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THE SENSITIVITY OF BACTERIA TO THE ACTION OF PENICILLIN

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INTRODUCTION

THE clinical use of penicillin in the treatment of infections is determined by the sensitivity of the causal organisms. It has been customary to distinguish bacteria as being either " penicillin sensitive " or " penicillin resistant." On the basis of some *in vitro* test, such as the " agar plate and gutter test," an organism is found to be " sensitive " or " resistant " according to whether or not its growth is inhibited by penicillin in a concentration of about 0·1 to 0·3 units per ml. This distinction is in many respects a useful one, for during systemic administration at normal dosage rates (*e.g.* 100,000 to 200,000 units per day) the penicillin content of the blood serum and of infective exudates is commonly about 0·05 to 0·1 units per ml. and is seldom greater than 0·3 units per ml. (McAdam *et al.*, 1944 and 1945). It is possible, however, to obtain much higher concentrations of penicillin in the body fluids ; for example, up to a few units per ml. in the serum during systemic administration at high dosage rates, from 10 to 500 units per ml. in the urine during systemic administration at normal dosage rates, and up to 10, 100 or 1000 units per ml. in the cerebrospinal fluid and in serous cavity, abscess cavity and wound exudates, when penicillin is introduced locally. Many of the organisms normally considered " penicillin resistant " are fully susceptible to these higher concentrations of penicillin ; for example, Strept. fæcalis (Helmholz and Sung, 1944 *a* and *b*), some Brucella strains (T'ung, 1944), and some of the Gram-negative bacilli (Thomas and Levine, 1945). The possibility is thus suggested that, in suitable cases, infections with these " resistant " organisms may respond to penicillin treatment.

Quantitative sensitivity measurements for *a number of strains of the same species* have been recorded by several authors : for Staph. aureus, by Rammelkamp and Maxon (1942), Spink, Ferris and Vivino (1944) and Plough (1945) ; for various streptococcal species, by Watson (1944) and Dawson, Hobby and Lipman (1944) ; for the gonococcus, by Cohn and Seijo (1944) and Lankford (1945) ; for the

Brucella group, by T'ung (1944); for B. ducreyi, by Mortara, Feiner and Levenkron (1944); for the Gram-negative intestinal bacilli, by Thomas and Levine (1945); for B. proteus, by Stewart (1945); for B. influenzæ, by Gordon and Zinnemann (1945); and for a variety of species, by Selbie, Simon and McIntosh (1945) and Meads, Ory, Wilcox and Finland (1945). Considerable variation, up to ten-fold or one hundred-fold, was commonly found in the sensitivity of different strains of a single species. Just as some strains of a normally " sensitive " species may be resistant to penicillin in concentrations of 0·1 to 0·3 units per ml., a few strains of a normally " resistant " species may prove sensitive to such concentrations; for example, two pathogenic strains of B. influenzæ (Forgacs and Hutchinson, 1945).

Comparison of the sensitivity measurements recorded by different authors is rendered uncertain because of differences in the size of the inoculum employed in the tests. It has been shown by Rammelkamp and Keefer (1943) and Hobby and Dawson (1944*a*) for Strept. pyogenes, by Rantz and Kirby (1944) and Kirby (1945*a*) for Staph. aureus, and by Shwartzman (1944) for B. coli, that greater concentrations of penicillin are necessary for the inhibition and killing of large numbers of organisms in a given volume of medium than for the inhibition and killing of small numbers in the same volume. Accordingly, in the present sensitivity examinations of different strains and species, the inoculum of organisms was kept, as far as practicable, at a constant size. The extent to which the results obtained are comparable is limited to the accuracy of the method for determining the inoculum size and, even more, where different conditions of culture had to be adopted.

Method

Except in the case of certain organisms which required special conditions for growth, all the tests were carried out in the following uniform manner. The inoculum was prepared from a twenty-four hours' culture; its size was controlled by taking a measured volume of an appropriate dilution of a bacterial suspension made up to a standard opacity. From 100,000 to 300,000 organisms were added to each of a series of tubes which contained, in 1 ml. of tryptic horse-flesh digest broth, the different concentrations of penicillin (as shown in Fig. 1). The penicillin was taken from a single batch of one commercial brand of the sodium salt (Pfizer); the contents of some ampoules of this batch had been assayed and found to contain the stated amount of penicillin. With each set of tests, a control test with the standard " Oxford " Staph. aureus was included. After twenty-four hours' incubation at 37° C., the tubes were examined for the presence of growth, as denoted by visible turbidity or deposit. In tests of B. pestis, the Brucella organisms, B. smegmatis, B. butyricus and B. phlei, a larger initial inoculum was used: from 1,000,000 to 3,000,000 organisms; the tubes were examined for growth after forty-eight hours as well as after twenty-four hours. Tests of the gonococcus, the meningococcus, B. influenzæ and B. pertussis were carried out in a different manner: a loopful of a suspension containing about 100,000,000 organisms per ml. was spread on the surface of each of a number of 10 per cent. blood agar plates which

incorporated the different concentrations of penicillin; after twenty-four and forty-eight hours' incubation the plates were examined for growth. In tests of the tubercle bacillus, a loopful of a culture suspension was spread on the surface of Lowenstein egg medium slopes; for each strain examined, two inoculated slopes were used as controls and other four were treated with penicillin in different concentrations, 0·15 ml. of solution containing 100,000, 32,000, 10,000 or 3200 units per ml. being introduced at the bottom of the sloped medium which was 1·5 ml. in volume. Assuming even diffusion of the penicillin throughout the medium, the final concentrations in the different tubes would be 10,000, 3200, 1000 and 320 units per ml. Fresh addition of penicillin was made every second day during incubation to allow for such inactivation as might occur. The penicillin concentration to which the tubercle bacilli were exposed must have varied considerably during the period of incubation; however, assays of the fluid at the bottom of the slopes suggested that, in the main, the concentration fluctuated about the initial level. The slopes were examined for growth at intervals during one month of incubation.

Sources of the Strains

Old stock cultures were examined in the case of the gonococcus, the meningococcus, the Brucella group (3 abortus, 2 suis and 4 melitensis strains), B. anthracis, B. pestis, B. influenzæ, B. pertussis, B. typhosus, B. paratyphosus A and B, B. dysenteriæ (2 Shiga, 2 Flexner and 2 Sonne strains), V. choleræ, B. smegmatis, B. butyricus and B. phlei. The strains of the other species examined had been freshly isolated from human sources, where they had been present in a pathogenic or commensal rôle; except for some of the staphylococcal strains, they had *not* been exposed to penicillin treatment. The strains of Strept. fæcalis, B. coli, B. ærogenes, B. proteus, and B. pyocyaneus had been isolated from infected wounds, fæces, milk and urine. The strains of Strept. pyogenes, all of which produced soluble hæmolysin, had been recovered from the throats of patients with scarlet fever or tonsillitis. The strains of Strept. viridans had been isolated from the saliva of normal persons and the blood of patients with subacute bacterial endocarditis. All of the strains of Staph. aureus were coagulase positive; about *half* were from infections such as osteomyelitis, breast abscess, hand suppuration and septicæmia, about *quarter* from the anterior nares, skin and clothing of healthy persons and about *quarter* from infected wounds of patients who had received a short prophylactic course (a few days in duration) of systemic penicillin treatment.

Results

The results of the investigation are illustrated in Fig. 1. This shows the number of strains of each species which were just susceptible to each of the different concentrations of penicillin (*e.g.* the Fig. " 2," for B. ærtryke, placed between the abscissæ for 10 and 32 units per ml., denotes that, in tests of *two* strains of B. ærtryke, growth took place in the presence of 10 units per ml., but not in the presence of 32 units

per ml.). In the case of several species, there was a great difference in sensitivity between the least sensitive and the most sensitive strains. This difference was one hundred-fold in the case of Staph. aureus, Strept. viridans, Br. melitensis and B. ærogenes, and was one thousand-

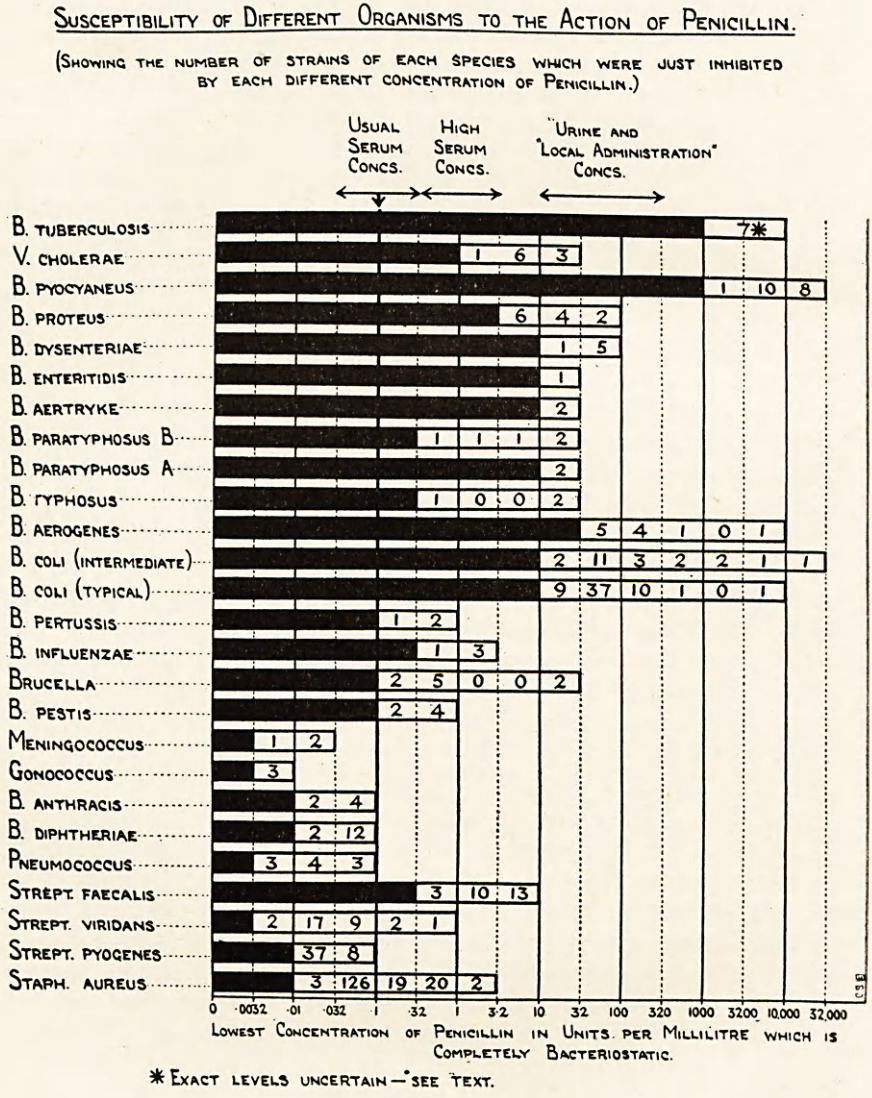

Fig. 1.

fold in the case of B. coli. Similar great variation in sensitivity might have been found in the case of some of the other species, if a larger number of strains of these species had been examined.

When the strains of Staph. aureus were grouped according to their source, it was found that the proportion of "resistant" strains was

not the same in each of the different groups. This differentiation was observed in a series of examinations by the " agar plate and gutter test " on a somewhat extended range of strains.* Of 95 strains isolated from closed infective lesions such as osteomyelitis, breast abscess and septicæmia, only one (1·1 per cent.) was " penicillin resistant " ; of 66 strains isolated from the anterior nares, skin and clothing of healthy persons, three strains (4·5 per cent.) were " penicillin resistant " ; and of 49 strains isolated from infected war wounds subsequent to systemic penicillin treatment, eighteen strains (37 per cent.) were " penicillin resistant." It is of interest to compare these results with the findings of Gallardo (1945) ; of 85 strains of Staph. aureus isolated from wounds prior to penicillin treatment, Gallardo found that 13 per cent. were " penicillin resistant," and that a further 9 per cent. acquired resistance during the subsequent penicillin treatment (see also Plough, 1945).

As will be seen from Fig. 1, penicillin in a concentration of 0·1 units per ml. was inhibitory for most strains of the species normally considered " penicillin sensitive," and was not inhibitory for any strains of the species normally considered " penicillin resistant." Some strains of Strept. fæcalis, B. pestis, the Brucella group, B. influenzæ, B. pertussis, B. typhosus and B. paratyphosus B were susceptible to penicillin in concentrations of 1 and 3·2 units per ml. As such concentrations are obtained in the serum during systemic administration of large doses, systemic infections with these partially resistant organisms may in some cases respond to penicillin treatment. Many strains of almost all the " penicillin resistant " species were found to be susceptible to penicillin in concentrations of from 10 to 320 units per ml. Penicillin concentrations of this order are readily attained in the urine and in the exudates of locally treated cavities and surfaces. It is possible, therefore, that urinary infections and accessible local infections, caused by the Gram-negative bacilli, may in many cases be amenable to penicillin treatment. It must be remembered, however, that the numbers of organisms present in infected exudates and urine may be much greater than the numbers of organisms initially present in the sensitivity test mixtures ; when this is the case, higher concentrations of penicillin will be required than those indicated by the results of the sensitivity tests. For example, a strain of B. coli which was susceptible to 32 units per ml. when the inoculum was 300,000 organisms per ml., was not susceptible to less than 100 units per ml. when the inoculum was 30,000,000 organisms per ml.

The most resistant organisms encountered, which were able to grow freely in penicillin concentrations of 320 units per ml. or higher, included a few of the strains of B. coli and B. ærogenes, and all of the tested strains of B. pyocyaneus, B. tuberculosis and the saprophytic acid-fast bacilli. Even these very highly resistant organisms, however, were found to be inhibited by penicillin when the concentration was

* Carried out in collaboration with Dr S. W. Challinor of this department.

sufficiently increased. The strains of B. pyocyaneus, B. coli and B. ærogenes were inhibited by 1000, 3200, 10,000 or 32,000 units per ml.; the acid-fact saprophytes were susceptible to 1000 units per ml.; the strains of B. tuberculosis failed to grow in the culture tubes to which 3200 or 10,000 units per ml. were added every second day. Thus, under the conditions of these tests, *absolute resistance to the action of penicillin was not found in the case of any species or strain examined.*

The Action of Penicillin on "Resistant" Organisms

It was necessary to consider the possibility that the inhibition of growth of the more resistant organisms by strong solutions of penicillin was not due to the specific activity of the penicillin, but was due instead to some non-specific activity of the penicillin or of the impurities present in the preparation used. It is not thought that this was the case; evidence was obtained in several ways to suggest that inhibition was indeed due to the specific activity of penicillin. *Firstly*, the inhibitory action of the penicillin solutions on "resistant" organisms was annulled by the addition of penicillinase. *Secondly*, the action of penicillin on "resistant" organisms was similar to its action on "sensitive" organisms, as regards its relationship to the size of the inoculum and the nutrient character of the medium. *Thirdly*, similar characteristic morphological changes were produced in the "sensitive" and in the "resistant" organisms by effective concentrations of penicillin.

The Effect of Penicillinase.—Experiments to show that inhibition was annulled by penicillinase, were carried out with sodium penicillin (the Pfizer penicillin, and also Glaxo penicillin of 669 units per mg.). The penicillinase * used was the filtrate of a B. subtilis broth culture prepared in the presence of penicillin. In parallel with the usual sensitivity test for each strain, a test was carried out in which each of the different penicillin concentrations was made up in a mixture of 0·1 ml. of penicillinase solution and 0·9 ml. of broth. The mixtures were allowed to stand for one hour before inoculating and incubating. Tests were carried out in this manner with twelve strains of B. pyocyaneus, nine strains of intermediate B. coli, two strains of typical B. coli, two strains of B. ærogenes and one strain each of the smegma bacillus, the butter bacillus and the timothy-grass bacillus. In every case the presence of penicillinase was found to allow growth of the organism in penicillin concentrations which otherwise would have been inhibitory. Thus, in tests with the Glaxo penicillin, the smegma bacillus, the butter bacillus and the timothy-grass bacillus were found to be inhibited by a penicillin concentration of 1000 units per ml. in the absence of penicillinase, while not to be inhibited even by a concentration of 3200 units per ml. in the presence of penicillinase; (some

* Prepared by Miss M. L. Campbell-Renton and Dr S. W. Challinor of this department.

inhibition, presumably non-specific, was given by 10,000 units per ml. in the presence of penicillinase). Six strains of B. pyocyaneus were tested against the Pfizer penicillin and another six against the Glaxo penicillin; in the absence of penicillinase, growth was inhibited by 10,000 or 32,000 units per ml., while in the presence of penicillinase no strain was inhibited by 32,000 units per ml. In tests with the Glaxo penicillin, the strains of B. coli and B. ærogenes were inhibited variously by concentrations of from 100 to 10,000 units per ml. in the absence of penicillinase; in the presence of penicillinase, no inhibition was given by 10,000 units per ml.

The Bactericidal Action of Penicillin.—The mode of action of penicillin on "resistant" organisms was found to be similar to its mode of action on "sensitive" organisms. When sub-inoculations (by "plating out") were made from the sensitivity test mixtures, first before and then after incubation, it was found that effective concentrations of penicillin killed the organisms initially present and did not merely inhibit growth; this was demonstrated in the case both of "sensitive" and of "resistant" organisms. Furthermore, when inocula of different sizes were tested in this way (*e.g.* 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 organisms per ml.), it was found that greater concentrations of penicillin were required to inhibit and kill the larger than the smaller inocula: a doubling of penicillin concentration was often required for a hundred-fold increase in the size of the inoculum. This was demonstrated both for the "sensitive" organisms Staph. aureus, B. anthracis and B. diphtheriæ, and for the "resistant" organisms B. pyocyaneus, B. phlei and B. coli (*cf.* Shwartzman, 1944). The dependence of bactericidal action and rate of killing by penicillin upon the nutrient character of the medium has been established in the case of "sensitive" organisms by several investigators (Bigger, 1944; Hobby and Dawson, 1944*b*; Lee, Foley and Epstein, 1944; Miller and Foster, 1944; and Todd, 1945). In the present investigation, the same relationship of the bactericidal action to the nutrient character of the medium was found in the case of a "resistant" organism, B. ærogenes. Changes in the viable count, during twenty-four hours' incubation in the absence of penicillin and in the presence of 1000 units per ml., were observed in cultures inoculated with 100,000 organisms per ml. When a highly nutrient medium was used (*e.g.* nutrient broth, peptone water, or a dextrose and ammonium salt solution), B. ærogenes multiplied *rapidly* in the absence of penicillin (an increase of one thousand to ten thousand-fold in the first eight hours) and was *rapidly* killed in the presence of penicillin (a reduction of one thousand to ten thousand-fold in eight hours). When a poorly nutrient medium was used (*e.g.* saline, tap water or Locke's solution), multiplication was very *slow* in the absence of penicillin (three to thirty-fold in the first eight hours); correspondingly, in the presence of penicillin, killing was also *slow* (ten to three hundred-fold in eight hours).

The Morphological Changes Produced by Penicillin.—Gardner (1940 and 1945) has described certain morphological changes which are produced by penicillin in both "sensitive" and "resistant" organisms. In the present investigation, a study was made of the morphological changes undergone by B. pyocyaneus, B. proteus, B. coli, B. ærogenes, B. paratyphosus B, V. choleræ, B. pestis, B. anthracis B. diphtheriæ, Staph. aureus and Strept. pyogenes. These organisms were observed directly (unstained) while growing on blocks of nutrient agar which incorporated different concentrations of penicillin and

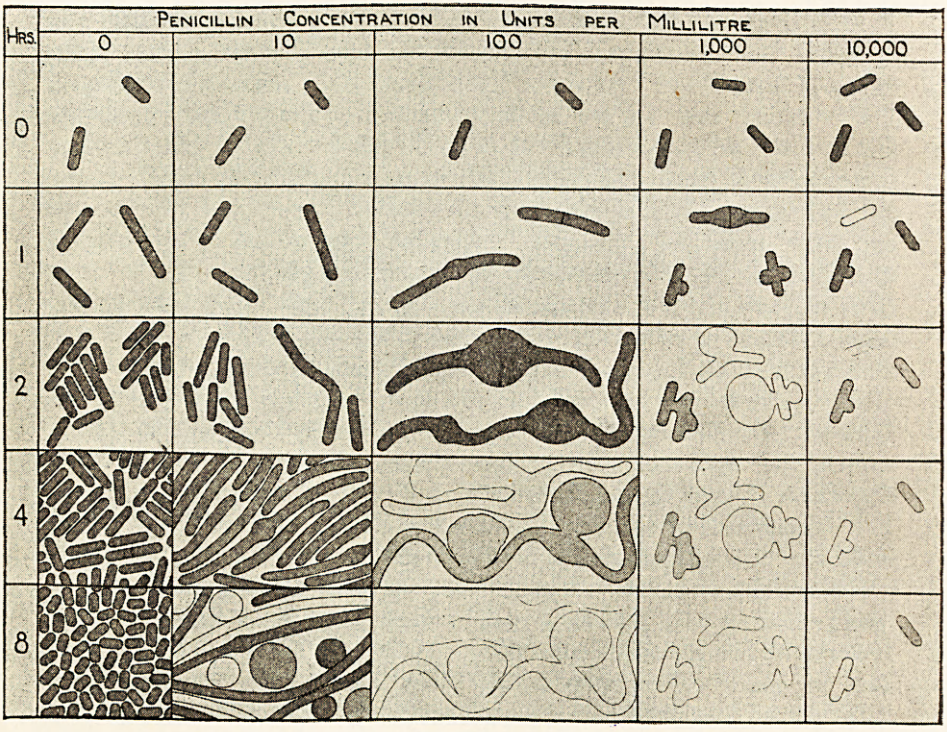

Fig. 2.—The effect of penicillin on growth of B. coli.

which were mounted between a slide and coverslip under an incubated microscope. Essentially the same sequence of changes was found to occur in the "resistant" as in the "sensitive" organisms, although, as might be expected, the exact features presented were to some extent dependent on the initial morphological type of the organisms. (See Fig. 2 for a diagrammatic representation of the changes shown by Gram-negative bacilli in the unstained state.)

(1) *At penicillin concentrations in the neighbourhood of*, e.g. *from one-third to thirty times, the lowest inhibitory concentration for the strain.* There was an initial period of growth which usually lasted for four, five or six hours, but which might be prolonged up to ten hours or even longer with the lower penicillin concentrations, and shortened

to two or three hours with the higher concentrations. During this period, some multiplication took place, together with considerable enlargement of the individual cells ; a single bacterium of the inoculum often gave rise to two, four or eight abnormal giant cells which were destined ultimately to die as a result of the action of the penicillin. Death of the organisms, which terminated the period of growth, was usually marked by visible lysis. In many species, lysis was initiated by the protrusion of " bubbles " of protoplasm. The organism would then become paler in appearance and either disappear entirely or remain visible as a delicately outlined, empty or slightly granular " ghost." Lysis sometimes occurred rapidly, in the course of a few minutes, and sometimes gradually, during the course of an hour or so.

In the case of the Gram-negative bacilli and V. choleræ, enlargement took the form of elongation into filaments of 10, 20, 30 or even 100 microns in length. A large proportion of the filaments developed, at one or more points in their length, spindle-shaped or spherical swellings, from 2 to 5 microns in breadth ; most commonly, a single swelling was found in the middle of a filament, at the site of what appeared to be a transverse septum incapable of developing to the stage of complete fission. In preparations stained by the method of Robinow (1944) for demonstrating cytoplasmic boundaries, the transverse equator of each swelling was seen to be occupied by a band or rounded body, which was intensely stained like the cytoplasmic boundaries ; deeply stained transverse bands were also seen at intervals along the filaments, representing perhaps imperfectly formed boundaries between the component bacterial units. When stained by Robinow's method for demonstrating chromatinic (" nuclear ") bodies, each filament was found to contain these bodies, usually as single or paired transverse rods, distributed in a fairly regular manner along its length ; in the swellings, the chromatinic bodies were clustered irregularly and were usually absent from a large central area which apparently corresponded to the equatorial cytoplasmic mass. Up to the stage of filament formation and swelling, the abnormal cells were apparently alive, for growth and " nuclear " division had been proceeding and normal motility was exhibited in the case of the motile strains (*e.g.* of B. proteus and V. choleræ). Lysis, and thus death, of the filamentous cell was in most cases initiated by the gradual or sudden protrusion, usually from the region of a swelling, of one, two or sometimes more " bubbles " of protoplasm, which varied in diameter from 1 to over 10 microns and which contained numerous chromatinic bodies ; following this, the filament became pale, or even disappeared entirely. Some filamentous cells underwent lysis without any visible protoplasmic protrusion, and some without even having developed a swelling.

In the case of B. anthracis (see Mackie, 1946 ; figure), single bacilli developed into short chains of from two to eight swollen, oval or spherical cells ; these were from 2 to 4 microns in transverse

diameter. Lysis was sometimes initiated by the protrusion of visible " bubbles " of protoplasm. The swollen bacilli shrivelled up, became pale and either disappeared or remained visible as empty " ghosts." In the case of B. diphtheriæ gravis, single bacilli developed into short filaments which consisted of from two to eight cells, marked off from each other by transverse septa, but seldom accomplishing complete fission and separating in the normal manner ; some cells became swollen, up to 2 or 3 microns in breadth. A proportion of the cells underwent lysis, becoming pale and empty looking ; there was no visible protoplasmic protrusion. In the case of Staph. aureus and Strept. pyogenes, swollen, spherical and oval forms were developed, of up to 2 or 3 microns in diameter ; multiplication was often marked by incomplete fission, resulting in the formation of clusters of incompletely separated staphylococci and spindle-shaped chains of closely fused, giant streptococci. A proportion of the cells underwent lysis ; these merely became pale and did not show any visible protoplasmic protrusions. The swollen cells which remained unlysed appeared to be dead ; when placed on fresh nutrient agar, they did not again begin to grow.

(2) *At penicillin concentrations much higher*, e.g. *over thirty times higher, than the lowest inhibitory concentration.* There was no initial period of growth. The cells did not enlarge by elongation or swelling, and they did not multiply except for the occasional completion of a single division. Lysis usually took place within one or two hours. The Gram-negative bacilli became pale or disappeared entirely, sometimes after the protrusion of small " bubbles " of protoplasm and sometimes without this occurring. B. anthracis became very pale, or disappeared entirely, without visible change in shape or protoplasmic protrusion. B. diphtheriæ, Staph. aureus and Strept. pyogenes showed little change.

This sequence of morphological changes exhibited both by " sensitive " and by " resistant " bacteria, appears to be related essentially to the bactericidal action of penicillin. None of the changes were due to the action of impurities present in the commercial penicillin preparations, for exactly the same changes were produced by pure crystalline sodium penicillin (1600 units per mg.) in tests with B. anthracis, B. proteus, B. ærogenes and B. coli.

Conclusions

It appears that all bacteria (within the limits of this investigation) are susceptible in some degree to the specific activity of penicillin. If this is so, the metabolic mechanism or cellular component upon which penicillin exerts its effect, must be possessed in common by all bacterial species. Variations in *degree* of sensitivity may be due to a variety of factors ; in some cases, a high degree of resistance may be due to penicillinase production (Bondi and Dietz, 1944 and Kirby,

1945*b*). Little is known about the mechanism of the bactericidal action of penicillin, beyond that only actively metabolising and growing cells are susceptible. The morphological changes described above as produced by the lower penicillin concentrations, in particular the failure of proper cell division and the ready occurrence of swelling and protoplasmic protrusion, suggest that penicillin in these concentrations interferes specifically with the formation of the outer supporting cell wall, while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and so undergoes lysis. In the higher concentrations, penicillin must act somewhat differently.

SUMMARY

1. Quantitative measurements of sensitivity to penicillin have been carried out on a number of strains of each of a variety of bacterial species.

2. Many strains of those organisms which are usually considered "penicillin resistant" were found to be susceptible to penicillin in concentrations obtainable in infected body fluids.

3. Under the conditions of the tests, absolute resistance to the action of penicillin was not found in the case of any of the strains or species examined.

4. Reasons are given for believing that the nature of the action of penicillin on "resistant" organisms is essentially the same as the nature of its action on "sensitive" organisms. The action of penicillin on "resistant" organisms is annulled by penicillinase. As with "sensitive" organisms, the action of penicillin on "resistant" organisms is bactericidal and not merely bacteriostatic; the lowest bactericidal concentration varies with the initial number of organisms present in a given volume of medium; the rate of killing varies with the nutrient value of the medium. Similar characteristic morphological changes are produced in "sensitive" and in "resistant" organisms by effective concentrations of penicillin.

I wish to express my thanks to Professor T. J. Mackie for his valuable advice and for his interest in this investigation.

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