin assay plates seeded with gram-negative organisms

REAGENT*	REACTION ON ASSAY PLATES					
	Escherichia coli			*Proteus vulgaris*		
	Color		Definition of boundary	Color		Definition of boundary
	Inside of zone	Outside of zone		Inside of zone	Outside of zone	
K-ferricyanide, ferric sulfate	Faintly blue-green	Deep blue-green	Very sharp, deep blue ring of enhanced growth	Bluish	Blue	Poor
Schiff's	Faintly pink	Pink	Poor	Pink	Deep red	Very sharp deep rose ring of enhanced growth
	Pink†	Red†	Sharp†			
Schiff's (after pretreatment with $HgCl_2$	Pink	Deep red	Sharp	Pink	Deep red	Extremely sharp
Osmic acid	Clear	Dark	Very sharp black ring	Clear	Dark	Very sharp black ring of enhanced growth
Azo-reaction (in alkaline solution)	Clear	Red	Sharp	Light orange	Red-orange	Sharp
Sakaguchi‡	Faintly pinkish	Pink	Poor	Faintly pinkish	Red-orange	Poor
Molybdate	Blue	Deep blue	Poor§	Blue	Light blue	Good
Hematoxylin after molybdate	Blue-violet	Deep purple	Good	Bright blue-violet	Deep blue (very little violet)	Sharp
Toluidine blue	Lavender	Purple	Sharp	Bluish lavender	Dark purple	Fair
$FeCl_3$	Light yellow-green	Greenish brown	Extremely sharp	Light yellow-green	Greenish-brown	Good
Methyl green (approximately 0.02% solution)	Green	Pink	Sharp; intense deep pink ring of enhanced growth	Green	Pink	Very sharp, intense ring of enhanced growth

* See table 1 for groups assumed to react and for authorities.

† Reaction on plates incubated 18 hours with no preincubation.

‡ Plates must be iced before reagents are applied.

§ Although definition of zones is poor when plates are viewed macroscopically, especially by reflected light, they are very clearly seen when plates are examined under high dry power of microscope. The cells within the zones of inhibition appear as long blue filaments and are sharply differentiated from the uninhibited cells in the normal background. Both filamentous and normal cells stand out clearly from the less intensely stained agar background.

remaining subbacteriostatic, does reach a critical level that is capable of stimulating metabolism and growth. It is well known that, like other antibacterial agents, penicillin in certain subbacteriostatic concentrations may exert a stimu-

lating effect on microorganisms *in vitro* (Miller, Green, and Kitchen, 1945; Eriksen, 1946; Curran and Evans, 1947).[3] The threshold concentration below which penicillin enhances and above which it inhibits metabolism is, however, many times greater for activity toward the gram-negative organisms than toward the gram-positive. For example, on plates seeded with *S. aureus* and treated as prescribed for the FDA cylinder plate assay (Federal Register, **10**, 11478–11485,

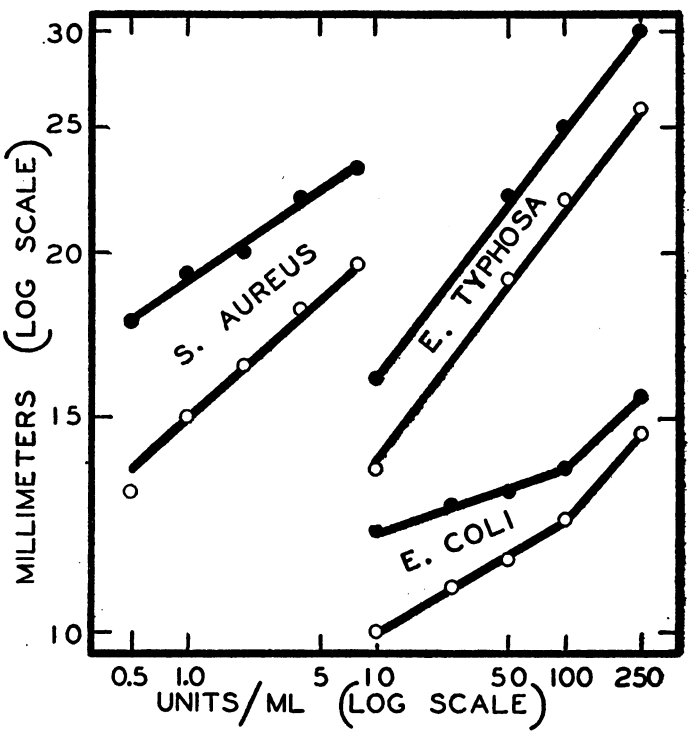

FIG. 1. DIAMETERS OF ZONES OF INHIBITION CORRESPONDING TO DIFFERENT CONCENTRATIONS OF PENICILLIN ON ASSAY PLATES SEEDED WITH S. AUREUS, E. TYPHOSA, OR E. COLI

Open circles are values for plates prepared with the standard test agar. Solid circles are for same agar with $COCl_2 \cdot 6H_2O$ added at the rate of 1 mg/L. All points are averages of values from at least 4 plates. Assays with *S. aureus* were performed by the 3-hour method, others by standard overnight procedure. For convenience in plotting, all values for *E. coli* have been raised 1 mm on the ordinate scale.

1945), a solution containing 1 unit of penicillin per milliliter produced inhibition zones approximately 21 mm in diameter; but a solution containing approximately 100 units of penicillin per milliliter was required to produce a zone of the same diameter on plates seeded with *E. typhosa* or *P. vulgaris*, while solutions containing 250 units per milliliter produced zones only slightly over 13 mm in diameter on plates seeded with *E. coli* (figure 1). It is noteworthy that the addition of

[3] Evidence of similar action of penicillin *in vivo* is less convincing. The authors know of no clear-cut demonstration of such an effect in animals or human patients infected with penicillin-susceptible organisms and treated with penicillin.

trace amounts of cobalt to the test agar markedly lowers the threshold concentration (Dufrenoy, Strait, and Pratt, 1947). Figure 1 shows that the response-dosage curve is shifted along the abscissa when *E. coli* or *E. typhosa* is used as the test organism instead of *S. aureus* and that the curve for a given organism is shifted upward on the ordinate scale when appropriate concentrations of $CoCl_2 \cdot 6H_2O$ are added to the test agar. A similar effect of cobalt in lowering the effective threshold for penicillin has been demonstrated *in vivo* (Pratt, Dufrenoy, and Strait, 1948). The enhancing action of trace amounts of cobalt on penicillin activity appears to be specific and is receiving special study in this laboratory. Salts of nickel, manganese, platinum, iridium, gold, zinc, and copper have been tested in a similar manner over a wide range of concentrations and have failed to produce any similar increase in the diameters of the inhibition zones around cylinders containing penicillin.

DISCUSSION

The tests that are most useful in revealing the chemical changes that occur on penicillin assay plates are those that result in clear-cut differential staining of test organisms vs. agar background and of inhibition zones vs. the general background of uninhibited growth, and that intensify the ring of enhanced growth. These staining reactions, emphasizing the contrasts between the regions of normal growth in the background of the plates, the marginal rings of enhanced growth, and the zones of inhibition, can be interpreted from the physicochemical point of view as manifestations of differential shifts in rH and concomitant shifts in pH in the corresponding parts of the plates. The various levels of rH and of pH can be estimated by the proper use of indicators. From the biochemical point of view these changes may probably be ascribed in large measure to changes in the relative rates of proteogenesis and proteolysis in cells exposed to bactericidal, inhibiting, stimulating, and ineffective concentrations of penicillin. Disturbance of the normal assimilative and growth metabolism might be expected to lead, in turn, to an unequal distribution of different proteins and other cellular components on different parts of the plates. As shown in tables 1 and 2 these changes can be revealed by use of reagents for detecting -SH groups, aldehydes, polyphenols, guanido groups, phosphate ion, polynucleotides, fatty acids and lipids, etc. Surface phenomena, due no doubt in part at least to changes in pH, cannot be ignored in interpreting these results and warrant a full discussion separately.

In our experiments it was convenient to work with inhibition zones approximately 15 to 20 mm in diameter. It was found in our work that to produce inhibition zones in this range on plates that were seeded with *E. typhosa*, or *P. vulgaris*, and to which no cobalt had been added, it was necessary to employ penicillin solutions 10 to 100 times as concentrated as when *S. aureus* was used for the test organism, and that, on plates seeded with *E. coli*, solutions containing as much as 250 units of penicillin per milliliter failed to produce zones of this size. However, when the concentration of penicillin was adjusted so that it fell within a bacteriostatic range, the reactions for the several active groups, levels of rH and pH, etc., were as pronounced and sharp on plates seeded with gram-negative

organisms as on those seeded with gram-positive organisms, and they demonstrated a homologous pattern. In fact, plates treated with a given reagent appeared macroscopically identical, irrespective of the test organism that was used, if the relative times of preincubation and of secondary incubation were chosen so that the plates were developed at the time when the sharpest differential could be achieved.

For clearest results it is essential that the interaction of the biological and the physical factors be properly balanced. Our experience indicates that failure of a reagent that has revealed a sharp definition of zones on plates seeded with a given organism to "develop" properly plates seeded with another organism may be ascribed primarily to too long a preincubation period. If the primary incubation period exceeds the duration of the lag period, growth of the test organisms on the plates becomes too dense before the penicylinders[4] are placed thereon and diffusion of penicillin is permitted to begin. Under these conditions the subbacteriostatic effect corresponding to "below threshold" concentrations of penicillin may fail to be differentiated from the bacteriostatic effect corresponding to "above threshold" concentrations. For example, the Sakaguchi test (tables 1 and 2) very clearly revealed the prevalence of substituted guanido groups in the zone of enhanced growth on penicillin assay plates seeded with *S. aureus*. The failure of the reagent to provide a sharp response on plates seeded with other test organisms may be ascribed to improper timing of preincubation or secondary incubation periods.

Methods that require flooding of the test plates with reagent solutions are subject to the criticism that the flooding operation may dislodge some of the test organisms from their initial position on the plates and that, consequently, the pattern which develops following the chemical treatment may fail to correspond to the original pattern of distribution of the several reactive groups. Therefore, to eliminate this objection, in the present experiments all results obtained with the several reagents were checked as to sizes of inhibition zones, distribution of enhanced growth, bacteriostasis, and bacteriolysis, on plates which, at the end of the second incubation period, were inverted over a watch glass containing a solution of osmic acid that was stabilized by chromic acid. It was seen that the results on plates so treated corresponded with the observations made on plates treated with the other reagents. The development of the plates exposed to the vapors of osmic acid can be watched easily as it progresses—first, the ring of enhanced growth darkens, and then it blackens as the general background darkens. Microscopical examination of such plates under oil immersion shows that osmic acid is reduced in the vacuolar solution of the test organisms. The reduction occurs more rapidly and is more evident in the zones of enhanced growth. Plates thus "fixed" by fumes of osmic acid may subsequently be stained by appropriate cytological stains, for further study of the cytochemical structure. Osmic acid is reduced rapidly to black osmium oxide in the vacuolar solution and

[4] Trade name for standard cylinders used in assaying penicillin solutions by the agar plate method. Penicylinders are available from Eimer and Amend, New York, and from other firms that supply laboratory apparatus.

in other parts of living cells that contain phenolic materials. To a certain extent it may also be reduced in contact with certain fatty materials. The formation of the black deposit in contact with phenolic compounds has never been clearly explained in terms of physical chemistry. In view of what happens with other metals chelation might be surmised.

The results of our experiments suggest that in penicillin-sensitive organisms the gram-positive complex, which is known to consist of a magnesium ribonucleate involving a sulfhydryl group (Henry and Stacey, 1946; Bartholomew and Umbreit, 1944), accelerates the action of the penicillin molecules in inactivating -SH groups which form essential links in the chain of metabolic reactions involved in growth. Under the effect of penicillin the "gram-positiveness" disappears. This is significant in view of the hypothesis that has been developed in this and in earlier papers (Dufrenoy and Pratt, 1947*a*; Pratt and Dufrenoy, 1947*b*), since it is known that the gram-positive complex loses its characteristics as its -SH groups become dehydrogenated to S-S (Henry and Stacey, 1946). In microorganisms which lack the gram-positive complex the concentration of penicillin must be increased many times to obtain the bacteriostatic effect; but irrespective of the minimum dosage required to produce bacteriostasis, the sequence of events is always the same: first the microorganisms undergo a phase of enhanced activity, during which they develop pronounced reducing power, and the cells at the margins beyond the range of diffusion of bacteriostatic concentrations of penicillin manifest the characteristic symptoms of the logarithmic phase of growth. This is the period during which the dehydrogenases are most active, and the rH of the medium tends to drop to the lowest value. This change is evidenced by reduction of Redox indicators. During this phase of growth the organisms store phenolic compounds in their vacuolar solutions which, therefore, acquire the aptitude to absorb (or adsorb) basic fuchsin, phenosafranine, neutral red, etc. The phenolic compounds can be demonstrated by the action of mild oxidants such as potassium dichromate or potassium iodate, which oxidize them to brownish yellow quinoid derivatives. Conversely they can also be demonstrated in the vacuolar solution of the bacteria by virtue of their reducing action toward osmic acid, silver nitrate, etc., or by the formation of darkly colored metallic complexes with ammonium molybdate (Marchal and Girard, 1947) or with ferrous salts. Where positive reactions can be obtained for phenolic compounds, a sharp positive reaction can be obtained for -SH groups through the formation of Prussian blue, by treatment with potassium ferricyanide followed by ferric sulfate.

One other point should be mentioned in connection with the observation that a ring of enhanced growth always surrounds the zones of inhibition on penicillin assay plates. It has been pointed out above that these rings of enhanced growth probably represent a visible manifestation of enhanced metabolism induced in cells in that region by subbacteriostatic concentrations of penicillin. It should be observed, however, that other factors may contribute to the enhancement of growth in those areas circumjacent to the areas where growth is inhibited and lysis of cells occurs. It is not impossible that as cells in the areas of inhibition

are affected by bacteriostatic concentrations of penicillin, some of their components are liberated into the agar, through which they may diffuse to regions of the plate in which the concentration of penicillin fails to reach a bacteriostatic level. A number of references in the literature indicate that products liberated by dying microorganisms may serve as growth factors for survivors (Nicolle and Faguet, 1947; Lasfargues and Delaunay, 1947; Cook and Cronin, 1941; Loofbourow, 1947; Webb and Loofbourow, 1947). Some of these substances, especially the nucleoproteins, might then be absorbed by the bacterial cells outside of the zones of inhibition and serve as metabolites or growth factors. Support for such a hypothesis is afforded by the experiments of Bonét-Maury and Perault (1945). By the use of a recording photometer they observed that when *S. aureus* was cultured in the presence of small amounts of penicillin in broth two waves of growth occurred. The results may be interpreted as indicating that when *S. aureus* cells are suspended in broth containing very small amounts of penicillin, the most sensitive organisms, which are first affected, release into the medium substances that promote a second wave of growth among the more resistant cells. Therefore, the possibility should not be overlooked that the enhancing action that is apparent as a "space" effect on assay plates is comparable in some measure to the action which the recording photometer demonstrates as a "time" effect in suitable broth cultures.

SUMMARY

A study has been made of physical and chemical changes that occur in different parts of penicillin assay plates seeded with gram-positive and with gram-negative test organisms. The techniques that were used were intended to reveal differential changes that occur in cells under the influence of bactericidal, lytic, inhibiting, and stimulating concentrations of penicillin as contrasted with the reaction of cells in the normal background where the concentration of penicillin remains ineffective.

It was found that the same pattern developed on all penicillin plates treated with a given reagent, irrespective of the test organism, provided the proper concentrations of penicillin were used and provided the proper balance of the biological and physical factors involved in the cylinder plate method of assay was achieved. The latter was found to be largely a matter of properly controlling the relative lengths of the primary incubation period, when the organisms were in the lag period, and of the secondary incubation period, during which the organisms were in the log phase of growth and during which penicillin was diffusing through the medium in the plates.

The evidence indicates that penicillin affects aerobic gram-positive and gram-negative organisms through the same chemical systems. The threshold concentration at which its effects become manifest is, however, many times greater on plates seeded with gram-negative organisms than on those seeded with gram-positive organisms.

The proper use of trace amounts of cobalt lowers the effective threshold on test plates, a fact which may have practical clinical importance as well as theoretical interest, since the same phenomenon has been demonstrated *in vivo*.

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