CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

VI. The Influence of Cobalt on the Optimal Bacteriostatic Concentration of Penicillin[1]

ROBERTSON PRATT and JEAN DUFRENOY[2]

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

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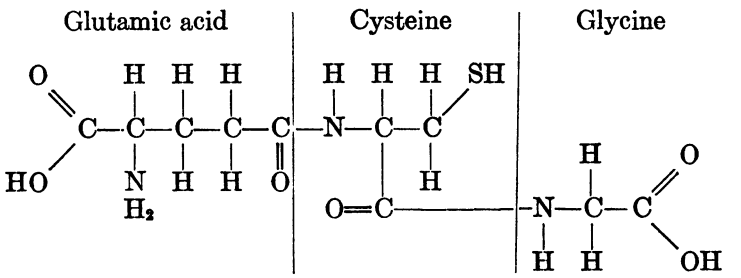
It has been shown that the antibacterial action of penicillin *in vitro* and *in vivo* may be markedly enhanced by the presence of appropriately low concentrations of cobalt that do not themselves inhibit proliferation of the test organisms (Pratt and Dufrenoy, 1947*b*; Pratt, Dufrenoy, and Strait, 1948; Strait, Dufrenoy, and Pratt, 1948). This paper presents further evidence of the enhancing action of cobalt on penicillin activity and offers an explanation of the phenomenon. It also attempts to define the factors that determine the threshold concentration below which penicillin fails to check and above which it checks the normal tendency of the test organism to proliferate. The question of an "optimal" concentration of penicillin, i.e., whether concentrations above the minimal effective level retard the action of penicillin or not, is also discussed.

Growth of *Staphylococcus aureus* is dependent on an external source of —SH groups, such as presumably may be converted to cysteine (Fildes and Richardson, 1937) and on an external source of glutamic acid (Gale and Taylor, 1946, 1947). The observations of Gale and coworkers (1946, 1947) as well as results of our own cytochemical studies (Dufrenoy and Pratt, 1947*a*,*b*; Pratt and Dufrenoy, 1947*a*,*b*, 1948) suggest that glutathione (γ-glutamyl-cysteinyl-glycine), resulting from the linkage of glutamic acid to glycine via cysteine, as follows,



GLUTATHIONE

may be involved in the mechanism of bacteriostasis of gram-positive organisms by penicillins, since the various penicillins may be written as stereochemical

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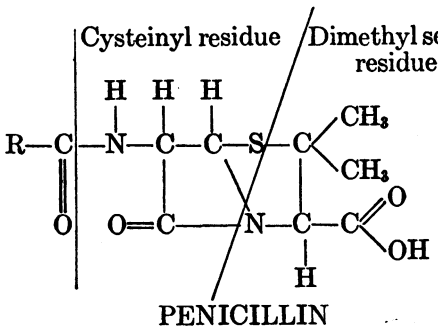
analogues of glutathione with glycine replaced by dimethyl serine, and the glutamyl fraction replaced by an R residue as follows,



The similarity in structure of glutathione and penicillin has been commented upon in several reports, including those of Fischer (1947) and Pratt and Dufrenoy (1948).

Gale and coworkers (1946, 1947) have shown that organisms that depend for their survival on absorption of glutamic acid from the substrate are penicillin-sensitive. Our own studies of staining reactions on assay plates seem to point directly to the involvement of glutathione in the action of penicillin. When assay plates are treated with the Prussian blue reagent for the detection of —SH groups, the ring of enhanced growth that circumscribes each zone of inhibition stains an intense blue (Dufrenoy and Pratt, 1947*a*; Pratt and Dufrenoy, 1947*b*); the positive reaction is not obtained, however, if the —SH groups have been blocked by bromacetate before addition of the ferricyanide-ferric sulfate reagent (Dufrenoy and Pratt, 1948). These observations suggest that the over-all effect of penicillin may be traceable ultimately to dehydrogenation of reduced glutathione to oxidized glutathione, resulting largely from the increased rate of metabolism that is induced by appropriately low concentrations of penicillin. The most efficient bacteriostatic or bactericidal concentration of penicillin may be visualized as one that promotes dehydrogenation faster than rehydrogenation can restore the —SH groups, thus shifting the aerobic respiratory system out of balance and depriving the microorganisms of the respiratory energy required to provide for absorption of essential metabolites from the substrate.

If these assumptions are correct, it follows that penicillin will be most effective at a concentration such that it accelerates the transfer of hydrogen from —SH groups without impeding the activity of hydrogen acceptors. Therefore, for each set of experiments *in vitro* or *in vivo* there would be expected a threshold value, below which the antibiotic speeds up metabolic processes without irreversibly shifting them out of balance, and above which it increases dehydrogenation beyond the rate compatible with maintenance of the proper redox systems and thereby causes irreparable damage to the cells. According to this hypothesis penicillin at an appropriate subthreshold concentration would be expected to act as a "growth factor," and it is noteworthy that such an effect

has been reported repeatedly (see review by Pratt and Dufrenoy, 1948). Concentrations greatly in excess of the threshold would be expected to impair the effectiveness of H acceptors and so partially to protect H donors against rapid, irreversible dehydrogenation. Therefore, it would be predicted that the most efficient and rapid bacteriostatic effect would follow use of the minimal concentration capable of inducing irreversible injury to the cells.

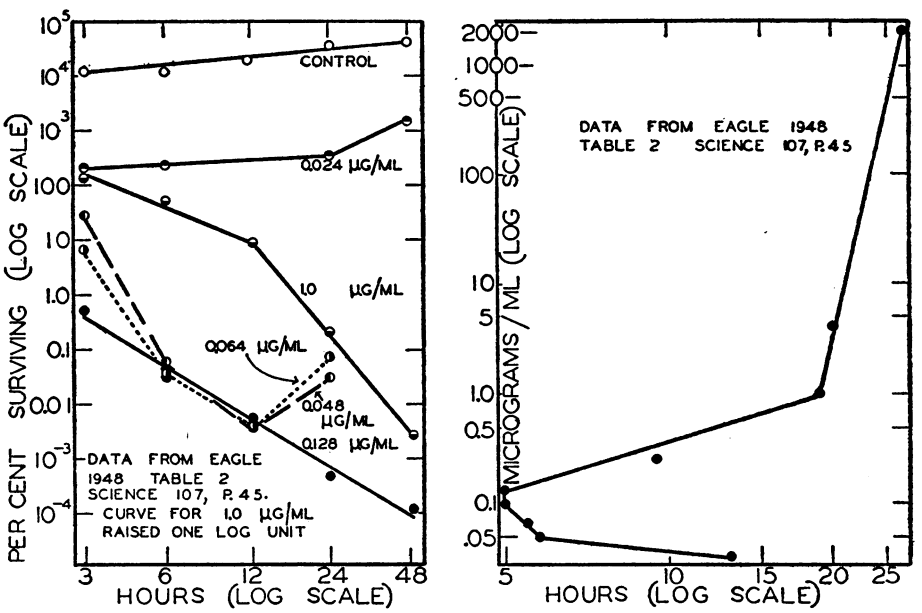
Experimental evidence to support this concept is provided by the data of Abraham and Duthie (1946) and Eagle (1948), and by the results of our own researches. Abraham and Duthie observed that "after 22 hours, more than twice as many bacteria were visible in the presence of 0.04 units per c. cm. as in the presence of 0.03 units per c. cm." Eagle showed that for the organisms he studied there are three critical concentrations of penicillin, including an "optimal" concentration at which the organisms are killed at a maximal rate. In figure 1, data for *S. aureus*, taken from Eagle's table 2, are plotted on a log-log scale, showing the percentage of organisms surviving (referred to original inoculum as 100) as ordinates and the hours of exposure to penicillin as abscissae. In the presence of 0.024 μg penicillin per ml, the curve is shifted downward on the ordinate. This represents a concentration that, in Eagle's words "serves only to reduce the rate of multiplication." At higher concentrations, 0.048 and 0.064 μg per ml, organisms die faster than they multiply during the first 12 hours, so that approximately a logarithmic order of death replaces the logarithmic order of growth, as was reported by Hobby, Meyer, and Chaffee (1942) and by Abraham and Duthie (1946).[3] For concentrations between 0.048 and 0.128 μg per ml, the percentage of survivors at 12 hours was approximately the same, but at concentrations below 0.096 μg per ml, the curves are V-shaped with an ascending branch from 12 hours on, evidencing a tendency toward restoration of the logarithmic order of growth, and corresponding to the "post-lytic waves of growth" reported by Bonét-Maury (1947) and Bonét-Maury and Pérault (1945) for cultures incubated with low concentrations of penicillin. At the optimal concentration (0.128 μg per ml in this experiment), however, the approximately logarithmic order of death continued throughout the 48 hours of the experiment. With further increase in concentration the rate of killing was strikingly reduced, as shown by the higher level of the curve for 1.0 μg per ml. The existence of an optimal concentration for maximal rate of killing is shown graphically in figure 2, which correlates the time required to kill 99.9 per cent of the test organisms with the concentration of penicillin.

Similar evidence of a maximal rate of killing with the minimal effective concentration of penicillin may be adduced also from the patterns that develop on assay plates processed by the 3-hour assay involving impregnation with silver and subsequent development (Goyan, Dufrenoy, Strait, and Pratt, 1947; Pratt, Goyan, Dufrenoy, and Strait, 1948). This technique is based on the principle that cells under the bacteriostatic influence of penicillin lose the ability, possessed by normal cells, to absorb silver nitrate. Thus, areas of the plate in which the

[3] For additional references see Pratt and Dufrenoy (1948).

cells have lost this ability appear clear following treatment with a solution of $AgNO_3$, exposure to light, and subsequent treatment with an appropriate developer, whereas areas of the plate containing cells that do absorb silver appear black.

The outer extremity of a zone of inhibition on such plates corresponds to the site where penicillin in its outward diffusion from the cylinder has reached the critical concentration that causes maximal bacteriostatic effect with the shortest time of exposure (probably corresponding to the time between two successive cell divisions). This region is that of least density of silver, and appears as a



FIGS. 1 and 2. SURVIVAL AT 37 C OF STAPHYLOCOCCUS AUREUS IN BROTH CONTAINING DIFFERENT CONCENTRATIONS OF PENICILLIN

Fig. 1. *Left*: Number of living organisms (expressed as percentage of initial inoculum) after different lengths of time. Fig. 2. *Right*: Time required to kill 99.9 per cent of organisms. (Note that concentrations of penicillin are expressed in µg per ml. To convert to Oxford units per ml divide by 0.6.)

clear ring on developed plates. Immediately outside this ring, the concentration of penicillin attained is such as only to enhance metabolism and growth. The deposition of silver is greatest in this region. Immediately inside the clear periphery of the inhibition zone is a region in which, despite (or because of) the fact that there is a higher concentration of penicillin, deposition of silver occurs, indicating less rapid impairment of cellular activity than occurs in areas where cells are exposed to lower concentrations for a shorter period of time. Thus, the test organisms that give the greatest response, as evidenced by physical development, are those that have been exposed for the shortest time to a minimal effective concentration. It should be pointed out that the shortest time re-

ported by Eagle (1948) to accomplish 99.9 per cent killing in broth cultures of *S. aureus* was 5 hours, which corresponds approximately to the minimum time of total incubation required for latent inhibition zones to become demonstrable on assay plates seeded with the same organism.

MATERIALS AND METHODS

The present experiments were performed with cultures of *Staphylococcus aureus* NRRL 313 and of *Bacillus subtilis* NRRL B-558 in broths of the following compositions:

	S. aureus	*B. subtilis*
Difco peptone	6.0 g	5.0 g
N-Z case peptone[4]	4.0 g	—
Difco yeast extract	3.0 g	1.5 g
Difco beef extract	1.5 g	1.5 g
Glucose	1.0 g	1.0 g
Distilled water to	1,000 ml	1,000 ml

Cultures were inoculated with 1 ml of an 18-hour suspension of cells for 200 ml of broth, and were incubated at 37 C. Turbidity of the suspensions was estimated visually. The penicillins that were used were a crystalline sodium salt of benzyl penicillin that assayed 1,549 units per milligram by the cylinder plate method and a 9-amino-acridine penicillin prepared from crystalline potassium benzyl penicillin.[5]

EXPERIMENTS AND RESULTS

Evidence for threshold and optimal concentrations of penicillin in broth. The threshold concentration of penicillin required to check the growth of *S. aureus* in serial dilution tests was repeatedly found to be 0.01 unit per ml under the conditions of our experiments, whereas when the broth contained 1 mg of $CoCl_2 \cdot 6H_2O$ per liter the threshold occurred at 0.001 units per ml. We define the threshold in this paper as the minimal concentration of penicillin in which the broth remains clear for 48 hours. The threshold determined by observation of turbidity may be confirmed colorimetrically by the use of appropriate rH and pH indicators such as resazurin or phenolsulfon-phthalein, which give sharp end points in the range involved. These end points can also be defined by microscopical examination of drops removed from the several tubes and mixed with an equal volume of a 0.005 per cent solution of neutral red.

As cells of *S. aureus* come under the influence of toxic concentrations of penicillin, they swell and lose their tendency to form normal 3-dimensional colonies; instead they may line up in 2-dimensional "streptococcuslike" chains or they may simply form "diplococcuslike" cells that evidence bipolar staining due to

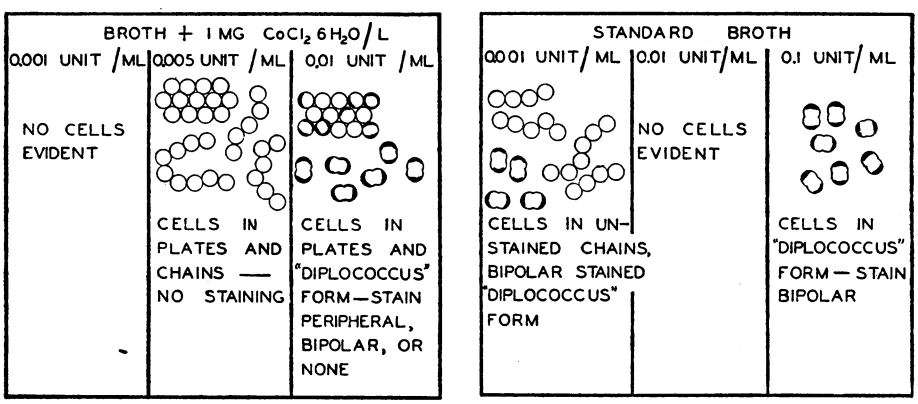
[4] Obtainable from Sheffield Farms Company, Inc., New York 19, New York.

[5] Prepared and kindly furnished by Dr. W. D. Kumler of this college. (Manuscript in preparation.)

displacement of the vacuolar material toward the periphery of the cell and its concentration at the poles (Pratt and Dufrenoy, 1947*a*). Concomitantly with these phenomena, the cells release their ribonucleic materials into the medium and lose their positive reaction to the gram stain.

The morphological and cytochemical changes found in broth cultures are shown schematically in figures 3 and 4. The characteristic tendency of cells irreversibly damaged by penicillin to deteriorate progressively, losing their normal colonial habit of growth and becoming concatenated, at the same time losing the ability to absorb and concentrate neutral red in their vacuoles, was evidenced at lower concentrations in the cobalt series than in the control series, although the cells in both groups passed through the same sequence of events.

The morphological evidence obtained tends to establish the validity of the concept of an optimal concentration of penicillin above which the antibacterial



FIGS. 3 AND 4. DIAGRAMMATIC REPRESENTATION OF MORPHOLOGICAL AND STAINING CHARACTERISTICS OF CELLS OF STAPHYLOCOCCUS AUREUS EXPOSED FOR 18 HOURS AT 37 C TO DIFFERENT CONCENTRATIONS OF PENICILLIN IN BROTH

Fig. 3. *Left*: Standard broth plus 1 mg $CoCl_2 \cdot 6H_2O$/L. Fig. 4. *Right*: Standard broth.

action is less rapid. Figures 3 and 4, respectively, show that after 18 to 20 hours no cells were apparent in tubes with 0.001 unit per ml in the cobalt series or in tubes with 0.01 unit per ml in the controls, although cells in various stages of degeneration were evident in higher concentrations. This suggests a slower rate of lysis, which may result in a lower over-all efficiency of penicillin in concentrations exceeding the minimal effective dose. Several references in the literature point to the role of lysis products released into the medium in stimulating the metabolism of neighboring cells and thereby rendering them more susceptible to the deleterious action of penicillin (Bonét-Maury and Pérault, 1945; Abraham and Duthie, 1946; Bonét-Maury, 1947; Pratt and Dufrenoy, 1947*b*, 1948).

A direct correlation exists between the availability of oxygen and the ease with which the threshold concentration can be defined by turbidimetric, morphological, or chemical means. This was shown by culturing the organisms in shallow layers where the diameter of the surface approximated the depth, and

in deeper layers where the depth was approximately 3 times the diameter of the surface. This observation confirms the earlier report of Mulé (1946), who reached the same conclusion by the use of different techniques.

Similar evidence of an optimal concentration for preventing growth has been found repeatedly when the 9-amino-acridine salt of penicillin has been tested against *Bacillus subtilis*. The results obtained by visual observations of turbidity in three representative tests are shown in table 1.

During the first 24 hours there was the conventional direct relation between concentration of drug in the broth and inhibition of growth of the organism. It is noteworthy, however, that longer periods of incubation revealed a distinct optimal concentration which completely checked proliferation, but below and above which growth occurred. The existence of the optimum was apparent

TABLE 1

Growth of Bacillus subtilis in different concentrations of 9-amino-acridine benzyl penicillin

CONCENTRATION OF ACRIDINE-PENICILLIN IN PHOSPHATE BUFFER (pH 6.9)	THEORETICAL PENICILLIN EQUIVALENT*	GROWTH† IN TUBES AFTER							
		18 Hours		24 Hours		42 Hours		62 Hours	
		Trial II	Trial III	Trial II	Trial III	Trial I	Trial II	Trial III	Trial I
	u/ml								
$1:10^5$	11.0	—	0	—	0	—	—	+	—
$1:10^6$	1.1	0	0	0	0	++-++	+++	++	++
$1:10^7$	0.11	0	0	0	0	++	++	0	+++
$1:5 \times 10^7$	0.022	0	0	0	0	++	0	0	+++
$1:7.5 \times 10^7$	0.015	0-+	0	++	0	0	+++	+	0
$1:10^8$	0.011	+	+	+++	+	—	++++	++	++
$1:2 \times 10^8$	0.0055	—	+	—	++	—	—	+++	+++
$1:5 \times 10^8$	0.0022	+++	—	++++	—	—	++++	—	—

* Calculated from the quantities of potassium benzyl penicillin (purity 1,503 units per mg) and of 9-amino acridine that were reacted.

† 0 = no growth, tubes clear; ++++ = maximum turbidity; — means no observation made.

not only by visual observation of turbidity, but was confirmed colorimetrically by the use of triphenyl tetrazolium chloride as an indicator of pH and also morphologically and cytochemically by microscopic observation of drops removed from the several tubes and mixed with solutions of neutral red.

It is noteworthy that on the basis of penicillin content, the 9-amino-acridine salt of penicillin is 5 to 25 times more effective than the sodium salt in checking proliferation of *B. subtilis*. Whereas at least 0.06 μg sodium penicillin (0.1 unit) per ml were required to inhibit proliferation to the eighteenth or twenty-fourth hours, and 0.3 μg per ml were required to prevent a secondary wave of growth between the twenty-fourth to the forty-second hours, inhibition was obtained with as little as 0.015 to 0.02 μg of any of the several lots of the 9-amino-acridine penicillin, embodying only 0.009 to 0.012 μg of penicillin. The concentrations of the acridine penicillin that are involved are far below the inhibitory concentra-

tions of 9-amino-acridine alone. Thus, the effect noted here represents enhancement of penicillin activity and is not to be considered merely the result of two antibiotics acting simultaneously.

Evidence for threshold and optimal concentrations of penicillin on assay plates. Since the preincubation technique, which permits cultures to reach the logarithmic phase of growth before coming under the influence of penicillin, makes it possible to expose the cells to the effect of diffusing penicillin at the phase of development when they are most reactive and the sharpest metabolic responses obtain, the sequence of events will be illustrated from seeded plates preincubated for 3 hours without penicillin and then reincubated for a second period of 3 hours dur-

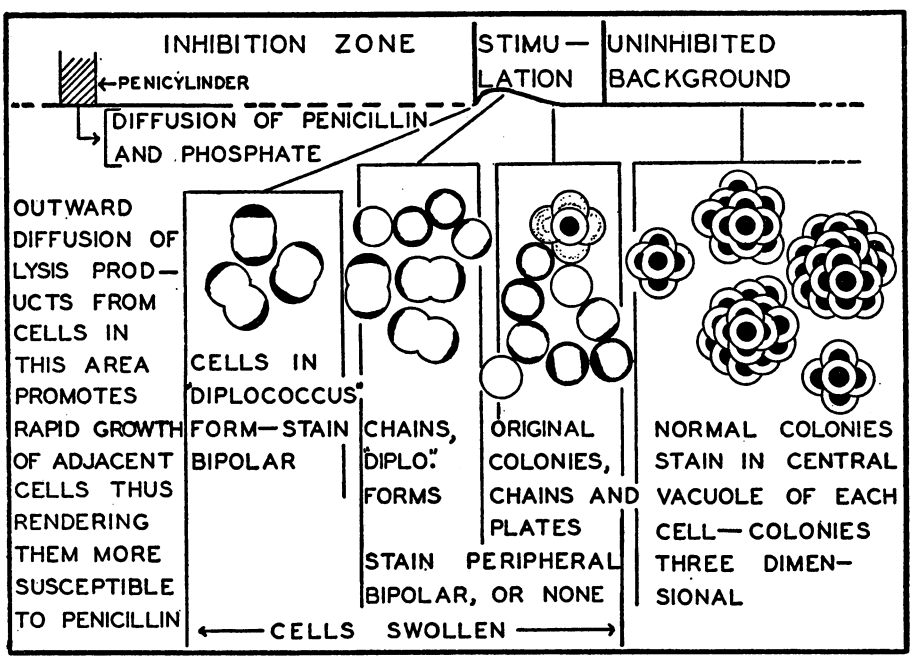


FIG. 5. SCHEMATIZED DIAGRAMMATIC REPRESENTATION OF MORPHOLOGICAL AND STAINING CHARACTERISTICS OF CELLS OF STAPHYLOCOCCUS AUREUS IN DIFFERENT REGIONS OF A PENICILLIN ASSAY PLATE INCUBATED AT 37 C

ing which penicillin was diffusing from the cylinders. Figure 5 is a schematic representation of the morphological and vital staining characteristics of *S. aureus* in different parts of such plates. The figure (not drawn to scale) represents a portion of an inhibition zone and adjacent regions of the assay plate. The original micro colonies that form during the preincubation continue during the second incubation period to proliferate three-dimensionally in the background where they do not fall under the influence of enhancing or depressing concentrations of penicillin. The background is made up of randomly distributed colonies comprising living cells, each of which is able to absorb and concentrate neutral red or other vital dyes in its vacuolar solution (black in diagram). Where enhancing

concentrations of penicillin obtain, the original colonies no longer proliferate three-dimensionally, mostly within the agar, but instead the newly formed cells spread in one plane, forming "streptococcuslike" chains that may lie close together forming platelike structures on the surface of the agar. This alteration in spatial arrangement of the cells reflects a modification of the surface properties of the cells (to be discussed in terms of electrostatic charge in a subsequent paper). These changes probably are correlated with the release of cellular constituents from the swelling organisms, and can be made evident microscopically by the use of vital stains, since the staining, when it occurs, is peripheral, as shown under the outermost region of stimulation in the diagram.

Under slightly higher, though still subbacteriostatic concentrations, the cells become more loosely associated and finally resolve into "diplococcuslike" structures that evidence bipolar staining. It will be seen that the profile of the agar surface indicates a slightly raised level in the ring of stimulation (enhanced growth) wherein the material diffusing from the lysed organisms in the inhibition zone is actively metabolized. The tendency of the proliferating chains and plates of cells in this area to crowd the surface, as contrasted with the habit of the colonies in the normal background, may reflect an "oxygen hunger" induced in such cells. This is probably the result of two factors, i.e., direct stimulation by an appropriately low concentration of penicillin and an accelerated rate of metabolism induced by the availability in this region of "growth factors" released from the lysed cells in the inhibition zone.

The features described and diagrammed above can be observed *in situ*, under the oil immersion objective, on plates dehydrated with methylal[6] and then flooded with cedar oil.

The same sequence of changes in morphology and staining reaction of cells exposed to different concentrations of penicillin can be observed in serial dilution broth cultures. By analogy (compare figures 4 and 5) one might estimate the threshold concentration of penicillin delineating the ring of enhanced growth from the zone of inhibition on assay plates as being between 0.001 and 0.01 unit per ml. Incorporation in the agar of trace amounts of cobalt that are not bacteriostatic per se results in considerable enlargement of the zone of inhibition, indicating a lower threshold concentration of penicillin as compared to that obtaining on standard agar. By analogy with serial dilution tests (figure 3) the threshold on such plates may be estimated as below 0.001 unit per ml.

DISCUSSION

The effectiveness of cobalt in lowering the threshold concentration of penicillin for bacteriostasis *in vitro* and possibly *in vivo* (Pratt, Dufrenoy, and Strait, 1948) may be ascribed to formation of complexes involving —SH groups, although the possibility of other groups of cell proteins also being involved should not be overlooked. This is in accord with the conclusion of Barron (1944), that the effects of cobalt on living systems are manifestations of inhibition of —SH groups of

[6] Obtainable from Celanese Chemical Corporation, 180 Madison Avenue, New York City.

enzymes, the functioning of which is so altered that the oxidation-reduction equilibria on which the survival of the cell depends are thrown irreparably out of balance.

It is interesting to note in this connection that in our tests, which included Ir, Fe, Zn, Sr, Cd, Li, Cu, Ag, Au, and Bi, cobalt has been outstanding in lowering the bacteriostatic threshold of penicillin.

As was pointed out by Young and Zelle (1946), to fulfill its function of coenzyme in the cell, glutathione must generally be in the reduced state (GSH), since the presence of —SH groups is necessary for the activity of such enzymes as glyoxalase, succinic dehydrogenase, phosphorylase, etc. These authors in studying the respiratory pathogenicity of *Bacillus anthracis* spores pointed out that the presence of heavy metals markedly accelerates the oxidation of reduced glutathione to the disulfide state.

It should be pointed out that the phenomenon of occurrence of an optimal range or ranges of concentration that check metabolic activity of test organisms, but above and below which normal or even accelerated activity may occur, is not peculiar to penicillin. A similar phenomenon has been reported by Welch, Price, and Randall (1946) for streptothricin tested against *S. aureus* and for streptomycin tested against two strains of *Eberthella typhosa*. They noted that "relatively high concentrations of streptothricin did not interfere with the ability of the organism to reduce nitrate to nitrite, while somewhat lower concentrations caused complete inhibition of nitrate reduction," and that "essentially the same results were obtained when streptomycin was substituted for streptothricin."

Moreover, such a phenomenon is not restricted to the action of antibiotics. That a given concentration of a metal may adversely affect measurable manifestations of metabolism more effectively than either lower or higher concentrations was considered a "general law" by Richet as early as 1906. It is pertinent to note that his results were obtained from a study of the effect of different concentrations of metals, including cobalt, on lactic acid fermentation, which is now known to involve glyoxalase, the activity of which depends on —SH groups.

ADDENDUM

Just as the proof of this paper was received, an additional pertinent report showing the existence of optimal concentrations of penicillin for antibacterial action came to hand.

Eriksen (1946), working with *S. aureus*, observed that "the highest degrees of lysis occur in the weaker concentrations (1/16–1/64 unit per cc.). In the strong concentrations (1–1/2 unit) lysis is very insignificant. . . . " He also noted that cells exposed to weak concentrations of penicillin undergo marked changes in morphology but that "in strong concentrations of penicillin (1–1/2 unit per cc.) there is no corresponding alteration in the morphology."

SUMMARY

Employing standard serial dilution techniques, it was found that the presence of trace amounts of cobalt in the broth markedly lowered the concentration of penicillin required to inhibit proliferation of *Staphylococcus aureus*. Concomi-

tant tests with suitable rH and pH indicators confirmed the "end point" concentrations that were determined on the basis of turbidity. Microscopical examination of cells from the several cultures showed that in the controls and in the cobalt series exposed to penicillin the same morphological and cytochemical changes occurred, viz., a tendency to change from the normal colonial habit of growth to the formation of "streptococcuslike" chains of swollen cells which may become arranged in plates and finally separate to form "diplococcuslike" structures that evidence bipolar staining with vital dyes instead of the normal tendency to accumulate such dyes in the central vacuolar material.

Similar phenomena were demonstrated on assay plates exposed to penicillin.

Evidence based on observations of turbidity, of response of the cultures to rH and pH indicators, and morphological changes in the cells, indicates the existence of an optimum concentration of penicillin above and below which inhibition of cellular activity is less pronounced.

Similar evidence of an optimum concentration was obtained in tests of the 9-amino-acridine salt of penicillin tested against *Bacillus subtilis*.

An approximation is made of the concentration of penicillin at the site of the periphery of inhibition zones on assay plates seeded with *S. aureus*.

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