THE INACTIVATION OF ANTIBACTERIAL AGENTS AND THEIR MECHANISM OF ACTION

C. J. CAVALLITO, JOHN HAYS BAILEY, T. H. HASKELL, J. R. McCORMICK, AND W. F. WARNER

Research Laboratories, Winthrop Chemical Company, Inc., Rensselaer, New York

Received for publication March 5, 1945

The observation by two of us (Cavallito and Bailey, 1944) that a variety of antibiotic substances of heterogeneous chemical nature are inactivated by cysteine and by certain other compounds containing very reactive sulfhydryl groups has led to further investigation of this reaction and to the formulation of a theory of the mode of action of these antibacterial agents. Several specific instances of sulfhydryl inactivation of antimicrobial agents are already in the literature. Eagle (1939) has shown that sulfhydryl compounds inactivated the arsphenamine type derivatives; Fildes (1940) described the inactivating action of thioglycollate, cysteine, and glutathione on the antibacterial activity of mercury; and Atkinson and Stanley (1943) have shown a similar inactivation for penicidine. More recently, Geiger and Conn (1945) have extended this observation to include clavacin, penicillic acid, and some α-β unsaturated ketones. That so many antibacterial agents are inactivated by highly reactive thiol compounds indicates that this is more than just a fortuitous action and leads to a working hypothesis for the mode of action of a large class of antibacterial agents.

Tests for antibacterial action and for inactivation of this activity were conducted by two methods, the Oxford cup test and titration in broth. Preliminary inactivation tests were conducted by treating relatively concentrated solutions of the antibacterial agent with the inactivators for various time intervals, followed by dilution of the mixture and testing by the cup method. Inactivation could be shown readily in this manner. Titration tests were conducted in two ways. In one group the reaction mixture of inactivator and antibacterial agent prepared as for the cup tests was diluted in broth so as to obtain the inhibiting concentration in broth cultures. In another group the inactivator was added to the antibacterial agent in broth after the final dilution. The tubes were incubated for 18 hours.

The antibacterial agents tested quantitatively for inactivation included mercuric chloride, pyocyanine, penicillin, the antibacterial principle of *Arctium minus*, $C_{15}H_{20}O_5$ (Cavallito, Bailey, and Kirchner, 1945), and the antibiotic from *Allium sativum*, C_3H_5—SO—S—C_3H_5 (Cavallito, Bailey, Buck, and Suter, 1944).

EXPERIMENTAL

For the cup tests the antibacterial agent was treated with several molecular proportions of the inactivating substance for given periods of time at room temperature, and then diluted on the basis of the antibacterial agent originally present to a point at which the inhibition zone was readily measurable. The

cups used were of aluminum with a 10 mm I.D. and 12 mm O.D. A 13-mm zone of inhibition indicated only trace activity; less than this is represented in the tables as zero mm inhibition. The antibacterial agents effective against both gram-positive and gram-negative bacteria were tested against both *Staphylococcus aureus* and *Salmonella paratyphi*. The results obtained with the two organisms were qualitatively the same. The action against *S. aureus*, however, was quantitatively greater; therefore, this organism was used for comparative inactivation tests. All reactions were conducted at pH 7 unless otherwise stated.

Penicillin

Cup tests. The penicillin used was a sodium salt of 750 units per mg. The quantities shown in the tables are calculated in terms of pure penicillin (1,650 ± units per mg) in the inactivation reaction mixture. The effects of time and concentration on the degree of inactivation produced by cysteine are shown in table 1. The ratios represent the lowest proportion of cysteine to penicillin which completely inhibited the action of penicillin when the reaction mixture was diluted to the equivalent of 3 Oxford units of penicillin per ml. With no inactivation a 30- to 32-mm diameter zone of inhibition against *S. aureus* was produced.

Sulfhydryl derivatives other than cysteine were also tested. Cysteine methyl and ethyl esters, β-aminoethanethiol, and β-(dimethylamino)-ethanethiol were as effective as cysteine; homocysteine was slower, whereas thioglycollic acid, glutathione, β-thiolethanol, N-acetylcysteine, and N-(*p*-toluenesulfonyl)-cysteine were very poor inactivators in high concentrations. Cystine, serine, methionine, S-methyl cysteine, and aminoethanol had no visible action under the test conditions.

The effect of reaction concentration of penicillin and β-(dimethylamino)-ethanethiol on the degree of inactivation is illustrated by the data in table 2. The concentrations indicated are those in the reaction mixture, all solutions being diluted so as to be equivalent to 2 units per ml prior to testing. The reaction time was 30 minutes.

These tests were conducted at pH 7. At pH 8 the sulfhydryl reagents produced much more rapid inactivation, whereas at pH 6 the inactivation was much slower.

Titration tests. The lowest concentration of penicillin producing complete inhibition of growth of *S. aureus* in broth after 18 hours' incubation was found, under the conditions of these experiments, to be approximately 0.03 Oxford units per ml. Solutions of penicillin containing between 0.03 and 10 units per ml showed no loss of activity when treated with 0.2 mg per ml of cysteine. However, when the concentration of penicillin was raised to 15,000 units per ml and was treated with 7 mg per ml of cysteine for a period of three hours, no penicillin activity could be detected in a dilution of this reaction mixture containing the equivalent of 300 units per ml. This is in agreement with the results obtained from the cup tests and shows that the rate of inactivation of

penicillin by sulfhydryl compounds is a function of the time of reaction and concentration of reactants. The lower the concentration of penicillin, the greater the time and excess of sulfhydryl reagent necessary to produce inactivation.

TABLE 1

Effect of time and concentration on inactivation of penicillin by cysteine

PENICILLIN CONCENTRATION	APPROXIMATE CONCENTRATION CYSTEINE, MG PER ML, FOR INACTIVATION IN:			
	1 hour	3 hours	6 hours	24 hours
0.5 mg per ml.....	2.0	1.0	0.50	
5.0 mg per ml.....	0.50	0.33	0.16	0.16

TABLE 2

Effect of concentration of β-(dimethylamino)-ethanethiol on inactivation of penicillin

THIOL CONCENTRATION	PENICILLIN CONCENTRATION	MM INHIBITION ON DILUTION
mg/ml	*units/ml*	
None	Control	27.5
2	2,000	20
20	20,000	0
15	20,000	0
12	20,000	0
10	20,000	13.5
8	20,000	17
5	20,000	22

TABLE 3

Cysteine inactivation of the antibacterial principle from Allium sativum

REACTION CONCENTRATION OF ANTIBIOTIC	MOLECULAR RATIO CYSTEINE:ANTIBIOTIC	MM INHIBITION
mg/ml		
5.0	No cysteine	34
5.0	1:1	28
5.0	2:1	15
5.0	3:1	0
0.5	No cysteine	32
0.5	1:1	26
0.5	2:1	14
0.5	3:1	0

Antibacterial Principle of Allium sativum

Cup tests. The reaction of this compound with cysteine is very rapid compared with the rate of cysteine inactivation of penicillin. After allowing the inactivator and antibiotic to react, the solution was diluted so as to be equivalent to a concentration of 0.25 mg of antibiotic per ml. If no inactivation had occurred, a 32- to 34-mm diameter zone of inhibition against *S. aureus* was observed.

Table 3 illustrates the inhibition zones produced after 15 minutes' reaction time with cysteine, followed by dilution as described, before testing. A 30 minutes' and 3 hours' reaction time gave the same results.

Homocysteine, thioglycollate, allyl mercaptan, glutathione, N-acetylcysteine, N-(*p*-toluenesulfonyl)-cysteine, β-(dimethylamino)-ethanethiol, β-aminoethanethiol, β-thiolethanol, and cysteine esters also produced inactivation, which proceeded equally well at pH 6.0 and at 7.5. S-methyl cysteine, methionine, cystine, alanine, and serine had no effect.

Titration tests. Approximately 0.0038 mg per ml of antibiotic was the lowest concentration producing complete inhibition of *S. aureus.* At a 0.02 mg per ml level, 0.2 mg per ml of cysteine produced complete inactivation of the compound. When to a solution containing 50 mg per ml of the antibiotic there were added 3 molar proportions of cysteine and the solution was diluted 1:10 with broth after 10 minutes' reaction time (precipitate filtered off), normal bacterial growth was observed.

Antibacterial Principle of Arctium minus

Cup tests. This substance reacted rapidly with cysteine at pH 6 or 7. The reaction mixtures were tested, without dilution, against *S. aureus,* and the zones of inhibition observed are recorded in table 4. The higher concentration contained 5 per cent alcohol.

N-acetylcysteine, β-thiolethanol, homocysteine, and glutathione also produced inactivation; S-methyl cysteine, alanine, and serine had no effect.

Titration tests. A concentration of 0.15 mg per ml of the antibacterial agent gave complete inhibition of growth of *S. aureus* in broth. A solution with 0.20 mg per ml no longer inhibited growth after treatment with one molar equivalent of cysteine in broth. It was possible to show cysteine inactivation in the final test concentration as a result of the relatively weak antibiotic activity of this substance and the relatively high concentrations required to inhibit growth.

Pyocyanine[1]

Cysteine, homocysteine, β-aminoethanethiol, β-(dimethylamino)-ethanethiol, β-thiolethanol, and N-acetylcysteine in high concentrations all reduced pyocyanine to a colorless reduction product. This reduction product was easily oxidized by exposure to air. For this reason, inactivation studies as measured by antibacterial action were not significant.

Penicillin and pyocyanine in dilute solutions both require high molecular ratios of thiol compounds for reaction. With pyocyanine any of the thiols tested reacted with the antibiotic, whereas with penicillin only specific thiols produced inactivation.

Mercuric Chloride

Cup tests. Inasmuch as preliminary tests with *S. aureus* and *S. paratyphi* showed the same reactions (*S. paratyphi* was somewhat less sensitive), extensive tests were conducted with only the former organism. A 1:10,000 solution of

[1] We are indebted to Dr. A. R. Surrey for the sample of synthetic pyocyanine.

mercuric chloride in water gave a 15- to 17-mm diameter zone of inhibition under the test conditions. The treatment of mercuric chloride solutions with sulfhydryl-containing compounds produced a different type of result from that observed with the organic antibacterial agents. The sulfhydryl compound strongly increased the effect of mercury in cup tests before decreasing it. A mixture of equimolar quantities of mercuric chloride and cysteine in aqueous solution (*p*H adjusted to 6.5) yielded a white precipitate, and upon addition of one more molar equivalent of cysteine the precipitate went into solution. The solution containing a 2:1 ratio of cysteine to mercuric chloride gave a much larger

TABLE 4

Cysteine inactivation of the antibacterial principle from Arctium minus

ANTIBACTERIAL AGENT	MOLECULAR RATIO CYSTEINE: ANTIBACTERIAL AGENT	MM INHIBITION AFTER REACTION TIME OF	
		10 min	60 min
mg/ml			
4	No cysteine	21	22
4	1:2	20	20
4	1:1	0	0
4	2:1	0	0
1	No cysteine	17.5	17
1	1:2	15	13
1	1:1	0	0
1	2:1	0	0

TABLE 5

Effect of cysteine on the antibacterial action of mercuric chloride (Oxford cup method)

MOLECULAR RATIO CYSTEINE: $HgCl_2$	MM INHIBITION
No cysteine	16 complete
1:1	21 complete
2:1	25 complete
4:1	25 partial
6:1	25 very weak
8:1	0
10:1	0

zone of bacterial inhibition than did mercuric chloride alone. As the cysteine to mercury ratio increased, the size of the growth inhibition zone remained nearly constant but inhibition became less complete; at sufficiently high ratios normal bacterial growth occurred. The data obtained are tabulated as averages (table 5). It must be remembered that readings in mm of diameter of zones of inhibition are not a linear function of the inhibition and that a difference of several mm represents considerable differences in potency. The effect of cysteine on the antibacterial action of a 1:10,000 aqueous mercuric chloride solution is shown in table 5.

The same results were obtained when the same molecular ratios were tested at 1:1,000 or 1:100 initial mercuric chloride concentration in the reaction with

cysteine, followed either immediately or after 3 hours by dilution so as to have 1:10,000 mercuric chloride in the test. Thus in the cup tests the reaction time or reaction concentration of mercuric chloride with cysteine does not affect the results.

N-acetylcysteine and N-(*p*-toluenesulfonyl)-cysteine behaved essentially as did cysteine. Thioglycollate, homocysteine, β-aminoethanethiol, β-thiolethanol, and glutathione all increased the activity of mercury but were much less effective in inhibiting the mercury antibacterial action at higher molecular ratios. The average inhibition produced by these substances is illustrated in table 6. The values are for 1:10,000 mercuric chloride solution, which, untreated, gave a 16- to 17-mm zone of inhibition.

Cystine, S-methyl cysteine, serine, alanine, di-*n*-propyl sulfone, di-*n*-propyl sulfite, ethyl sulfide, thiourea, and aminoethanol had no effect.

The increase in antibacterial action of mercuric chloride produced by addition of two molar equivalents of cysteine could not be observed with titration tests.

TABLE 6

Effect of thiols on the antibacterial action of mercuric chloride (Oxford cup method)

COMPOUND	MM INHIBITION AT MOLECULAR RATIO COMPOUND: $HgCl_2$			
	1:1	2:1	4:1	20:1
Thioglycollic acid	19	22	26	26
Glutathione	17	23	24	15
Homocysteine	25	27	25	22
β-Aminoethanethiol	24	26	26	21
β-Thiolethanol	31	32	31	20
n-Propyl mercaptan	23	28	30	30
β-(Dimethylamino)-ethanethiol	26	26	26	26

The increased activity observed by the cup test appears to be the result of a greater diffusibility in agar of the mercurial complex formed in the reaction of mercury salts with the sulfhydryl compounds. Agar, casein, gelatin, and starch, when added to mercuric chloride solution alone, decreased the antibacterial effect on the agar plate. If these substances were added to the mercuric chloride cysteine solution, no significant change was produced in the size of the inhibition zone.

Titration tests. The lowest concentration of mercuric chloride in broth which completely inhibited growth of *S. aureus* for 18 hours was approximately 0.001 mg per ml. When cysteine was added in molecular ratios ranging from 5:1 to 50:1, the minimum growth-inhibiting concentration varied from 0.2 to 0.7 mg of mercuric chloride per ml. A 2:1 or 1:1 cysteine to mercuric chloride ratio produced no appreciable change in end point from that of the mercuric chloride control.

Source of Chemicals

Cysteine hydrochloride, glutathione, methionine, β-thiolethanol, thioglycollic acid, *n*-propyl mercaptan, serine, alanine, di-*n*-propyl sulfone, di-*n*-propyl sul-

fite, and ethyl sulfide were obtained from the Eastman Kodak Company; allyl mercaptan was obtained from the Edwal Laboratories.

Methyl and ethyl esters of cysteine were prepared by standard procedures. N-(*p*-toluenesulfonyl)-cysteine (m.p. 163 to 165 C) was prepared by reduction of N,N^1-di-(*p*-toluenesulfonyl)-cysteine (McChesney and Swann, 1937) with sodium in liquid ammonia. N-acetylcysteine (Pirie and Hele, 1933), S-methyl cysteine (du Vigneaud, Loring, and Craft, 1934), β-aminoethanethiol hydrochloride (Gabriel and Leupold, 1898), homocysteine (Riegel and du Vigneaud, 1936), and β-(dimethylamino)-ethanethiol (Williams, 1933; Renshaw *et al.*, 1938) were prepared by procedures taken from the literature.

DISCUSSION

Sulfhydryl-containing compounds inactivate a certain class of antibacterial agents; some agents, such as penicillin, are highly specific in being inactivated by only certain types of sulfhydryl compounds, whereas C_3H_5—SO—S—C_3H_5 is inactivated by R—SH compounds in general. The penicillin and pyocyanine types of antibiotics react with sulfhydryl compounds at significant rates only in fairly concentrated solutions or in the presence of a large excess of sulfhydryl reagent. If the sulfhydryl antibacterial agent reaction is an indication that the mechanism of action of these agents involves their reaction with —SH groups in the organism, it would appear that in order for bacteriostasis to take place it is necessary for the antibacterial substance to be concentrated in the vicinity of the sulfhydryl groups essential for cell functioning so that reaction with these groups can occur. This concentration could result from an adsorption of the antibiotic at the surface of an —SH active enzyme where reaction could occur with the protein —SH groups or with —SH groups of simple molecules adsorbed by the protein.

The degree of activity and specificity of an antibiotic could be the function of a number of factors, such as (1) diffusibility of the antibiotic into the microbial cell structure; (2) degree of adsorption of the antibiotic by various enzyme systems; (3) ability of the antibiotic to react with sulfhydryl groups of the enzymes or with other sulfhydryl-containing substances adsorbed by the enzyme.

If specificity of action of an antibiotic is a function of the types of R—SH systems with which it can react, a nonspecific agent should react with most types of —SH active enzymes. The substances reacting less specifically with —SH active enzymes might also be expected to show higher toxicity to animals, as is the case with heavy metals. The highly specific penicillin, which shows very low animal toxicity, is probably not capable of being adsorbed or of reacting with animal enzymes but only with certain sensitive or highly adsorptive microbial enzymes. Most antibiotics would fall between these two extremes.

In many respects mercury salts resemble the naturally occurring antibiotics. Mercuric chloride is an active bacteriostatic agent, showing an effect up to a dilution of approximately 1:1,000,000; but it manifests relatively weak bactericidal action (effective in a dilution of approximately 1:1,000). Mercury is effective against both gram-positive and gram-negative bacteria and is also toxic

to animals. In this respect it resembles those antibiotics active against both groups of microorganisms, which usually are more toxic to animals than are the antibiotics effective against gram-positive bacteria only. In general, antibacterial agents active against gram-negative bacteria demonstrate much the same order of activity against gram-positive organisms, whereas many which are effective against the latter show slight or almost no activity against the gram-negative group.

The reaction with sulfhydryl reagents might serve as a criterion for classification of a group of antibacterial agents. It is possible for these agents to react with R—SH in a number of ways:

(1) Oxidation. The action of inorganic oxidizing agents, including the positive-halogen derivatives, C_3H_5—SO—S—C_3H_5, quinones, and certain dyes, might serve as examples of this type.

(2) Formation of heavy metal complexes. The action of mercury, silver, and other metals in forming mercaptides and complex addition products are examples.

(3) Metathesis. In this class we have alkylating agents such as iodoacetate.

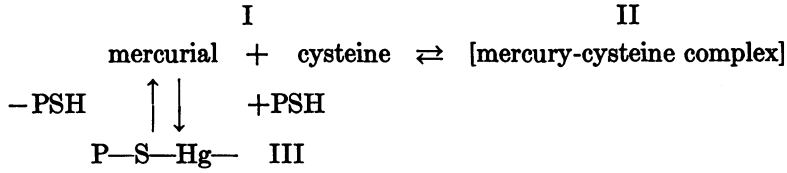
(4) Condensation. Aldehydes and ketones can react to form thioacetals and thioketals.

(5) Addition to unsaturated structures. A large number of the antibiotics may act by this method, including clavacin, penicillic acid, and anemonin.

The antibiotics reacting with R—SH may be a class exclusive of the surface-active or detergent antibacterial agents, or of those acting by exclusion of simple essential metabolites.

A possible explanation of the fact that growth is resumed upon dilution of a culture inhibited by a bacteriostatic agent is that the enzyme-antibiotic reaction product breaks down by dissociation, hydrolysis, or reduction, with regeneration of the enzyme-SH groups.

The behavior of mercury might be explained by the following scheme, in which P—SH represents an —SH active enzyme (P is a protein residue), and P—S—Hg— represents the mercaptide derivative of the enzyme:



When a mercurial is brought into the presence of an —SH active enzyme, an equilibrium is probably approached between the P—S—Hg— and free mercurial concentrations. If cysteine is added to such a system, the tendency is for the free mercurial to react to form a mercury-cysteine complex II. As the concentration of cysteine is increased, the equilibrium is shifted from III to I to II, resulting in the liberation of free P—SH from III. The liberation of the active enzyme P—SH allows the organism to resume normal growth. In this way it is possible to explain the ability of cysteine to reduce the antibacterial action of mercury. In the cup tests, complex II may be capable of diffusing farther than

can the mercuric chloride and demonstrate antibacterial activity by dissociation to state I. This would explain the increased zones of inhibition obtained with II. As the cysteine concentration is increased, the shift in favor of II increases and less antibacterial action is demonstrated. The relative stability of the mercury-cysteine and analogous mercury complexes with other sulfhydryl reagents might account for the relative ability of these sulfhydryl compounds to inhibit the mercury antibacterial action.

SUMMARY

The effects of time of reaction, relative concentrations of reactants, and type of sulfhydryl compound on the rate and degree of inactivation of mercuric chloride, penicillin, pyocyanine, and the antibacterial principles of *Allium sativum* and *Arctium minus* have been presented. It is postulated that a large class of antibacterial agents acts by reacting with the sulfhydryl groups of enzymes and that the differences in antibacterial action of various agents are dependent, among other factors, upon the ability of these agents to come into contact with the essential sulfhydryl groups.

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