CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

I. Oxidation-Reduction Levels[1]

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The present investigation was undertaken because it was thought that the results of the experiments might help to elucidate the mechanisms of action of penicillin on susceptible organisms. Although development of new assay methods was not the first objective of the experiments, several of the techniques described below were found to afford reliable and rapid methods of assay that retain the advantages of the cylinder plate technique but eliminate its relatively long incubation period. One of these has already been described (Goyan, Dufrenoy, Strait, and Pratt, 1947). A report of others is in preparation.

Assays of antibiotic agents by means of cylinder plate or cup plate techniques, or their modifications, depend upon a correlation between the concentration of antibiotic in the preparation under test and the diameter of the zone of inhibition that is produced. The zones of inhibition represent areas in which a "static" or "cidal" concentration of the antibiotic exists when the test organisms are in a susceptible stage of growth.

Since there must be a diffusion gradient, outward from the central cylinder or other reservoir of antibiotic, it is reasonable to expect subbacteriostatic concentrations to exist beyond the zones of inhibition. Two observations are of interest in this connection: first, subbacteriostatic concentrations of penicillin have been shown to enhance metabolic activity and growth of *Staphylococcus aureus* (Miller, Green, and Kitchen, 1945; Eriksen, 1946*b*); second, a narrow band of enhanced growth, immediately surrounding the zones of inhibition, is generally evident on test plates and can be seen on published photographs (Eriksen, 1946*b*). A similar zone of markedly enhanced growth immediately surrounding an area of inhibited growth, effected through use of another antibiotic, has been clearly pictured by Burkholder (1944, figure F). In the experiments described below it has been possible to demonstrate by appropriate techniques that these bands of increased growth on penicillin test plates coincide with an area of relatively intense reducing power. It may be suggested that these areas represent regions in which the test organisms have been subjected to subbacteriostatic concentrations of penicillin without subsequent exposure to bacteriostatic, bacteriocidal, or bacteriolytic concentrations, and that, in these areas, the organisms have been stimulated to a state of intense metabolism and growth, perhaps characterized by an abnormally high rate of respiration,

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such as has been ascribed to the "climacteric stage" induced in cells of various tissues by traces of different chemicals (Hansen, 1946). Of course, these bands of increased growth become much more evident and more easily demonstrable with increased time of incubation. This is probably due to the longer time of exposure to subbacteriostatic concentrations of penicillin. The fact that sharp boundaries can be demonstrated between areas of inhibition and noninhibition suggests that a threshold effect operates in these regions of the plates. We shall present convergent lines of evidence from staining experiments that indicate that such thresholds for a number of chemical groupings can be demonstrated on penicillin assay plates. The experiments reported in the present paper are concerned with tests for oxidizing and reducing substances and systems. Other papers describe the results obtained in tests for different constituents that might be expected to be liberated by the dissociation of cellular complexes and the role of detergents in the mechanism of action of penicillin on susceptible organisms.

The use of the techniques employed in the present work was based on the observation that in normal aerobic respiration of the test organism, as of other organisms, a balance exists between the rate of dehydrogenation of —SH groups effective in hydrogen transfer and the rate of restitution of such groups (Genevois and Cayrol, 1939). Therefore, a deficit of hydrogen donors or of oxygen acceptors eventually develops, and dienol groups become irreversibly dehydrogenated into diketones; concomitantly, the removal of hydrogen provides an opportunity for the development of carbon to carbon linkages, so that dehydrogenated phenolic compounds are likely to polymerize to gummy, quinoid derivatives. Evidence for the presence of quinoid groups will be presented in connection with the indophenol blue reagent.

It is noteworthy also that Wood and Cruikshank (1944) showed the climacteric increase in respiration in plant tissues to be followed by a "decrease in organization resistance," making cell proteins more readily subject to hydrolysis. Thus, at least a partial explanation is suggested for the observation (Gardner, 1940; Fisher, 1946; Eriksen, 1946*a*) that an early evidence of the action of penicillin on susceptible organisms is marked swelling of the cells. The breaking down of cell organization might be expected to expose —SH groups which are normally protected within protein molecules, such as glutathione or the nucleoproteins that play a role in dehydrogenase systems. Such exposed —SH groups should be expected to become more readily accessible to dehydrogenation or to blocking agents. Thus we may have a partial explanation for the fact that, in the presence of detergents, which help unfold protein molecules, and in the presence of an —SH-binding substance such as bismuth, penicillin becomes bacteriostatic at a lower concentration than in their absence (Treffers, 1946; Levaditi and Vaisman, 1946).

MATERIALS AND METHODS

The penicillin used in most of the present work was a crystalline preparation of sodium penicillin G that assayed 1,560 Oxford units per mg. Similar results were obtained with commercial sodium and calcium penicillins assaying about

1,100 to 1,200 units per mg and with an impure preparation of calcium penicillin assaying about 300 units per mg.

The test organisms that were used were *Staphylococcus aureus* NRRL strain no. 313 (the same as FDA strain no. 209P) and the rough form of *Bacillus subtilis* NRRL strain no. B-558. Seeded plates were prepared as prescribed in the specifications of the Food and Drug Administration, which are similar to the recommendations of Schmidt and Moyer (1944). Then they were treated as described below under the individual experiments. In general, after the plates were seeded, one of two procedures was followed before addition of the several stains and reagents: either plates were treated as in the standard FDA assay or they were incubated as for the 3-hour cylinder plate assay described by Goyan, Dufrenoy, Strait, and Pratt (1947). Except as noted below, the procedure used before addition of the dyes or reagents made no qualitative difference in the reactions that were obtained with a given stain or reagent and a given test organism.

EXPERIMENTS AND RESULTS

As long as sources of —SH groups remain in the reduced state, or as long as —SH groups are rehydrogenated as rapidly as they are dehydrogenated there remains in the aerobic cell a reservoir of hydrogen available to rehydrogenate diketones to dienols. Glutathione may be considered an ultimate source of —SH groups. Once all the available —SH groups have been converted to S—S, dienols are no longer protected from irreversible dehydrogenation; on the contrary, they tend to polymerize to more and more irreversibly oxidized quinoids that may tend to catalyze further dehydrogenations. Such a picture of "decompensated aerobic respiration" as might be expected to be brought about by the shift of glutathione, or other sources of sulfhydryl groups, from the reduced to the oxidized condition can be revealed experimentally by evidence obtained from three different but convergent lines of attack that are based on the use of techniques for the detection of reduced —SH or of dienol groups, for the estimation of dehydrogenase activity, and for the estimation of indophenoloxidase activity.

For convenience, the several reagents that were used, the presumed active groups reacting with them, and their effects on different parts of penicillin assay plates are presented in table 1.

Development with Reagents for Reduced Substances

Ferricyanide reagent. Mason (1930) recommended this reagent for the "determination of reduced glutathione in tissues." In this test the reduction of ferricyanide to ferrocyanide is considered to be brought about by the —SH groups, which are thereby dehydrogenated to S—S. Certain other reducing agents may accomplish the same result, but they would not be expected to be encountered in living tissues. Therefore, he suggested that the test makes it possible to locate free sulfhydryl groups as contrasted with disulfide groups and, more specifically, reduced glutathione as contrasted to oxidized glutathione.

When standard assay plates (incubated 16 hours according to FDA specifications) are flooded for 1 minute with a 2 per cent aqueous solution of potassium ferricyanide, drained, and then reflooded for 1 minute with a saturated aqueous

TABLE 1

Reagents used in experiments

REAGENT	GROUP ASSUMED TO BE ACTIVE	AUTHORITY*	REACTION ON ASSAY PLATES			
			Color			Definition of boundary†
			Inside of zone	Boundary	Outside of zone	
K-ferricyanide Ferric sulfate	—SH	Mason, H. L. 1930	Faintly bluish	Deep blue	Blue	Very sharp
Cd-acetate	—SH	Binet, L., and Weller, G. 1934; Joyet-Lavergne, Ph. 1938	Clear	Opaque	Cloudy	Not practical
Cobalt nitrate	—SH		Clear	Opaque	Cloudy	Not practical
Schiff's	Aldehyde	Oster, K. A. 1946	Clear	Red	Red	Very sharp
Osmic acid	Dienol (*o*-polyphenols)	Dufrenoy, J. 1945	Clear	Black	Smoke, gradually darkening to black	Very sharp
Chromaffin 1 {K-bichromate, Hg-bichloride}	Dienol (*o*-polyphenols	Lison, L. 1936	Clear	Orange	Brown	Sharp
2 {NH_4-molybdate in acetic acid}	Dienol (*o*-diphenols)	Lison, L. 1936	Clear	Dark brown	Brown	Very sharp
$FeCl_3$	Dienol (*o*-diphenols)	Lemoigne, M. 1928	Clear	Green	Green	Not practical
Azo reaction (in alkaline solution)	Dienol (*o*-polyphenols)	Lison, L. 1936	Faintly pink	Vivid red	Red	Sharp
Methyl green Malachite green Janus green	Dehydrogenase systems	Prevot, A. R. and Ferly, A. 1946	Green		Faintly pink	Moderately sharp
"Thydi" (*p*-phenylenediamino chlorhydrate and thymol, buffered at pH 9)	Indophenol oxidase	Lison, L. 1936	Blue		Clear	Not practical

* For complete citation see list of references at the end of the paper.

† Reagents that give boundaries designated as "sharp" or "very sharp" are considered of potential value in the development of rapid cylinder plate methods of assay. Those designated "not practical" are useful in experimentation but are considered unsuitable for routine work.

solution of ferric sulfate, the uncolored inhibition zones are seen to be bounded by a well-defined deep blue ring which stands out clearly on the less intensely colored general background. The blue color is taken to represent a deposit of Prussian blue where ferri- has been reduced to ferrocyanide. The intensity

of color indicates the relative abundance of reducing substances. *S. aureus* plates after 3 hours of secondary incubation, as described in a previous paper (Goyan *et al.*, 1947), and *B. subtilis* plates after 2 hours of secondary incubation can be developed promptly by similar treatment. This technique gives a very sharp definition of the inhibition zones.

Cadmium acetate reagent. Standard assay plates treated with a solution of 1 per cent cadmium acetate rapidly develop a densely opaque ring, such as might be expected to evidence the formation of cadmium glutathionate immediately around the clear zone of inhibition. This ring is coincident with the ring of enhanced growth surrounding the zone of inhibition and suggests that in this region glutathione is present in the reduced condition. Glutathione can also be detected through the formation of complexes with salts of other metals, such as cobalt nitrate.

Schiff's reagent. This reagent, prepared by decolorizing a hydrochloric solution of fuchsin with sodium sulfite, has been used to detect the presence of aldehydic groups. When plates seeded with *B. subtilis* and incubated 3 hours or more with penicillin are flooded with the sulfite-fuchsin mixture, a deep red color develops immediately in the background and clearly outlines the unstained inhibition zones. A sharp delineation of the zones is produced on plates seeded with *S. aureus* and flooded with this reagent, however, only when the plates are first pretreated with a 1 per cent aqueous solution of $HgCl_2$, according to the procedure recommended by Oster (1946), to liberate bound aldehydes. We believe the color developed on the penicillin test plates is to be ascribed to the restoration of basic fuchsin where aldehyde groups exist freely (as on plates seeded with *B. subtilis*) or are liberated by $HgCl_2$ (as on plates seeded with *S. aureus*). It should be pointed out, however, that the development of the red color outside the zones of inhibition might also be ascribed to adsorption phenomena, which will be discussed in another paper. Colonies can be stained red by the sulfite-fuchsin mixture, and also on plates which have been submitted to physical development according to the procedure described previously (Goyan *et al.*, 1947).

The techniques which are considered in this paper differ from that of physical development, reported earlier, in that physical development involves reactions in both the agar medium and the bacterial colonies, whereas in the present techniques the bacterial colonies are stained without any staining action on the agar. Thus the present techniques may be truly considered cytochemical, since they involve only the cells themselves.

Development with Reagents for Phenolic Compounds

The preceding sections show that the areas inside the zones of inhibition differ from the areas outside in failing to give a positive response for —SH groups. Since phenolic compounds within the organisms can be expected to be protected from irreversible dehydrogenation only as long as —SH groups are available, a threshold for diketones vs. dienols should coincide with that for S—S vs. —SH. By diketones vs. dienols we mean the active groups of quinoid derivatives vs.

those of the original *ortho*-diphenols. Evidence for such a threshold can be revealed experimentally by several techniques that have been discussed by Lison (1936) as to their specificity in revealing prescribed active groups. These techniques may be classified as involving (a) argentaphil (or "argyrophil"), osmiophil, and chromaffin reactions, and (b) azo reactions. The argentaphil, osmiophil, and chromaffin reactions have been used for many years for histochemical studies.

Argentaphil. A method of silver impregnation (essentially an argentaphil reaction) has been described previously (Goyan *et al.*, 1947) and will not be reviewed here.

Osmiophil. It should be recalled that the osmiophilic reaction, often incorrectly ascribed to fats, indicates the reduction of osmic acid through the mediation of reducing agents. (In living cells these are likely to be phenolic compounds.) The development of plates by exposing them to the fumes of osmic acid, therefore, may depend fundamentally on the same mechanisms, i.e., processes of reduction that are brought into operation in the method of physical development previously reported, and which account for the precipitation of metallic copper outside the inhibition zones when plates are flooded with Fehling's reagent.

In our experiments, after the cylinders were removed from the plates a few drops of a 2 per cent aqueous solution of osmic acid (stabilized by chromic acid) were placed in the cover of the petri dish, the bottom was replaced, and the dish was kept in an inverted position. When this is done, within a few minutes dark rings outline the outer margins of the clear inhibition zones, thus revealing the zones of enhanced metabolic activity where the test organisms might be expected to be at the climacteric stage. Progressively, the blackening extends to the background, outside the inhibition zones, and, of course, eventually even the inhibition zones themselves darken. As in other methods of development, exposure to the reagent must be timed properly to give the sharpest definition. An advantage of this method for research purposes is that it eliminates the danger of mechanically washing colonies off the test plates.

Chromaffin reaction. Lison (1936) pointed out that the chromaffin reaction, which involves dehydrogenation of phenolic compounds in the tisue itself to colored quinoid derivatives, is the only one which can be considered specific for *ortho*-diphenols in histochemical or cytochemical work. At the boundary of the inhibition zone, a region of enhanced growth, the organisms might be expected to be most active in synthesizing and storing phenolic compounds. These would be likely to yield colored derivatives under the action of mild oxidants, such as are employed in the so-called chromaffin reaction.

A chromaffin reaction is obtained easily by flooding the assay plates with a mixture of a saturated aqueous solution of potassium bichromate and mercury bichloride containing 10 per cent formaldehyde, whereupon a conspicuous orange ring promptly develops around the clear inhibition zones. Gradually the entire background outside the inhibition zones colors while the interiors of the zones remain relatively uncolored.

An even sharper definition of inhibition zones may be observed when plates are treated with a saturated solution of ammonium molybdate in acetic acid. In animal tissues a similar color reaction with this reagent has been ascribed to the formation of a deep brown complex between ammonium molybdate and *ortho*-diphenols. The reaction is not specific, however, since conceivably it might be merely the result of the dehydrogenation of those diphenols to the corresponding brown quinoids.

In 1928 Lemoigne reported that *B. subtilis* produces an *ortho*-phenolic compound that yields a colored derivative with saturated solutions of $FeCl_3$. We have observed that when assay plates seeded with *B. subtilis* are flooded with a saturated solution of $FeCl_3$, green rings that outline the zones of inhibition appear promptly as the general background of the plate outside the inhibition zones darkens. The color develops more vividly and persists longer if a dilute solution (about 1 per cent) is employed. This is especially true for plates seeded with *S. aureus*. It is well known that *ortho*-diphenols, such as catechol, form colored complexes with $FeCl_3$. This reaction, however, is not unequivocal evidence of the presence of such phenolic compounds, since various other molecular structures that might be present in living tissues (such as hydroxamic acids, for example) may also give rise to colored complexes with $FeCl_3$.

Azo reaction. Lison (1936) described a technique which has been considered specific for the determination of intracellular phenolic compounds. The reaction depends upon the formation of an azo dye when phenols react with the reagent, which consists of a freshly prepared, cold, alkaline solution containing an aromatic amine and sodium nitrite. We have used naphthylamine. When assay plates seeded with *S. aureus* are flooded with such a reagent, the general background almost immediately develops a bright red color which sharply outlines the relatively unstained zones of inhibition.

Development with Reagents for Dehydrogenase Activity

Reactions obtained with ferricyanide and with cadmium acetate have been discussed as evidence of threshold effects with respect to —SH groups within and without the zones of inhibition. Reactions obtained with reagents for phenolic compounds have been discussed as evidence of threshold effects with respect to the presence of dienols outside the zones as contrasted to their absence inside the zones. These results can be correlated with evidence for decreased dehydrogenase activity and correspondingly increased oxidase activity within the zones.

When assay plates seeded with *S. aureus* are flooded with a 0.1 per cent aqueous solution of methyl green, the inhibition zones immediately appear as vivid green areas on a faintly pink background. This can be interpreted to mean that the dye fails to be reduced by the organisms under the influence of bacteriostatic concentrations of penicillin, but that it is promptly reduced to the pink or leuco derivative by those cells outside the sphere of inhibiting concentrations. Experiments with methyl green have been cited as an example, but other dyes (malachite green, Janus green, etc.) currently used as rH indicators may be used to detect the upward shift of rH where colonies of *S. aureus*

have been exposed to the bacteriostatic action of penicillin. This is in contrast to the preservation of the normal aerobic level of oxidation-reduction potential outside the range of diffusion of bacteriostatic concentrations.

Similarly, results obtained with various pH indicators suggest that the zones of inhibition are sites of a downward shift in pH from approximately 7 to pH 6 or lower. It should be pointed out, however, that such staining reactions or changes of color may also be interpreted as manifestations of a differential adsorption of molecules or of ions in the inhibition zones and in the background. Evidence concerning the interference of surface effects will be presented in another paper, dealing with adsorption phenomena. In the present paper we are confining our attention to converging lines of evidence which seem to suggest a lessening of dehydrogenase activity, and a concomitant increase of oxidase activity inside the inhibition zones.

Although impairment of dehydrogenase activity can be inferred from the results of a diversified set of reactions, the only biochemically reliable test for oxidase appears to depend on the use of freshly prepared "thydi" reagent. When plates are flooded with this reagent, nascent indophenol blue develops more rapidly and more intensely in the larger zones of inhibition, around cups containing higher concentrations of penicillin, and correspondingly more slowly and less intensely in the smaller zones, surrounding cups containing lower concentrations. The formation of indophenol blue may be considered to be catalyzed by quinoid derivatives arising from irreversible oxidation of phenolic compounds by phenoloxidases. Phenoloxidases are known to remain active after cellular organization has been destroyed. Consequently, they might be expected to remain active in regions where dehydrogenases have been dispersed or otherwise inactivated. Dehydrogenases are known to be inhibited by various agents at concentrations far below lethal levels (Bach and Lambert, 1937, 1938).

DISCUSSION AND CONCLUSIONS

All of the techniques reported in this paper intensify the boundaries between areas of inhibition and noninhibition on penicillin assay plates, whether the zones are clearly discernible without further treatment (as in the standard FDA test) or are very faintly visible (as after only 2 or 3 hours of incubation and diffusion of penicillin). The techniques that have been described all agree in locating the margin of the zone of inhibition at the same distance (within the limits of experimental error) from a cylinder from which a given concentration of penicillin has been permitted to diffuse for a given length of time. This is of some practical interest since it opens up several new methods of conducting rapid assays for penicillin without the use of expensive or elaborate equipment.

The theoretical implications of these techniques are of greater interest, however, since they all provide convergent lines of evidence that may be interpreted to mean 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 the inhibition zones may be an expression of the shift of —SH to S—S. Reagents stated to be specific for —SH groups in living cells and tissues failed to reveal such groups in the zones of inhibition but

indicated their presence outside the zones. Several reagents for di- and polyphenols indicated their relative abundance in the areas of normal growth and their relative scarcity in the areas of inhibited growth. Other reagents for detecting the activity of dehydrogenase systems indicated that, in comparison with areas of normal growth, the areas of inhibited growth were deficient in dehydrogenase activity. This is pertinent, since it is well known that many dehydrogenase systems depend for their activity in hydrogen transfer on the presence of reduced sulfhydryl groups. Use of the "thydi" reagent indicated a higher oxidase activity in areas of inhibition than in areas of normal growth.

Koschtojanz and Turpajew (1946) stressed the potential importance of sulfhydryl groups in the processes of irreversible denaturation of protein structures under the conditions prevailing in a living cell. In another paper we shall present cytochemical evidence of dissimilation of nucleoprotein complexes in bacterial cells under the influence of penicillin, and we will show how these processes are intimately related to the sulfhydryl-disulfide picture that is indicated by the present work.

SUMMARY

A study of penicillin assay plates has been made by means of techniques intended to delineate the cytochemical picture that develops on such plates when the test organisms are subjected to the action of penicillin.

The present paper is concerned with techniques intended to yield information about oxidation-reduction levels in different parts of the plates.

All of the tests that were used (14) agreed in indicating that the zones of inhibition are characterized by relatively high levels of rH as compared with the areas of noninhibited growth.

Evidence is presented to show that the very sharp demarcation between areas of inhibition and of normal growth is due to the development of a narrow band of increased growth and metabolism immediately outside the zones of inhibition. This band is attributed to the stimulation of cells subjected to appropriate subbacteriostatic concentrations of penicillin without subsequent exposure to concentrations able to induce stasis, death, or lysis.

The sharp separation of the zones from the background on test plates is taken as evidence for a threshold effect.

The patterns developed on the plates subjected to the different stains and reagents are interpreted largely in terms of relative concentrations of —SH and S—S groups.

Several of the techniques employed are adaptable as new procedures for rapidly assaying penicillin by the cylinder plate method.

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