CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

II. Changes in Reactions of Staphylococcus aureus to Vital Dyes[1]

ROBERTSON PRATT AND JEAN DUFRENOY[2]

University of California College of Pharmacy, The Medical Center, San Francisco 22, California

Received for publication April 2, 1947

In a previous paper we described the gross appearance of penicillin assay plates after chemical treatments which reveal sharp boundaries around the zones of inhibition (Dufrenoy and Pratt, 1947). Most of the tests that were described are effective following exposure of the test organisms to penicillin for periods as short as 2 to 3 hours, i.e., before zones of inhibition are discernible on untreated plates. Several of the tests may find application as rapid cylinder plate assay methods (Pratt and Dufrenoy, 1947). The sharp delineation of the inhibition zones was interpreted as an expression of a threshold effect; positive reactions for —SH groups and for OH═C—C═OH groups were obtained outside the zones of inhibition, but not inside. The evidence suggested an increase of rH within the zones and that this increase was correlated with inhibition of dehydrogenase systems. Since there is strong evidence in the literature that dehydrogenase activity depends on the structural integrity of the microorganism (Guggenheim, 1945), it seemed of interest to study the changes in response to various reagents in different parts of the cells of test organisms following exposure to penicillin. In this paper cytochemical structure will be interpreted from microscopical study of living cells of *Staphylococcus aureus* treated with vital stains, and from observations made after treatment of the organisms with various other chemical reagents. Our experimental data confirmed the data available in the literature to the effect that the part within the living cells of *Staphylococcus aureus* which responds most evidently to various reagents represents the vacuolar materials.

The accumulation of vital dyes in the vacuole may be correlated with the presence and nature of phenolic compounds within the vacuolar solution in a twofold manner: first, because phenolic compounds, and in general dienol compounds, play a fundamental role in the respiratory systems providing the energy for active absorption; secondly, because some actual linkage may occur. Historically it may be noted that as early as 1900 Nakanishi recognized that living and dead staphylococci respond differently to staining with methylene blue.

Imsenecki (1946) has pointed out that "many authors, Piekarski, Peshov, Robmorv,—found in long cells of *Proteus vulgaris*, chromophilic granules which they regard as nuclei . . . the structures mistaken for nuclei are polar granules,

[1] The execution of the work reported in this paper was made possible by a generous research grant from the Cutter Laboratories, Berkeley, California.

[2] With the Laboratory Assistance of Toinie Juntunen.

an excellent picture of which was given by Migula in 1897." Migula indeed clearly understood the significance, if not the chemical nature, of polar granules in bacteria, and their relation to vacuoles, which he discussed in his *System der Bakterien.* These vacuolar granules are now recognized as involving Feulgen-negative material in contrast with the nuclear part of the cell which is Feulgen-positive. In addition to the Feulgen-negative bodies, the vacuolar material contains phenolic compounds.

Among the vital stains used, some accumulate almost exclusively in the vacuolar solution, others may be partly adsorbed by other cell constituents. Accumulation of a dye in the vacuolar solution is influenced by the quantity and nature of the phenolic compounds that are present.

MATERIALS AND METHODS

Assay plates were prepared according to the standard method prescribed by the FDA. Some were incubated according to the standard 16-hour method. Others were seeded, preincubated for 3 hours, cylindered, and then subjected to a second incubation period of 3 hours with penicillin, according to the modified cylinder plate method involving physical development (Goyan, Dufrenoy, Strait, and Pratt, 1947). The test organism used was *Staphylococcus aureus* NRRL strain 313 (same as FDA strain 209P).

For cytological examination, organisms were removed with a platinum loop from different parts of test plates and were suspended in a drop of a solution of a vital dye or other reagent without previous killing and fixing. Cells collected from the uninhibited background outside the zones of inhibition (normal cells) were compared with those taken from different locations within zones surrounding cylinders containing solutions with concentrations of penicillin ranging from 0.5 to 8 Oxford units per ml.

These cells were compared with cells removed from similar locations on plates which subsequent to incubation with penicillin were flooded with the several reagents. The various regions of the treated plates were also examined directly under the oil immersion to study the pattern of response to the several reagents within cells retaining their original position in relation to the gradient of penicillin diffusing from the cups. These studies *in situ* confirmed in every case the cytological observations made on test organisms transferred from homologous sites on the plates to glass slides before addition of the reagent. The vital dyes used most extensively were neutral red, methylene blue, and methyl green.

EXPERIMENTAL RESULTS

Fisher (1946) documented his "Study on the mechanism of action of penicillin as shown by its effect on bacterial morphology" by means of excellent photographs, some of which illustrate the results of staining with a dilute solution of carbol fuchsin—a technique which apparently was initiated by Nakanishi (1900) and which was used by Stoughton in his attempts to demonstrate the presence of a nucleus in *Bacterium malvacearum* (1929, 1932). Fisher's photographs show

that each "control" cell contains a stained body. We interpret this stained body as representing a drop of vacuolar solution, such as has been shown to respond to vital staining as well as to staining with the carbol fuchsin technique (Dufrenoy, 1931), and such as we have found in our present work with penicillin.

Our own researches with vital staining showed that when cells of the test organisms are exposed to penicillin, the vacuolar solution tends to be displaced from its central position toward the margin of the cell, and generally toward one pole. Consequently, dyes which normal staphylococcus cells would concentrate in the central vacuole tend to accumulate more and more toward the periphery, and preferentially toward one pole, in cells under the influence of penicillin. Eventually the swollen cells may assume the appearance of a stained shell (figure 1).

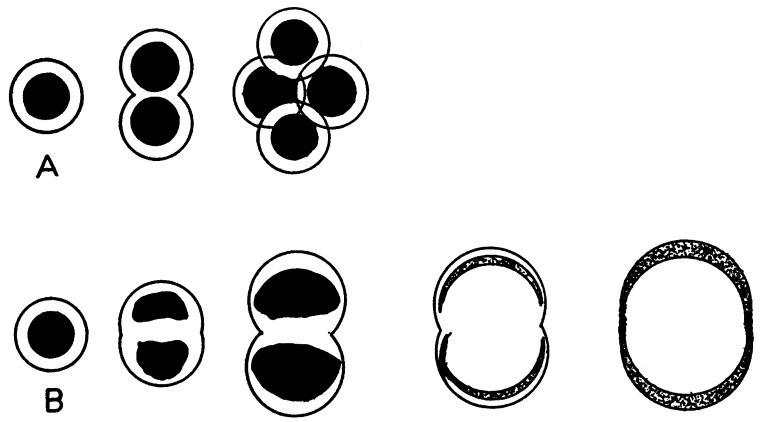

FIG. 1. DIAGRAMMATIC REPRESENTATION OF STAPHYLOCOCCUS AUREUS STAINED VITALLY WITH NEUTRAL RED

A. Phases of cell division in normal *Staphylococcus aureus*. Vacuolar material which selectively stains red is shown as solid black.

B. Phases of unsuccessful attempt toward a first division of *Staphylococcus aureus* under the influence of a bacteriostatic concentration of penicillin. Successive diagrams from left to right show that the cell swells; material staining with neutral red diffuses from the vacuole to the periphery; the cell fails to divide and eventually swells into an empty shell, which exhibits diffuse bipolar peripheral staining (stippled area).

It is recognized that penicillin acts on *S. aureus* as it is actively dividing and may allow a first division to occur. Figure 1A represents diagrammatically the behavior of a normal, living cell of *S. aureus* exposed to a solution of a vital dye. It may be seen that the dye accumulates in the vacuole and that cell division proceeds without hindrance. Figure 1B is a similar representation of the behavior of cells subjected to a vital dye following exposure to a bacteriostatic concentration of penicillin. The initial stages are about normal, but, instead of each of the two resulting vacuoles being distributed to daughter cells, the vacuolar material (staining with basic dyes) tends to move to the periphery of the swelling parent cell, which often fails to complete division.

It will be recalled here that the vacuolar accumulation of vital dyes against a concentration gradient in normally functioning aerobic organisms is a physiologi-

cal process entailing expenditure of energy that is derived from respiration; as respiration becomes unbalanced, the vacuolar solution not only becomes unable to absorb materials from the external environment, but even becomes unable to retain the solutes it already contains. Thus its solutes are free to seep out. This condition, which is one of the early symptoms of the effect of penicillin on susceptible cells, occurs concomitantly with the disorganization of the cellular nucleoproteins and the liberation of lipids and fatty acids.

The disorganization of cellular constituents is notably revealed by the "lipophanerese," defined by Lison (1936) as the unmasking of the reactive groups of the fatty components from the liponucleic complex. Hurst (1945) pertinently remarked that "lethality usually involves an irreversible increase in phenoloxidase activity, produced by the displacement of protective lipoids from the tissue receptors."

Data previously reported from assay plates (Dufrenoy and Pratt, 1947) demonstrated that bacteriolytic or bacteriostatic effects of penicillin are correlated with a relative increase of phenoloxidase activity that occurs concomitantly with an inactivation of dehydrogenase systems. This inactivation may be cytochemically visualized as resulting from displacement of the protective lipoids from the lipoproteins in the dehydrogenase systems.

In the present investigation it has been shown experimentally by evidence from standard 16-hour assay plates treated with appropriate reagents (Nile blue, copper acetate, or fat-soluble dyes) that lipids are displaced from cells undergoing lysis in the inhibition zones surrounding cylinders containing solutions of penicillin ranging in concentration from 0.25 to 8 units per ml, and that the liberated lipids are hydrolyzed into fatty acids. It is well known that basic dyes which are able to combine with fatty acids form soaps which have the color of the salt of the dye. The sharpest response was obtained with Nile blue, which Knaysi (1941) recommended for the discrimination of neutral fats from fatty acids.

When standard 16-hour assay plates are flooded for 1 minute with a saturated aqueous solution of Nile blue, and then are rinsed with distilled water, normal colonies (uninhibited areas of the plates) stain a deep blue and stand out boldly from the agar substrate. Where lysis has occurred, within the zones of inhibition, a purple color develops. This area of lysis is surrounded by a clear blue ring corresponding to the region of enhanced growth just beyond the threshold of bacteriostatic concentration of penicillin.

A convergent line of evidence that fatty acids are liberated at the site of bacteriolysis is furnished by flooding 16-hour assay plates with a saturated aqueous solution of copper acetate and further incubating them for 6 hours at 37 C. When this is done, a thick, opaque layer of bluish copper salts of fatty acids develops covering entirely the areas of the inhibition zones. The areas of uninhibited growth do not appreciably react with the reagent. The reactive and nonreactive regions are sharply delineated.

Further support for the conclusions drawn from the results obtained with Nile blue and copper acetate was provided by experiments with FD and C yellow

no. 3,[3] a fat-soluble dye which stains neutral fat a bright yellow and imparts a deep orange color to fatty acids. Plates flooded with a saturated solution of the dye in methylal develop a bright orange color where lysis has occurred. Each zone of lysis is surrounded by a bright yellow ring delineating the region of enhanced growth.

DISCUSSION AND CONCLUSIONS

In previous papers (*loc. cit.*) we demonstrated that suitable reagents applied to penicillin assay plates under appropriate conditions sharply delineate the general background of uninhibited growth from the zones of inhibition surrounding cylinders containing penicillin. The results were interpreted as evidence of a threshold for—SH vs. S-S groups or for dienols vs. diketones in the uninhibited and inhibited areas. It has been known for some time that—SH groups are essential metabolites for *Staphylococcus aureus* and that the blocking of —SH groups inhibits the growth of the staphylococci (Fildes, 1940). Our experimental data from cylinder plate assays, therefore, suggested that penicillin may act through this mechanism, i.e., by blocking —SH groups, thus lending biological support to the chemical evidence presented by Cavallito (1946).

We have also observed (unpublished experiments) that after exposure to bacteriostatic concentrations of penicillin cells of *S. aureus* are no longer gram-positive. This result is consistent with the findings of Henry and Stacey (1946) concerning the significance of the —SH group in the gram-positive complex. Further evidence that —SH groups may be involved in the interference of penicillin with the growth of *S. aureus* can be marshaled from the observation of Gale and Taylor (1946) that penicillin prevents the assimilation of glutamic acid, one of the constituents of glutathione.

In the present paper discussion is confined to phenomena that are revealed by staining and that may be considered to be incidental to changes affecting the sulfhydryl compounds. Active absorption of solutes by living cells may be assumed to entail expenditures of energy provided for by aerobic respiration which depends upon the cytochemical integrity of liponucleoproteins involving —SH. Therefore, interference of penicillin with —SH components of the respiratory systems might be postulated to effect (1) changes in the rate of absorption of solutes, and (2) swelling of the organisms coincident with the disorganization of the liponucleoproteins and liberation of lipids and fatty acids.

The results of our experiments tend to support these hypotheses. Our evidence obtained from vital staining of cells of *S. aureus in situ* on the assay plates or after transfer from different regions of the plates to a drop of the dye solution on a slide shows that, following exposure to bacteriostatic concentrations of penicillin, the cells lose their ability to accumulate neutral red, methyl green, or meythlene blue within the vacuolar solution, and that they swell to at least twice their original diameter. Evidence was also obtained that concomitant

[3] Sold by National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, New York.

with or subsequent to the swelling of the cells, fatty acids appear. These may account for the downward shift of pH revealed by the use of indicators (Dufrenoy and Pratt, 1947).

The cytological observations reported in this paper are mainly from plates seeded and preincubated for 3 hours on which penicillin was subsequently allowed to diffuse during a 3-hour secondary incubation period, since in that short time bacteriostatic effects were obtained without extensive bacteriolysis in the inhibition zones. The standard 16-hour plates were not used routinely for the cytological observations, since, because of the extensive bacteriolysis that occurred in that length of time, it was difficult to find material suitable for study. The 3-hour technique described above more readily provided cells appropriate for our different studies. The longer diffusion period, on the other hand, was found to provide the best material for the study of lipids and fatty acids arising from bacteriolysis.

SUMMARY

In continuation of work previously reported, studies of penicillin assay plates have 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 concerns cytochemical changes that occur in different parts of bacterial cells exposed to bacteriostatic or bactericidal concentrations of penicillin.

The first evidence of the effect of penicillin on *Staphylococcus aureus* was observed to be the failure of the dividing organism to apportion vacuolar material to daughter cells.

This was followed by failure of the vacuoles to retain material normally encompassed therein.

These changes were manifest in cells under the influence of penicillin, first by loss of the ability to accumulate vital dyes in the vacuolar solution, and second by dispersion of the vacuolar solution, originally located in a central body, toward the periphery of the swelling cell.

This results, in such cells, in diffuse staining with vital dyes, with a relatively high concentration of the dye at the periphery of each cell.

The use of appropriate reagents showed that lipids are displaced from cells undergoing lysis under the influence of penicillin and that the liberated lipids are hydrolyzed into fatty acids.

REFERENCES

CAVALLITO, C. J. 1946 Relationship of thiol structures to reaction with antibiotics. J. Biol. Chem., **164**, 29–34.

DUFRENOY, J. 1931 Sur le vacuome des bactéries. Compt. rend. soc. biol., **108**, 617–618.

DUFRENOY, J., AND PRATT, R. 1947 Cytochemical mechanisms of penicillin action. I. Oxidation-reduction levels. J. Bact., **53**, 657–666.

FILDES, P. 1940 The mechanism of antibacterial action of mercury. Brit. J. Exptl. Path., **21**, 67–73.

FISHER, A. M. 1946 A study on the mechanism of action of penicillin as shown by its effect on bacterial morphology. J. Bact., **52**, 539–554.

GALE, E. F., AND TAYLOR, E. S. 1946 Action of penicillin in preventing the assimilation of glutamic acid by *Staph. aureus*. Nature, **158**, 676–678.

GOYAN, F. M., DUFRENOY, J., STRAIT, L. A., AND PRATT, R. 1947 A three-hour physical development cup plate assay for penicillin. J. Am. Pharm. Assoc., Sci. Ed. **36**, 65–68.

GUGGENHEIM, K. 1945 The effect of various chemical and physical agents on the dehydrogenating enzymes of *Eberthella typhosa*. Biochem. J., **39**, 419–423.

HENRY, H., AND STACEY, M. 1946 Histochemistry of the gram-staining reaction for microorganisms. Proc. Royal Soc. (London), B, **133**, 391–406.

HURST, H. 1945 Enzyme activity as a factor in insect physiology and toxicology. Nature, **156**, 194–198.

IMSENECKI, A. A. 1946 Some remarks on the report of M. A. Peshkov on "The caryology of the development cycle of *Bact. proteus vulgaris* Hauser." Mikrobiologiya, **15**,229–232.

KNAYSI, G. 1941 On the use of basic dyes for the demonstration of the hydrolysis of fat. J. Bact., **42**, 587–589.

LISON, L. 1936 Histochemie animale. Méthodes et problems. Gauthier-Villars, Paris.

MIGULA, W. 1897 System der Bakterien. 2 vol. Jena.

NAKANISHI, K. 1900 Vorlaüfige Mittheilung über eine neue Färbungsmethode zur Darstellung des feineren Baues der Bacterien. Münch. med. Wochschr., **47**, 187–188.

PRATT, R., AND DUFRENOY, J. 1947 Practical three-hour and two-hour cylinder plate assays for penicillln. Nature, **159**, 576–577.

STOUGHTON, R. H. 1929 The morphology and cytology of *Bacterium malvacearum*. Proc. Roy. Soc. (London), B, **105**, 469–483.

STOUGHTON, R. H. 1932 The morphology and cytology of *Bacterium malvacearum*. II. Reproduction and cell fusion. Proc. Roy. Soc. (London), B, **111**, 46–52.