PROPERTIES OF A PENICILLIN INACTIVATOR EXTRACTED FROM PENICILLIN-RESISTANT STAPHYLOCOCCI [1]

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An enzyme-like substance capable of destroying penicillin was produced from *E. coli* and certain other saprophytic and commensal bacteria in 1940 (1), but attempts to extract a penicillin inactivator from sensitive or resistant staphylococci were unsuccessful (2). More recently, acetone-ether extracts of para-colon bacilli which were more effective penicillin inhibitors than were extracts of *E. coli* were prepared (3).

In this country, it was found (4) that Clarase, a diastatic enzyme preparation, readily destroyed penicillin, and this substance is now used widely for sterility tests during penicillin production. Apparently, the ability to inactivate penicillin is possessed by only certain lots of Clarase (5), and this action is attributed in a later publication (6) to the presence of bacterial products, especially of *B. subtilis* and related gram-positive organisms.

A penicillin-destroying staphylococcus has been found contaminating a culture of *Aspergillus flavus* (7), and independently, in this laboratory, a highly potent penicillin inactivator has been extracted from penicillin-resistant staphylococci obtained from clinical sources (8). Details of the extraction and properties of this substance are presented in the present paper.

DESCRIPTION OF RESISTANT STAPHYLOCOCCI

During a study of several hundred strains of staphylococci isolated from patients (9, 10), a few were found which were completely resistant to the lytic action of penicillin; 5 of these were selected for the present study. Two additional strains were cultured from a secondary outgrowth of highly resistant staphylococci following the lysis of susceptible organisms by 0.1 U. per ml. of penicillin (9). All 7 strains produced yellow pigment, and there were no abnormalities of growth or colony characteristics. Details of the sensitivity tests have been described elsewhere (9, 10).

METHOD OF EXTRACTION

The acetone-ether method of Harper (3), devised for the para-colon bacillus, was equally satisfactory for the staphylococcus. Twenty-four-hour plate cultures were washed with a minimum of saline and precipitated with 7 volumes of acetone. After standing 2 hours, the precipitate was treated with a fresh supply of acetone for an hour, washed with 2 changes of ether, and dried in the anaerobic jar. During extraction, the coarse precipitate was crushed repeatedly with a glass rod to insure maximum contact between the acetone and the organisms. The fine dried powder was stored in a desiccator at room temperature.

MEASUREMENT OF PENICILLIN INACTIVATION

The powder was suspended in broth, usually in a concentration of 1 mgm. per ml. The ability of various amounts of this suspension to inactivate penicillin was measured by recording the growth of a Group A hemolytic streptococcus which was inhibited by less than 0.05 U. per ml. of penicillin. To tubes containing the powder and a heavy inoculum (about one million organisms per ml.) of the test bacterium, penicillin was added in concentrations of 0.1, 1.0, 5.0, and 10.0 U. per ml., and optical densities (turbidities) were measured every few hours on a Coleman universal spectrophotometer during 24 hours, incubation. The exact amounts of broth, penicillin, etc.,

TABLE I

Outline of test for measurement of penicillin inactivation

Tube	Broth plus hemolytic streptococcus	Penicillin	Penicillinase	Sterile broth
	ml.	*1.0 U. per ml.*	*1 mgm. per ml.*	
1	10	0	0	0
2	9	1 ml.	0	0
3	8	1 ml.	1 ml.	0
4	8	1 ml.	1 ml.	0
5	8	1 ml.	1 ml.	0
6	8	1 ml.	1 ml.	0
7	0	0	1 ml.	9

[1] The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

are listed in Table I. The results of a typical experiment are presented in Figure 1.

Penicillin was destroyed gradually, with the length of time required for complete destruction roughly proportional to the amount of penicillin present. One hundred units were completely destroyed by 1 mgm. of the powder within 6 to 8 hours. By decreasing the amount of powder, it was found that 0.6 mgm. would cause complete destruction of 100 units of penicillin in 24 hours, whereas 0.4 mgm. would not (Figure 2).

Using this arbitrary endpoint, it could be said that 1 mgm. of the powder would destroy 167 units within 24 hours. Although there were some variations, this same high degree of potency was shown by the extracts of all 7 resistant strains.

Penicillin sensitive staphylococci were extracted and tested in the same manner. Extracts of 7 strains, isolated from blood cultures of patients with acute infections, all failed to cause any demonstrable inactivation of penicillin, even with 2 mgm. of the powder and only 1 unit of penicillin.

PROPERTIES OF THE PENICILLIN-DESTROYING SUBSTANCE

Heat stability. Broth suspensions of the powder could be kept in the incubator (37° C.) for at least 48 hours, or at room temperature for several weeks with no detectable diminution in their ability to destroy penicillin. At 56° C., however, the ability to inactivate penicillin was completely lost in 5 minutes or less.

Rate of destruction of penicillin at different temperatures. Broth suspensions of the powder (1 mgm.) and penicillin (100 units) were left at 2° C., 22° C., and 37° C. for 2, 3, 4, and 5 hours, and then tested for penicillin destruction in the usual manner. In every instance, growth of the test organism occurred at the same rate for the mixtures left at 2° C., 22° C., and 37° C. after any given time interval, indicating that penicillin was destroyed as rapidly at 2° C. as it was at room temperature or in the incubator.

Nature of penicillin inactivation. The following experiment was performed to determine whether penicillin was actually destroyed, or whether it merely entered into a chemical com-

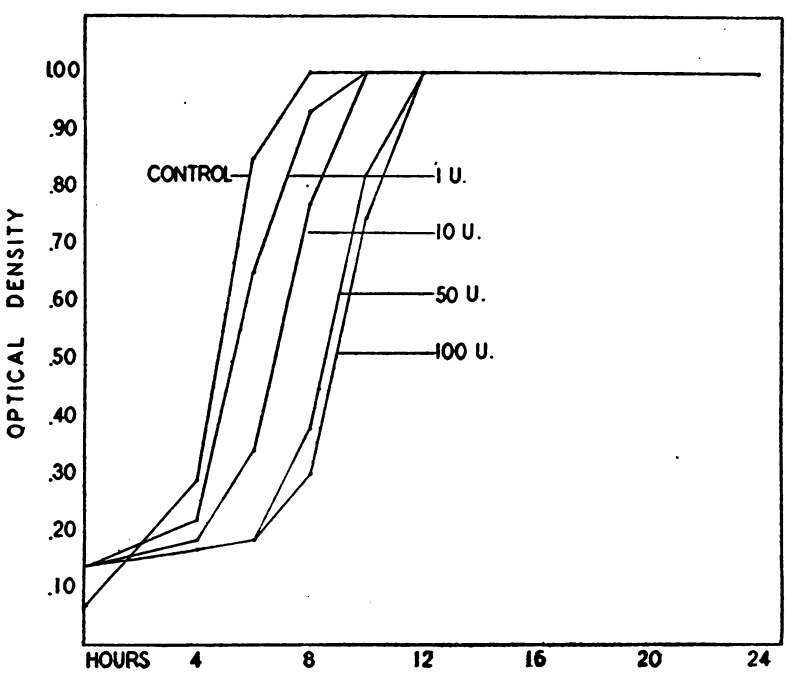

FIG. 1. DESTRUCTION OF VARIOUS AMOUNTS OF PENICILLIN BY 1 MGM. OF THE STAPHYLOCOCCUS EXTRACT

Complete destruction is indicated by growth of the organism which was inhibited by 0.05 U. per. ml. of penicillin.

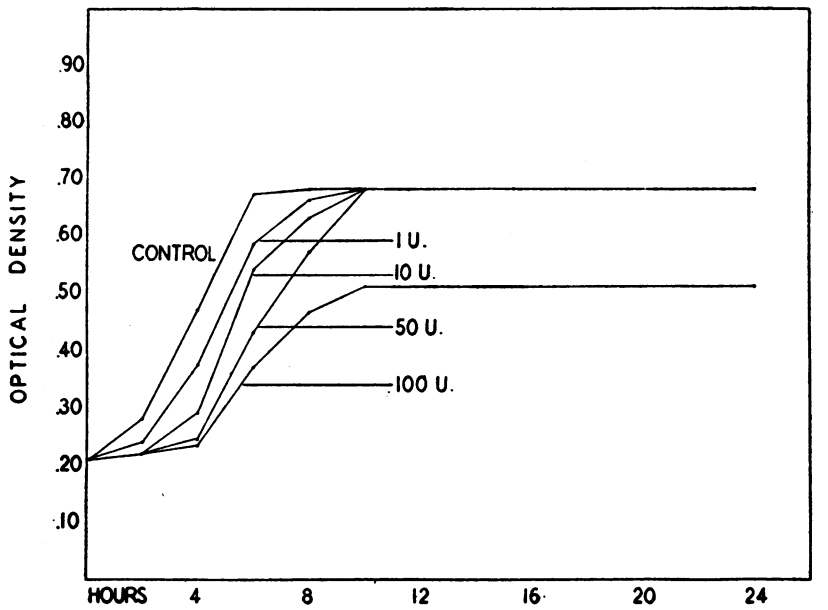

FIG. 2. INACTIVATION OF PENICILLIN BY 0.4 MGM. OF THE DRIED POWDER

Fifty units were destroyed, whereas 100 were not, giving an arbitrary endpoint for titrating the potency of the powder.

bination with some constituent of the bacterial extract. One mgm. of the dried powder was added to 100 units of penicillin, and left at room temperature overnight. The next morning the mixture was placed in a 56° C. waterbath for 15 minutes to destroy the penicillin inactivator. The solution was then tested for penicillin, and none could be demonstrated. Fifteen minutes at 56° C. caused no loss of potency of a control sample of penicillin. This suggests that the penicillin was actually destroyed, although the possibility of a chemical combination not disrupted by heat cannot be definitely excluded. The observation that penicillin could not be extracted with amyl acetate after inactivation by penicillinase is further evidence in favor of actual penicillin destruction (3).

Penicillin inactivation by living cultures. Growth of resistant staphylococci in the presence of penicillin was associated with destruction of the penicillin. During this destruction, definite bacteriostasis occurred but the organisms resisted lysis and multiplied so that after 24 hours, growth was equal to that of controls containing no penicillin. This is illustrated in Table II. Complete destruction of penicillin was confirmed by testing Seitz filtrates of the culture fluid for penicillin; none was present.

Seitz filtrates of cultures of penicillin-resistant staphylococci or of broth suspensions of the powdered extract did not destroy penicillin; *i.e.*, the penicillin was intracellular, and did not pass out into the culture fluid.

COMPARISON WITH CLARASE

In contrast to the staphylococcus extract, Clarase (lot. No. 1351) was dissolved in phosphate buffer at pH 7, and the penicillin inactiva-

TABLE II

Turbidimentrically recorded growth of a penicillin-resistant S. aureus

Although there was definite bacteriostasis, the penicillin was destroyed and growth after 24 hours was equal to that of the control. Turbidity is expressed in terms of optical density.

Penicillin	Hours			
	0	4	6	24
0	0.168	0.57	0.745	0.77
10 U. per ml.	0.168	0.376	0.52	0.77
100 U. per ml.	0.168	0.285	0.376	0.77

tor passed readily through a Seitz filter. Ability to destroy penicillin was tested in the manner described for the dried powder. Using a 1 per cent solution of Clarase, destruction of 100 units of penicillin by 0.6 ml. (6 mgm.) was equivalent to 0.6 mgm. of the staphylococcus extract; *i.e.*, in terms of weight of the original powder, the staphylococcus extract was 10 times as potent as Clarase. With equivalent quantities, the rate of destruction of penicillin at 2° C., 22° C., and 37° C., was identical with both substances. Clarase was quite stable at room and incubator temperatures and was more stable at 56° C. than the staphylococcus extract, activity being only partially destroyed by exposure to that temperature for 30 minutes.

DISCUSSION

A highly potent penicillin inactivator has been extracted from 7 strains of *S. aureus* which were naturally resistant to penicillin; 7 sensitive strains did not produce a penicillin-destroying substance. The resistant bacteria were not lysed, but were by no means completely resistant to the bacteriostatic action of penicillin, as indicated in Table II. This differentiation between resistance to lysis and resistance to bacteriostasis is discussed elsewhere (10).

It is of fundamental importance to determine whether the several penicillin inactivators so far described are identical, or whether penicillin can be destroyed by substances whose chemical structures are unrelated. Properties of the known penicillin inactivators are summarized in Table III. Presumably they are all products of bacterial metabolism, but whether they are produced by all penicillin-resistant organisms has not yet been definitely determined. The differences in properties are relatively superficial, and possibly largely due to differences in technical methods. Further chemical studies are necessary for final clarification.

Resistant staphylococcus extracts were 10 times as effective as Clarase in their ability to destroy penicillin, but since the potency of Clarase is presumably due to the presence of unknown amounts of bacterial products, no exact comparison could be made.

Penicillin inactivators are used for sterility tests, and for cultures from patients receiving penicillin. A brief description of the technic and results of the addition of staphylococcus extracts to culture media has been published elsewhere (11).

The presence of a penicillin inactivator in extracts of resistant but not of sensitive staphylococci is of interest in connection with the recent observation (12) that the development of sulfonamide-resistant strains of staphylococci is

TABLE III

Summary of the properties of the known penicillin inactivators

Source	Apparent ability to inactivate penicillin	Heat stability	Destruction of penicillin by living cultures	Presence of inactivator in culture fluid	Rate of penicillin destruction at 2° C., 22° C., and 37° C.
E. coli (Abraham and Chain)	+	Destroyed by 90° C. for 5 min.	Partial (Harper)	Absent	Slower at 25° C. than 37° C.
Unidentified gram-negative rod (Abraham and Chain)	+	Not known	Indefinite	Present	Not known
Paracolon (Harper)		No destruction at 50° C. for 30 min.	Complete	Present	Not known
Clarase (Lawrence)		Partial destruction at 56° C. for 30 min.		Present	Same with all temperatures.
Staphylococcus (McKee, Rake, and Houck)	Indefinite	Not known	At least partial	Present	Not known
Staphylococcus (Kirby)		Complete destruction at 56° C. for 5 min.	Complete	Absent	Same with all temperatures

related quantitatively to the production of para-aminobenzoic acid. The evidence is too incomplete to suggest the possibility of competitive enzyme systems as a basis for the explanation of the mode of action of penicillin, but the analogy is striking. Unfortunately, staphylococci made resistant *in vitro* have not been tested in this laboratory for penicillin inactivation. There is a need for refined methods of extraction to search for the presence of penicillin inhibitors in sensitive staphylococci, as well as for intensive study of the chemical nature of the inhibitors themselves. It seems not unreasonable to suppose that, as with the sulfonamides, a more fundamental understanding of the mode of action of penicillin will come as a result of a study of its inhibitors.

SUMMARY

A penicillin inactivator has been extracted from 7 strains of penicillin resistant *Staphylococcus aureus* obtained from clinical sources; 7 sensitive strains did not produce a penicillin-destroying substance. Properties of the penicillin inactivator are described, and its relation to other inhibitors and to the mode of action of penicillin in general is considered.

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