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J Clin Invest. 1947;**26**(3):379-393. <https://doi.org/10.1172/JCI101820>.

Research Article

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PENICILLIN-RESISTANT STAPHYLOCOCCI: MECHANISMS INVOLVED IN THE DEVELOPMENT OF RESISTANCE

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(Received for publication September 9, 1946)

While staphylococci are among the species of gram-positive bacteria considered to be susceptible to the antibacterial action of penicillin, a number of investigators have reported that there are some strains of coagulase-positive staphylococci which are naturally resistant to penicillin (1 to 11). Of added clinical importance is the fact that pathogenic strains originally sensitive to therapeutic concentrations of penicillin may become resistant to the antibiotic in patients who have received treatment with penicillin (7, 12 to 19). In an attempt to define the nature of the resistance of staphylococci to penicillin, the results of several studies have appeared in which sensitive strains have acquired a high degree of resistance by *in vitro* methods (4, 11, 13, 18, 20 to 24). Todd and Turner (25) succeeded in adapting 2 strains of staphylococcus resistant to penicillin by making repeated subcultures of the cocci in increasing quantities of penicillin. When the strains had acquired resistance, they subcultured the organisms daily in broth without penicillin and observed that there was a rapid decline in resistance. As a result of these observations, they assumed that the resistance of staphylococci which developed in the body was not a permanent characteristic of the organisms and that, upon withdrawal of the drug, the bacteria would quickly revert to a state of sensitivity. On the other hand, Spink and Ferris (26) showed independently that staphylococci which had acquired resistance *in vitro* lost this resistance when grown and transferred in the absence of penicillin, but that penicillin-resistance attained in the body appeared to be a permanent characteristic of the bacteria. This distinction between acquired *in vitro* and *in vivo* resistance has been confirmed by others (11, 18).

One of the major problems related to penicillin-resistance is whether penicillinase plays a role in

the resistance displayed by staphylococci. Abraham and Chain (27) first showed that *E. coli* produced an enzyme, designated as penicillinase, which destroyed penicillin, but in a later observation by Abraham and others (4), a strain of *Staph. aureus* adapted by *in vitro* methods to grow in the presence of high concentrations of penicillin did not produce penicillinase. McKee, Rake, and Houck (28) encountered a strain of *Staph. aureus* which was resistant to penicillin and which formed a filter-passing enzyme capable of destroying penicillin. Bondi and Dietz (8) concluded that penicillinase was responsible for the resistance to penicillin of naturally resistant strains of staphylococci, but they (21) also pointed out that other species of bacteria, notably gram-negative organisms, did not owe their resistance to the production of penicillinase. Kirby (29) demonstrated that strains of staphylococcus which were naturally resistant to penicillin possessed a potent inactivator for penicillin, whereas this property was absent in sensitive strains. This observation was extended by Spink and Ferris (26, 30), who showed that strains of staphylococcus made highly resistant to penicillin by *in vitro* adaptation did not produce an inactivator for penicillin; and, in addition, there appeared to be a quantitative relationship between the degree of resistance and the production of an inactivator for penicillin for naturally resistant strains or strains which had acquired resistance *in vivo*; that is, the more resistant a strain, the more potent the inactivator. Demerec (24) has approached the problem of penicillin resistance with a different type of experiment than that used by the foregoing investigators. Starting with a strain of staphylococcus sensitive to the action of penicillin, subcultures were made on agar plates containing penicillin. In this manner, he observed that the sensitive strain gave rise to small numbers of variants which were resistant to low concentrations of penicillin. When these variants were permitted to grow, there was

¹ Aided by grants from Sharp and Dohme, Inc.; the Committee on Scientific Research of the American Medical Association; and the Graduate School, University of Minnesota Medical School.

a gradual increase in variants which were highly resistant to penicillin. Demerec concluded that acquired *in vitro* resistance occurred independently of penicillin and involved a number of mutations and genic changes. In other words, resistance is an inherited characteristic of a given strain, and not acquired through the interaction of the bacteria and penicillin. Although he stated that the resistance persisted for the period of the experiments, it is not known from the data presented whether the resistance was of a permanent nature. Furthermore, it is not known whether the resistant mutants produced an inactivator of penicillin. In a follow-up study of Demerec's work in the same institution, Luria (31) has indicated that there are two types of penicillin-resistance as far as the staphylococcus is concerned, namely, resistance that develops as a result of mutation and resistance that is associated with penicillinase-producing strains. In other words, penicillinase apparently plays no role in the inherited and permanent type of resistance, a conclusion which is at variance with the observations of others and the results in this report. Luria has emphasized that if a small inoculum of organisms is used in the *in vitro* test for sensitivity, penicillinase-producing strains have individual cells which are sensitive to penicillin. However, as will be pointed out shortly, the degree of resistance is related to the potency of penicillinase produced by a given strain.

Because there appears to be a lack of general agreement concerning the mechanism or mechanisms whereby penicillin-resistant strains of staphylococcus are established, the present report includes a series of studies which have been carried out over a period of 4 years. Two fundamentally different types of resistance have been observed repeatedly. First, resistance that is acquired *in vitro* by adapting the organisms to grow in increasing concentrations of penicillin. This type of resistance is only a temporary property of the bacteria and is not associated with the formation of penicillinase. The second type of resistance appears to be a permanent characteristic of the strains and is always associated with the production of penicillinase. Furthermore, the degree of resistance is quantitatively related to the potency of penicillinase produced by a given strain. This type of resistance is an inherent property of some

naturally resistant strains and of strains which have acquired resistance in the human body as a result of treatment. The methods for obtaining penicillinase from staphylococci and the properties of this penicillinase will be described.

MATERIALS AND METHODS

Strains of staphylococcus

A total of 45 strains of coagulase-positive strains of staphylococcus was studied. All of the strains were recovered from human patients. Of these strains, 25 were sensitive to the action of penicillin; 9 showed a natural type of resistance; and 5 had apparently acquired resistance in the body as a result of treatment.² Each of the strains was cultured on veal infusion or tryptose phosphate agar slants and stored in the refrigerator and subcultures were made monthly.

Penicillin

Nineteen lots of commercial sodium penicillin as marketed by 8 different companies were utilized. In the majority of instances, serial and comparative studies of penicillin-resistance and similar studies on the mode of action of penicillinase, were carried out with the same lot of penicillin. It was observed that sodium penicillin could be dissolved in sterile physiological saline, frozen, and then stored in a deep-freeze refrigerator for weeks without loss of potency. Comparative studies were made between commercial sodium penicillin and crystalline sodium penicillin supplied by Merck & Company.

Determination of in vitro penicillin resistance

The sensitivity of each strain for penicillin was determined by adding a standard inoculum of organisms to test tubes with liquid medium containing increasing concentrations of penicillin, and then selecting as the end point that tube which showed no turbid growth with the lowest concentration of penicillin. Three types of medium were used for growing the staphylococcus. In the initial experiments, Gladstone's synthetic medium was employed, the preparation of which has been described elsewhere (32). In later studies, veal infusion broth and tryptose phosphate broth were utilized because these media were prepared with less difficulty than Gladstone's medium. The results of the sensitivity tests were essentially the same with all 3 media. The details of determining the *in vitro* sensitivity of each strain to penicillin were as follows: a loopful of culture was taken from an agar slant and grown for 2 to 3 generations in liquid medium. A standard inoculum of 0.1 ml. of 10^{-8} dilution of a 24-hour culture was seeded into each of several test tubes containing one of the foregoing liquid media.

² Dr. Donald G. Anderson of Boston kindly supplied us with 3 of the 5 strains which had developed *in vivo* resistance.

Such an inoculum contained between 300,000 to 900,000 organisms. Then 1 ml. of freshly prepared aqueous solution of sodium penicillin was added in ascending concentrations to each of the tubes with the bacterial suspensions. The final volume in each tube was 10 ml. Incubation was carried out for 48 hours at 37° C. and then growth of the organisms was determined by the presence of turbidity. The results of previous studies had emphasized the importance of using a standardized and small inoculum of organisms in performing tests for penicillin sensitivity (5). A large inoculum of organisms required a higher concentration of penicillin for the inhibition of growth than a small inoculum.

Development of in vitro resistance to penicillin

Strains of staphylococcus were made resistant to penicillin by exposing succeeding generations of organisms to increasing concentrations of the same lot of commercial sodium penicillin in Gladstone's medium or veal infusion broth. The method was as follows: to each of several tubes containing 9 ml. of liquid medium and 1 ml. of sodium penicillin dissolved in isotonic saline solution was added a standard loopful of a 24-hour culture which had been grown in liquid medium. The initial concentration of penicillin permitted turbid growth when the cocci were incubated for 24 hours at 37° C. When maximum growth was obtained with a given concentration of penicillin after loopful transfers every 24 to 48 hours, the concentration of penicillin was increased and the serial transfers repeated. Of considerable importance was the simultaneous transfer of control organisms from each strain in liquid medium without the presence of penicillin.

Detection of penicillinase production

The presence of crude penicillinase was determined for strains sensitive to the action of penicillin, strains made resistant *in vitro* to penicillin, naturally resistant strains, and strains which had become resistant in the human body. The method of obtaining penicillinase was that of Harper (33) as used for the paracolon bacillus, and as adopted for the staphylococcus by Kirby (34). Essentially, the method consisted of growing each strain on the surface of several agar plates for 24 hours at 37° C., preparing a thick emulsion of the organisms with distilled water, extracting the cocci with acetone and ether, and then drying the bacterial residue *in vacuo*. After testing for sterility, the dried material was stored in the refrigerator.

The potency of the penicillinase was ascertained in the following manner: a standard strain of staphylococcus sensitive to penicillin was grown for 24 hours in Gladstone's medium or broth. Then 0.1 ml. of a 10⁻⁸ dilution was added to each of several tubes containing synthetic medium or broth with 0.001, 0.01 or 0.1 mgm. per ml. of dried, extracted cells and increasing concentrations of penicillin. The mixtures were incubated at 37° C. for 48 hours and that tube which showed turbid growth with the highest concentration of penicillin indicated the number of units of penicillin destroyed by penicillinase.

RESULTS

I. Penicillinase from staphylococci

Kirby (34) first designated the material obtained from the cells of staphylococci which destroyed penicillin as a "penicillin inactivator," and subsequently described some of the properties of this substance (29). Broth suspensions of the inactivator were completely inactivated at 56° C. in 5 minutes. The inactivator could not be obtained in a cell-free filtrate. The rate of destruction of penicillin by the inactivator was the same at 2° C., 22° C., and at 37° C. In contrast to one of these properties, McKee, Rake, and Houck (28) obtained a strain of *Staphylococcus aureus*, appearing as a contaminant in a culture of *Aspergillus flavus*, which produced an enzyme capable of destroying penicillin. This enzyme was obtained in Seitz filtrates of broth cultures. More recently, Luria (31) reported that no inactivator for penicillin was obtained from filtrates of cultures of resistant staphylococci when sintered glass, Seitz, or Mandler filters were used. It appeared desirable to obtain more information concerning the properties of the inactivator of penicillin produced by staphylococci and to study its mode of action. In the following discussion this inactivator will be referred to as penicillinase.

Intracellular nature of staphylococcic penicillinase

It was observed repeatedly that bacterial cells from cultures of staphylococci with the permanent type of resistance contained a potent penicillinase after the cells had been extracted with acetone and ether and then dried. Several attempts were made to obtain the penicillinase in cell-free filtrates by growing the organisms in broth and filtering portions with Berkefeld, fritted glass and Seitz filters. The sterile filtrates contained no active agent against penicillin, thus confirming the findings of Luria (31). Penicillin-resistant organisms were grown in broth for 24 hours, and then the flask shaken vigorously on a shaking machine for 8 hours. When this material was filtered, the sterile filtrate did not contain penicillinase.

Sterile supernatants of penicillin-resistant staphylococci were obtained only after considerable difficulty. Organisms were grown for 24 hours in broth and then the majority of the cells removed

by centrifuging in the usual type of laboratory centrifuge. The supernatant was then centrifuged in a high speed centrifuge (13,000 r.p.m.) in the cold for 1 hour. The top layer of the supernatant was carefully drawn off and this material was centrifuged for another hour. This sterile supernatant did not contain penicillinase.

One hundred milligrams of dried bacterial cells from strain Rosen contained highly active penicillinase. The finely dried powder was added to 100 ml. of tryptose phosphate broth in a flask and the contents shaken vigorously. The powder did not go into solution but was dispensed through the broth as a fine suspension. After equal parts of the broth were filtered through Berkefeld, fritted glass, and Seitz filters, no active penicillinase was present in the filtrates.

The foregoing observations indicate that staphylococcal penicillinase is intimately associated with the bacterial cells and active material could be investigated only with dried cells.

Stability of staphylococcal penicillinase

Sterile dried preparations maintained their potency for several weeks when kept at room temperature or in the refrigerator. When the powder was suspended in sterile physiological saline solution and quickly frozen, there was no diminution in activity. Though no observations were made over an extended period of time, there was no loss of activity of aqueous or broth suspensions kept at room temperature for several days. Penicillinase was found to be heat labile. After 0.01 mgm. per ml. of dried powder prepared from a highly resistant strain was suspended in physiological saline solution and heated at 56° C. for 30 minutes only, 1 unit per ml. of penicillin was destroyed by the heated product, whereas the unheated control inactivated 40 units. After heating at 80° C. for 30 minutes, only slight inactivation of penicillin was noted by the same amount of material.

Effect of pH on action of staphylococcal penicillinase

One mgm. per ml. of a dried preparation from a resistant strain was suspended in physiological saline solution in each of 3 tubes. The pH was adjusted to 2.04, 6.90, and 11.30 and the contents incubated at 37° C. for 30 minutes and for 3 hours.

At the end of these periods, the solutions were brought back to neutrality and the activity of the preparations tested against penicillin. The results are tabulated in Table I. It is to be noted

TABLE I
Effect of pH on activity of 1 mgm. per ml. of penicillinase

pH	Per cent reduction in activity after incubation at 37°C.	
	30 minutes	3 hours
2.04	50	90
6.90	0	0
11.30	50	100

that there was a 50 per cent reduction in activity at the end of 30 minutes. In 3 hours, there was complete inactivation at a pH of 11.3 and a 90 per cent reduction in activity at 2.04.

In the second experiment, the same concentration of material was incubated at 37° C. for 4 hours at a pH of 3.0, 7.4, and 9.0. Table II shows a 50

TABLE II
Effect of pH on activity of 1 mgm. per ml. of penicillinase

pH	Per cent reduction in activity after incubation at 37°C. for 4 hours
3.0	50
7.4	0
9.0	100

per cent reduction of activity on the acid side and complete inactivation in an alkaline medium.

These results indicate that penicillinase from staphylococcus is more susceptible to changes in hydrogen ion concentration than the penicillinase obtained from an aerobic spore-forming bacillus belonging to the *B. subtilis* group as reported by Woodruff and Foster (35). They observed no change in activity of the enzyme over a range of pH from 3.0 to 11.0. On the other hand, staphylococcal penicillinase appears to be more resistant to changes in hydrogen-ion concentration than the penicillinase studied by McQuarrie and his associates (36) prepared from a gram-negative rod.

Factors influencing the action of staphylococcal penicillinase on penicillin

The inactivation of penicillin by penicillinase was found to be dependent upon 2 main factors. First, the *potency* of the penicillinase utilized in a given

observation, and, second, the period of time in which penicillinase was in contact with penicillin. The significance of these 2 factors is illustrated by the following experiments. Preparations of strain Bernardo II in concentrations of 1 mgm. per ml. and 0.1 mgm. per ml. were mixed with 100 units per ml. of penicillin in physiological saline solution and permitted to stand at room temperature for 5 hours. At the end of this period, the penicillinase was removed by filtration and the filtrate tested for penicillin activity. There was only a slight diminution in the potency of penicillin which had been in contact with 0.1 mgm. per ml. of dried cells, whereas there was almost complete destruction of activity with the mixture containing 1 mgm. per ml. When mixtures containing the same quantities of penicillinase and penicillin were allowed to stand for 18 hours at room temperature, there was complete loss of penicillin activity with both preparations.

Penicillinase prepared from strain Rosen always yielded a more potent material than that obtained from strain Bernardo II. When 0.2 mgm. per ml. of Rosen extracted cells were mixed with penicillin and tested for penicillin at the end of 1 and 3 hours, it was observed that there was no loss of

penicillin activity in 1 hour, but the potency of penicillin was considerably reduced in 3 hours.

In a preliminary communication (30), this relationship of the potency of penicillinase and the time necessary for inactivation of penicillin was illustrated in another type of experiment. A constant amount of extracted cells was added to each of several tubes containing increasing concentrations of commercial penicillin dissolved in Gladstone's medium. A small inoculum of a penicillin-sensitive strain of staphylococcus was seeded to each of the tubes and the mixtures incubated at 37° C. Growth curves of the organisms were determined by measuring the density of the bacterial cultures with a photoelectric colorimeter at appropriate time intervals. It was shown that with increasing concentrations of penicillin a longer period of time was necessary for inactivation of it. The same type of experiment was repeated in the present study comparing the effect of 0.005 mgm. per ml. of dried Bernardo II cells upon increasing concentrations of commercial penicillin and a purified preparation of crystalline sodium penicillin (Merck). The results are illustrated in Figure 1. The test strain used in this experiment was inhibited in growth by 0.05 unit per ml. of penicillin and

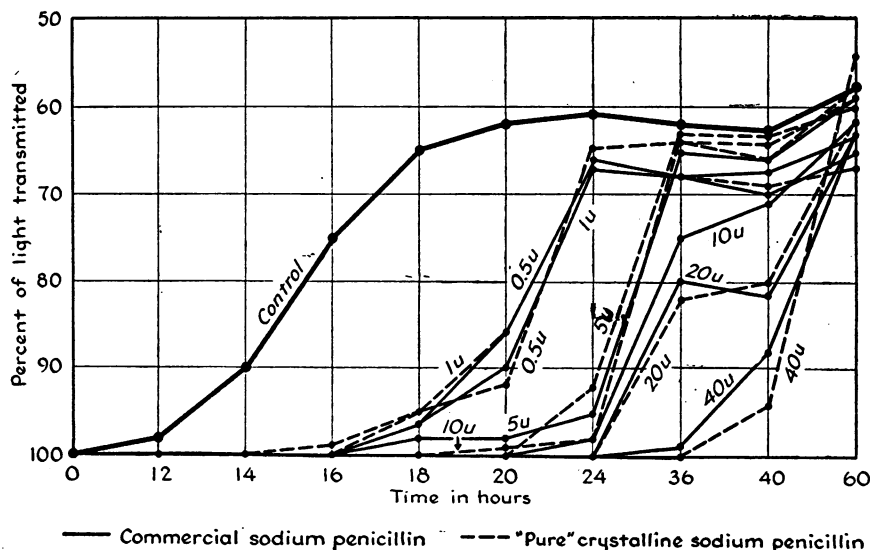


FIG. 1. COMPARATIVE EFFECT OF 0.005 MG. PER ML. OF STAPHYLOCOCCIC PENICILLINASE ON THE GROWTH CURVES OF A PENICILLIN-SENSITIVE STRAIN OF STAPHYLOCOCCUS IN THE PRESENCE OF INCREASING CONCENTRATIONS OF COMMERCIAL SODIUM PENICILLIN AND "PURE" CRYSTALLINE SODIUM PENICILLIN

Concentrations of penicillin expressed in units per cubic milliliter.

yet, if 0.005 mgm. per ml. of extracted cells containing penicillinase was allowed to remain in contact with penicillin for 40 hours, this strain finally grew out in the presence of 40 units per ml. of penicillin.

Several attempts were made to determine if there was a linear relationship between the inactivation of penicillin by penicillinase. However, no close correlation between these 2 factors was found, probably due in part to the fact that only crude preparations of penicillinase were available. The results of 1 experiment are given in Table III.

TABLE III

Growth of penicillin-resistant strain of staphylococcus in relation to increasing concentrations of penicillin and penicillinase

Incubation at 37°C. for 48 hours

Mgm. per ml. of dried bacterial cells with penicillinase	Units of penicillin per ml.						
	0.05	1	5	10	20	40	60
0.002	+	0	0	0	0	0	0
0.004	+	+	+	+	0	0	0
0.006	+	+	+	+	+	+	0
0.008	+	+	+	+	+	+	+
0.010	+	+	+	+	+	+	+

+ = turbid growth.

0 = no turbid growth.

Each of several tubes contained a standardized small inoculum of a penicillin-sensitive strain of staphylococcus in Gladstone's medium to which were added increasing concentrations of extracted cells and sodium penicillin. The mixtures were incubated for 48 hours at 37° C. and then bacterial growth determined by the presence of turbidity.

Continued action of staphylococcic penicillinase

It was of importance to determine if a preparation of penicillinase would continue to inactivate penicillin after one or more exposures to the antibiotic. This was determined by adding 1 mgm. per ml. of extracted cells to a flask containing tryptose phosphate broth with 100 units per ml. of penicillin. After standing for 24 hours a portion of the mixture was filtered to remove penicillinase, and tested for penicillin activity. Then fresh penicillin was added to the flask and the mixture allowed to stand for another 24 hours, and a filtrate again tested for the presence of penicillin. It was observed that the preparation of penicillinase continued to inactivate penicillin completely until

sampling of the mixtures had reduced the concentration of penicillinase to ineffective levels. The contents of a control flask containing only broth and added penicillin did not reveal any loss of penicillin activity when handled in the same manner.

II. Comparison of properties of resistant strains

Staphylococci with resistance acquired by in vitro methods

Four strains of staphylococcus isolated from patients were adapted to grow in increasing concentrations of penicillin. Strain Rutgers was cultured from a suppurative skin lesion, while strains Nelson, Ked, and Eden were isolated from patients with osteomyelitis. The initial sensitivity of these strains and the degree of resistance obtained after multiple transfers in Gladstone's medium containing penicillin are shown in Table IV. A significant

TABLE IV

Development of resistance after multiple transfers in Gladstone's medium with increasing concentrations of penicillin

Strain	No. of transfers in increasing concentrations of penicillin	No. of units per 1 ml. required to inhibit growth	
		Initial sensitivity	After acquisition of penicillin resistance
Rutgers	117	0.05	6
Nelson	117	0.05	6
Ked	115	0.10	120
Eden	113	0.10	80

feature is that the degree of resistance acquired by the strains varied. Strains Rutgers and Nelson required only 6 units per ml. of penicillin to inhibit growth while strains Ked and Eden necessitated 120 and 80 units respectively. It was assumed at the time these observations were made that this acquired penicillin resistance was a relatively permanent property of the strains. Subcultures of these strains were made on veal agar slants and stored in a refrigerator, and transfers were carried out at monthly intervals. At the end of 11 months, the strains were tested for their sensitivity to penicillin and repeated observations revealed that the acquired resistance was not present.

In order to confirm these observations, the foregoing strains which had acquired and lost their resistance to penicillin were again adapted to

TABLE V

Strains which had acquired resistance to penicillin (Table II) and then had lost this property adapted a second time to grow in the presence of increasing concentrations of penicillin

Strain	No. of transfers in increasing concentrations of penicillin	No. of units per 1 ml. required to inhibit growth	
		Initial sensitivity	After acquisition of penicillin resistance
Rutgers	72	0.05	80
Nelson	72	0.05	40
Ked	72	0.10	600
Eden	72	0.10	20

grow in increasing concentrations of penicillin. This time, these strains became resistant in a shorter period of time and, with the exception of strain Eden, a much greater degree of resistance was attained. The comparative resistance of these strains after 72 transfers is shown in Table V. The strains were then transferred to veal agar slants and placed in the refrigerator for 80 days. At the end of this period, sensitivity tests were carried out and the results revealed no loss of resistance after this one subculture and storage. But after multiple transfers, the resistance was lost.

During the period when the 4 strains were being adapted to penicillin, investigations were made with strain Ked. After 29 transfers in increasing concentrations of penicillin, the culture was plated out on agar, and 10 colonies were picked off the plate and tested for their sensitivity to penicillin. The results are presented in Table VI. It is apparent that in such a culture there are cells possessing different degrees of resistance. This is of

interest in view of the fact that this resistance is of only a temporary nature.

In an endeavor to ascertain whether the presence of body fluids, such as human plasma, would influence the properties of staphylococci made resistant by *in vitro* methods, strains Ked and Eden were transferred in a medium with penicillin and equal parts of human plasma and veal infusion broth. Although resistance to penicillin was acquired in this manner, the resistance was of only a temporary nature. These results are in agreement with the findings of Blair and his associates (11) who used human serum.

Repeated attempts were made to obtain penicillinase from the cells of those strains with acquired resistance, but in no instance was the material obtained.

In summary, the resistance to penicillin of staphylococci, established by *in vitro* methods is characterized by a temporary type of resistance, and the resistance is not associated with the production of penicillinase.

Staphylococci with natural resistance

Nine strains of coagulase-positive staphylococcus were isolated in 1942, or before, from the lesions or blood of patients, subcultured on veal agar slants and stored in the refrigerator. These strains, then, were isolated before penicillin was available for clinical use. In 1943, sensitivity tests were carried out with these strains and it was observed that more than 0.5 unit and less than 1 unit per ml. were required to inhibit growth. These strains were subcultured on agar slants every 2 to

TABLE VI

Sensitivity tests with 10 colonies of strain Ked after 29 transfers in increasing concentrations of penicillin

	Colony number									
	1	2	3	4	5	6	7	8	9	10
Control without penicillin	+	+	+	+	+	+	+	+	+	+
Control with 0.05 unit per ml. of penicillin	+	+	+	+	+	+	+	+	+	+
Control with 0.10 unit per ml. of penicillin	+	+	+	+	+	+	+	+	+	+
Control with 0.50 unit per ml. of penicillin	+	+	+	+	+	+	+	+	+	+
Control with 5.00 units per ml. of penicillin	+	+	+	+	+	+	+	+	+	+
Control with 10.00 units per ml. of penicillin	+	+	+	+	+	+	0	+	+	+
Control with 20.00 units per ml. of penicillin	+	+	+	+	+	+	0	+	0	+
Control with 40.00 units per ml. of penicillin	+	0	+	0	0	0	0	0	0	0
Control with 60.00 units per ml. of penicillin	0	0	0	0	0	0	0	0	0	0

+ = turbid growth.
0 = no turbid growth.

3 months and stored in the refrigerator. In 1945, tests for sensitivity still showed the same degree of sensitivity. In addition, these strains all produced penicillinase. It was observed that a concentration of 0.01 mgm. per ml. of extracted bacteria destroyed 20 to 40 units of penicillin per ml.

Briefly, then, staphylococci possessing a natural resistance for penicillin have 2 properties different from strains which have been adapted by *in vitro* methods to resist penicillin. The resistance appears to be a permanent characteristic of the strains and is associated with the production of penicillinase.

Staphylococci with acquired resistance from patients treated with penicillin

Over a period of 2 years, cultures isolated from 5 patients have been studied extensively. In 4 of the 5 patients staphylococci were obtained before and after treatment with penicillin. Because of the significance attached to these coagulase-positive strains in the present studies, the history of these cultures is presented. Strain Eden was cultured in 1942 from the blood of a young child who had staphylococcic bacteremia and acute osteomyelitis of the tibia. Strain Eden II was obtained from a draining osteomyelitic sinus in the same year after the child had received slightly over 2 million units of penicillin. Strain Long was isolated from a draining lesion in the right costovertebral region originating from a perinephritic abscess. Strain Long II was cultured from the same area after 2 million units of penicillin had been administered. The strains from patients Janson, Bernardo, and Rosen were supplied to us in 1944 through the courtesy of Dr. Donald G. Anderson of Boston. Strain Janson was isolated from an osteomyelitic sinus of a young adult in 1942. After approximately 2 million units of penicillin, strain Janson II was cultured from the same sinus. Strain Bernardo was also isolated from an osteomyelitic lesion at approximately the same time as Janson, and after the administration of 600,000 units of penicillin Bernardo II was cultured from the sinus. It is significant, as Anderson and his associates (14) have pointed out, that cultures of lesions made a year after the completion of treatment with penicillin showed the presence of resistant organisms. Strain Rosen was isolated from the blood

stream of a patient who had received about 5 million units of penicillin before the onset of bacteremia. With the exception of Rosen, the comparative increase in resistance of the foregoing strains is given in Table VII.

TABLE VII

Sensitivity of strains of staphylococcus isolated from patients before and after treatment with penicillin

No culture obtained from patient Rosen before treatment

Strain	Units per ml. of penicillin inhibiting growth before treatment	Units per ml. of penicillin inhibiting growth after treatment
Eden	0.10	0.8
Long	0.05	1.0
Janson	0.05	1.0
Bernardo	0.05	2.0
Rosen		20.0

One of the outstanding features of these strains is that the resistance has persisted for up to 4 years, although repeated subcultures have been made. In fact, as will be pointed out shortly, there has been an increase in the degree of resistance. In addition to the characteristic of a persistent resistance, these strains with acquired *in vivo* resistance produce penicillinase. The parent sensitive strains did not yield demonstrable penicillinase.

Attempts to increase resistance of staphylococci with permanent type of resistance by in vitro methods

The preceding studies have shown that sensitive strains of staphylococci may be adapted to grow in increasing concentrations of penicillin but that this acquired resistance was only temporary. In view of this, strains with the permanent type of resistance were adapted to grow in increasing amounts of penicillin by methods already described. While these strains were being adapted, simultaneous transfers were made in culture medium without the presence of penicillin.

The first series of observations were made with strain Long II, which had acquired resistance *in vivo* in association with treatment of the patient with penicillin. On 3/3/45, a culture of Long II was divided into 2 parts. Daily transfers were made with one part thereafter in Gladstone's medium, while the other part, known as Long III, was transferred daily in the same medium contain-

ing penicillin. At intervals, sensitivity tests with penicillin were performed with Long II and Long III. The results of these tests are presented in Table VIII. On 8/1/45, both cultures were

TABLE VIII

Subculture of strain II, known as Long III, adapted to increasing concentrations of penicillin

Beginning 3-3-45 daily transfers of Long II in Gladstone's medium without penicillin, and Long III in same medium with penicillin. Sensitivity tests carried out on dates indicated.

Date	No. of units of penicillin per ml. required to inhibit growth	
	Long II	Long III
3-3-45	0.8	0.8
4-30-45	0.4	6.0
5-26-45	0.4	20.0
7-12-45	0.8	60.0
9-3-45	2.0	60.0

placed on agar slopes and stored in the refrigerator for 29 days. Tests for penicillin sensitivity were carried out without any demonstrable loss of resistance being manifested. It is to be noted that the growth of Long II required from 0.4 to 2.0 units of penicillin per ml. to inhibit growth, whereas the growth of Long III necessitated the presence of 60 units per ml. Subsequently, Long III was subcultured repeatedly in the absence of penicillin and there was no significant change in resistance over a period of 6 months. No entirely satisfactory explanation is offered for the deviations in resistance shown for the control strain Long II. It may be related to the fact that 3 different lots of commercial penicillin were employed for performing the sensitivity tests during the course of the observations. The results of the studies with these 2 strains would indicate that the resistance of Long III was increased because of the presence of penicillin. Subsequent observations with 2 other strains revealed another possible explanation.

Strain Bernardo II, another strain with acquired *in vivo* resistance, was found to produce significant quantities of penicillinase and was used as a source of the material for the preceding studies on the properties of penicillinase. Over a period of several weeks, a subculture of Bernardo II was transferred at frequent intervals in Gladstone's medium and later in tryptose phosphate broth. In the preparation of penicillinase, it was more ad-

vantageous to have a broth culture at hand than to utilize the stock culture which was stored in the refrigerator. It was noted that the potency of penicillinase prepared from the broth subcultures of Bernardo II, labelled Bernardo IIA, was increasing markedly and for this reason the sensitivity of this subculture for penicillin was carried out. When compared with the original stock culture kept on agar slants, it was found that the concentration of penicillin necessary to inhibit growth had increased from 2 units per ml. to 60 units per ml. In other words, frequent transfers of this strain in Gladstone's medium and then in broth *without the presence of penicillin* had resulted in a marked increase in resistance. These results were comparable with those obtained with the Long II strain which was subcultured in the presence of penicillin. From these observations made with the Bernardo II strain, it would appear that the culture contained resistant variants which multiplied rapidly with frequent subcultures, resulting in a strain with uniformly high resistance.

In an attempt to confirm this observation under more controlled conditions, the strain Rosen was selected. A subculture in tryptose phosphate broth was divided into 2 parts, and daily subcultures were made in broth with and without penicillin for 40 days. Subcultures made in the presence of penicillin were designated as Rosen I. At the termination of this period, sensitivity tests revealed that the culture carried through in broth *without penicillin* required 400 units of penicillin per ml. for the inhibition of growth, whereas the culture which was transferred in broth containing penicillin required 600 units. It is to be recalled that the original resistance of this strain was a requirement of 20 units per ml. of penicillin for growth inhibition. These observations, then, confirmed those made with the Bernardo strain, namely, that the rapid transfer of a penicillin-resistant strain without the presence of penicillin resulted in a considerable increase in the resistance of the strain. During the ensuing 6 months, this increase in resistance persisted although repeated subcultures were made.

It was noted that after the Rosen and Rosen I strains were transferred daily for several days in tryptose phosphate broth, the 24-hour growth was quite granular and a pellicle formed on the surface.

This was in contrast to the original smooth growth exhibited by these strains. Granular growth with pellicle formation also took place when the strains were grown in Gladstone's medium. The possibility that this acquired growth characteristic was associated with the acquisition of increased resistance was ruled out when control sensitive strains showed the same type of granular growth after multiple and rapid transfers in broth. It should be emphasized that this phenomenon of increased resistance to penicillin developing in the absence of penicillin occurred only with the Bernardo and Rosen strains. Comparable studies showed that an increase in resistance was not established with 7 sensitive strains, including a subculture of the Oxford H strain.

Bernardo II strain, which had acquired an increased resistance in the absence of penicillin so that 60 units of penicillin per ml. instead of 2 units were necessary to inhibit growth, was subcultured on an agar slant and stored in the refrigerator. Then the strain was subcultured daily in tryptose broth for 2 months, but this was not associated with any further increase or loss in resistance.

In summary, it would appear that an occasional strain of staphylococcus manifesting resistance to penicillin may acquire a further increase in resistance in the absence of penicillin when the strain is permitted to multiply rapidly. This increase is probably due to the presence of resistant variants which reproduce rapidly with frequent subcultures. This phenomenon has not been observed with sensitive strains by the methods used, though the observations of Demerec (24) and Kirby (9) would indicate that sensitive strains do possess resistant variants.

III. Relationship between the resistance of staphylococci to penicillin and the production of penicillinase

Characteristics of strains with resistance acquired in vitro and strains naturally resistant or with resistance acquired in vivo

As has already been pointed out, 2 of the outstanding features of strains of staphylococcus, which have been adapted *in vitro* to grow in high concentrations of penicillin, are the temporary nature of this acquired resistance and the failure of these strains to produce penicillinase. In addition,

strains with the temporary type of resistance have been found to be avirulent for animals (11) (18).

In an attempt to define the relationship of penicillinase production to resistance, further comparative studies were made with strains representing the 2 different types of resistance. After ascertaining, by means of the standardized test in which a *small inoculum* of organisms was used, how resistant to penicillin the selected strains were, growth curves of the strains were carried out in order to determine the effect of penicillin upon a *large inoculum* of organisms. The concentrations of penicillin used were 0.1, 1, 10, and 100 units per ml. Thus, instead of an inoculum of fewer than 1 million organisms as was employed in the routine test for sensitivity, an inoculum containing from $\frac{1}{2}$ to 1 billion organisms was utilized. Observations were made with 2 strains which had acquired resistance by *in vitro* methods. The growth of strain Nelson was initially inhibited by 0.05 unit per ml. of penicillin, and, after adaptation, 40 units per ml. were required for the inhibition of growth. Strain Eden required 0.1 unit initially for growth inhibition and 0.8 unit after adaptation. When a large inoculum of these 2 strains was used, growth curves in the presence of penicillin are shown in Figure 2. With the more resistant strain, Nelson, it is to be noted that even a concentration of 0.1 unit per ml. of penicillin had a bacteriostatic effect upon growth and with all concentrations of penicillin, there was a definite inhibition of growth of the organisms. Neither of these 2 strains produced penicillinase. These results are to be compared with those obtained with 4 strains which had become resistant *in vivo*, all of which produced penicillinase. Resistance studies showed that strain Janson II required 0.6 for growth inhibition; strain Bernardo II, 2 units; strain Long II, 1 unit; and strain Rosen, 20 units. With the exception of the least resistant strain, Janson II, penicillin did not have a bacteriostatic effect on the growth of a large inoculum of organisms, even when a concentration of 100 units per ml. was employed. These results are shown in Figure 3. It is apparent that the presence of relatively large amounts of penicillinase protected the growth of these organisms against the action of penicillin in contrast to the strains which did not produce penicillinase.

Observations were also made to determine

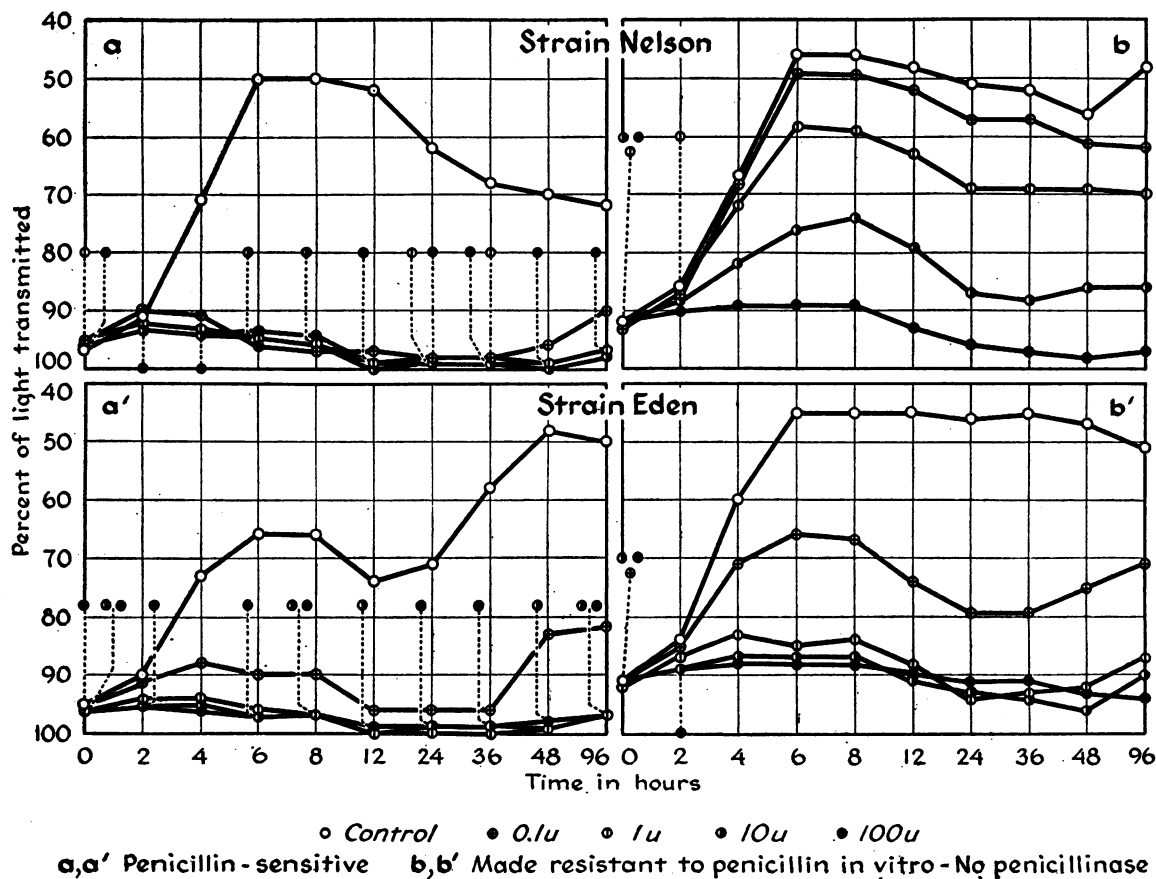


FIG. 2. INHIBITORY EFFECT OF VARYING CONCENTRATIONS OF PENICILLIN UPON GROWTH CURVES OF LARGE INOCULUM FROM STRAINS OF STAPHYLOCOCCUS WITH TEMPORARY TYPE OF RESISTANCE AND WITHOUT PROPERTY OF PRODUCING PENICILLINASE

whether strains of staphylococcus sensitive to penicillin destroyed penicillin when a heavy inoculum was used. A subculture of the Oxford H strain was grown for 24 hours on an agar plate and then a thick suspension was made and added to a flask containing 50 ml. of tryptose broth with 100 units per ml. of penicillin. The mixture was incubated for 24 hours at 37° C. and then a filtrate tested for the presence of penicillin. There was no diminution in the activity of penicillin. On the other hand, when the same experiment was carried out with a strain that produced penicillinase, such as Bernardo II, there was almost a complete destruction of penicillin activity.

Relationship between in vitro resistance to penicillin and inactivation of penicillin by penicillinase

In a previous report by Spink and Ferris (30) it appeared that there was a quantitative relation-

ship between the *in vitro* resistance of strains of staphylococcus to penicillin and the inactivation of penicillin with dried preparations of these strains. The observations were made with strains that were not highly resistant to penicillin. These observations have been confirmed in the present studies and further experiments were carried out with more resistant strains. The strains selected for study were Long, Bernardo, and Rosen. Extracted cells of the parent strains of Long and Bernardo did not inactivate penicillin and no parent strain was available for Rosen. Following treatment of the patients with penicillin, strains Long and Bernardo manifested an increase in resistance and these strains produced penicillinase. Strain Long was the least resistant of the 3 strains and produced the least potent penicillinase. The relative resistance of these strains and the relationship of this resistance to the potency of penicil-

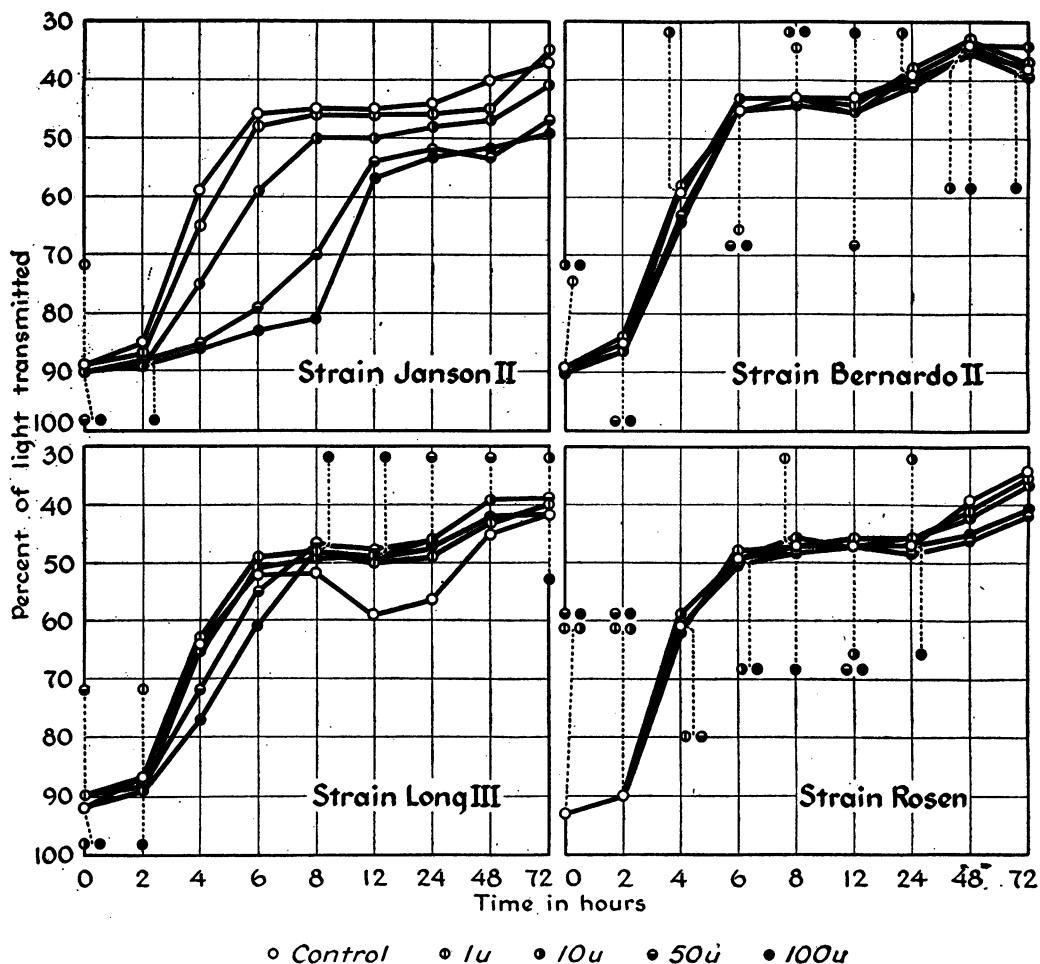


FIG. 3. WITH EXCEPTION OF STRAIN JANSON II, FAILURE OF VARYING CONCENTRATIONS OF PENICILLIN TO INHIBIT GROWTH OF LARGE INOCULUM FROM STRAINS OF STAPHYLOCOCCUS WITH PERMANENT TYPE OF RESISTANCE AND WITH PROPERTY OF PRODUCING PENICILLINASE

linase produced by them are shown in Table IX. After the 3 strains had acquired further resistance to penicillin by methods already described, there was an associated increase in the potency of penicillin

linase production as seen in Table IX. Thus, strain Rosen with an acquired resistance which required 400 units of penicillin to inhibit growth produced such a highly active penicillinase that 0.0001 mgm.

TABLE IX
Relationship between the in vitro resistance of staphylococci and the inactivation of penicillin by penicillinase

Strain	Strains isolated from patients after treatment with penicillin		Strains isolated from patients after treatment with penicillin and which had acquired further resistance to penicillin <i>in vitro</i>	
	No. of units of penicillin per ml. required to inhibit growth	No. of mgm. of dried bacteria which inactivated maximum no. of units of penicillin per ml.	No. of units of penicillin per ml. required to inhibit growth	No. of mgm. of dried bacteria which inactivated maximum no. of units of penicillin per ml.
Long	0.8	0.01 mgm.— 40 units	60	0.001 mgm.— 400 units
Bernardo	2.0	0.01 mgm.— 200 units	60	0.001 mgm.— 400 units
Rosen	20.0	0.01 mgm.— 600 units	400	0.001 mgm.— 1,800 units 0.0001 mgm.— 400 units

of dried cells inactivated 400 units per ml. of penicillin. The important feature of these observations is that resistance is correlated with penicillinase. The more resistant a strain, the more potent is the penicillinase produced by that strain. Furthermore, relatively small amounts of penicillinase inactivated large concentrations of penicillin. It should be pointed out, however, that the results as given in Table IX show only a relative quantitative relationship between the degree of resistance and the inactivation of penicillin. This is very probably due to the fact that the method of preparing penicillinase permitted only the use of crude material.

DISCUSSION

These studies emphasize the distinction between the 2 types of resistance to penicillin manifested by staphylococci. A clarification of these differences has been desirable, particularly from the viewpoint of the development of resistant strains of staphylococci in patients as a result of treatment with penicillin. The type of resistance which results from adapting strains *in vitro* to grow in increasing concentrations of penicillin is not a permanent characteristic of these strains, and this resistance is not associated with the production of penicillinase. It is not definitely known whether this type of temporary resistance occurs in the human body following therapy with penicillin. If the phenomenon does occur, it is probably of little clinical significance, since such strains have been shown to be less virulent than the sensitive parent strains (22, 11, 18). The underlying mechanism whereby penicillin-sensitive strains acquire a temporary resistance to the action of penicillin is not clear.

The second type of penicillin resistance, which appears to be a permanent characteristic of the strains and is accompanied by the production of penicillinase by these strains, is of considerable importance in the clinical use of penicillin. The most acceptable explanation for the origin of strains with the permanent type of resistance is that afforded by Demerec (24) and, independently, by Kirby (9), namely, that cultures of penicillin-sensitive strains contain resistant variants capable of mutating toward a state of a high degree of resistance if these cells are given an opportunity to

multiply rapidly. The present studies also show that penicillin-resistant strains may acquire a much greater resistance to penicillin if the cells are permitted to multiply rapidly, even in the absence of penicillin.

In describing the development of penicillin-resistant staphylococci by the mechanism of mutation, Demerec (24) did not state whether the resistant cells produced penicillinase. On the other hand, Kirby (29) isolated cells from penicillin-sensitive strains of staphylococci which proved to be resistant to penicillin and which produced penicillinase. In a follow-up study of Demerec's work, Luria (31) discounted the role of penicillinase in the mechanism of penicillin-resistance by emphasizing that the individual cells of resistant and penicillinase-producing strains were sensitive to small concentrations of penicillin. The fundamental question is not whether a given strain produces penicillinase but rather how quickly and how potent is the penicillinase produced by a strain in relation to the concentration of penicillin and the time necessary for the antibiotic to destroy the cells. It is to be recalled that in their original report, Abraham and Chain (27) found that *M. lysodeikticus* produced penicillinase, and yet this strain was sensitive to penicillin. The present investigations show that strains of staphylococci with a relatively high degree of resistance produce a potent inactivator of penicillin; the greater the resistance, the more potent the penicillinase. This relationship of penicillinase production to the resistance of penicillin has been demonstrated for other species of bacteria. Woodruff and Foster (35) described an aerobic spore-forming bacillus belonging to *B. subtilis* group which at pH 6.0 or above was not inhibited in its growth by several hundred units of penicillin because of the penicillinase produced by that strain. However, when the same strain was grown at pH 5.5, no penicillinase was produced, and growth was inhibited by 10 units per ml. of penicillin. McQuarrie and his associates (36), working with 3 strains of spore-forming gram-negative rods, noted a quantitative relationship between penicillin-resistance and the production of penicillinase. The present discussion relates primarily to the mechanism whereby staphylococci resist the action of penicillin because of penicillinase. It is well established that other

mechanisms are responsible for resistance with other species of bacteria since many gram-negative bacteria are highly resistant and yet do not produce penicillinase (21).

The observations presented here and those of others (35 to 39) indicate that the penicillinases from different species of bacteria are not identical but possess different physico-chemical properties, though they all have the common property of inactivating penicillin. It is of interest that Perlstein and Liebmann (40) produced an anti-penicillinase immune serum by injecting rabbits with purified penicillinase. The significance of this observation must await further investigations.

Clinically, the potentiality of penicillin-resistance in staphylococci should be recognized, with the object of treatment being to destroy all the organisms within the shortest possible time. If inadequate concentrations of penicillin are present in the body fluids or tissues of patients, the more susceptible bacterial cells may be eradicated, providing an opportunity for the resistant variants to multiply.

SUMMARY

1. Two types of resistance to penicillin have been described for staphylococci. The first type is an adaptation that may be reproduced *in vitro*, and the resistance is of a temporary nature and not associated with the production of penicillinase.

2. The second type which occurs in patients as a result of treatment with penicillin results in the establishment of strains with a permanent resistance to penicillin, and these strains produce penicillinase.

3. The mechanism whereby temporary resistance takes place is not clearly understood. The permanent type of resistance involves the presence of resistant cells in a penicillin-sensitive strain, which, when permitted to multiply rapidly, establish a uniformly resistant strain.

4. The magnitude of the resistance to penicillin manifested by the permanent type of resistance is quantitatively related to the potency of penicillinase produced by the strains.

5. The establishment of resistant strains of staphylococcus in the human body can be prevented by the prompt use of adequate quantities of penicillin.

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