THE REVERSAL OF ANTIBIOTIC ACTION

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Previous reports from this laboratory (Cavallito and Bailey, 1944; Cavallito *et al.*, 1945) have shown that many antibiotics may be inactivated with various thiol compounds, particularly cysteine. We have interpreted this as indicating that a large group of antibacterial agents acts by combining with protein sulfhydryl groups essential to growth. Barron and Singer (1945) and Singer and Barron (1945) have shown that many of the enzymes involved in growth and respiration contain this group or require it for their activation. Hellerman *et al.* (1943) have shown the importance of —SH in the activity of urease. These workers have shown that crystalline urease may be inactivated by mercuric chloride and the activity restored by treatment of the inactivated enzyme with cysteine.

Data are here presented that show that treatment of certain bacteriostatic systems with thiol compounds, particularly cysteine, will reverse bacteriostasis; i.e., will allow the inhibited bacteria to take up oxygen again and multiply in the presence of a bacteriostatic concentration of the antibacterial agent.

Eight antibiotics have been studied for the property of reversibility: penicillin, streptomycin, pyocyanin, the active principle of *Asarum canadense* (Cavallito and Bailey, 1946), phenyl mercuric acetate, mercuric chloride, gliotoxin, and the active principle of *Allium sativum* (allyl 2-propene-1-thiolsulfinate, referred to hereafter as the thiolsulfinate).

The technique generally employed to show reversal of bacteriostasis was as follows: the culture was prepared by adding 0.1 ml of an 18- to 20-hour culture in beef extract broth to 50 ml of sterile beef extract broth and incubating, depending on the organism employed, for 2 or 3 hours at 37 C. Two-ml portions of the 2-hour culture were placed in the reaction chamber of sterile Warburg flasks having 40 per cent KOH in the center well. The antibacterial agent was added in 0.5-ml portions to the reaction chamber of all the vessels excepting those to be used for culture control, which received a similar volume of phosphate buffer of pH 6.5. The thiol compounds were placed in the side arms of the Warburg flasks in the experimental vessels, whereas the control vessels contained phosphate buffer of pH 6.5 or sterile distilled water. Oxygen uptake in air by the bacteria was determined by the usual Warburg technique, observations being made every 10 minutes.

The antibacterial agents were prepared in the following concentrations: thiolsulfinate, 0.06 mg per ml; mercuric chloride, 0.01 mg per ml; active principle of *Asarum canadense*, 0.06 mg per ml; penicillin, 0.036 μg per ml; pyocyanin, 0.12 mg per ml; gliotoxin, 0.01 mg per ml; streptomycin, 24 μg per ml; and phenyl mercuric acetate, 0.0012 mg per ml. These concentrations are below the bactericidal levels of the agents for the organisms used.

Of the antibiotics tested, the bacteriostatic action of mercuric chloride, gliotoxin, and the thiolsulfinate could be reversed. The results of an experiment using the thiolsulfinate and *Salmonella paratyphi* that demonstrates this characteristically for the group may be described in greater detail. The results are summarized in table 1. In this experiment the vessels of the Warburg were divided into 5 groups (A, B, C, D, and E) of 3 vessels each. The reaction chambers contained the culture and antibacterial agent as described above, the vessels

TABLE 1

Reversal by cysteine of the bacteriostatic action of allyl 2-propene-1-thiolsulfinate on Salmonella paratyphi A

	CONTENTS OF VESSELS	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20-MINUTE INTERVAL				
		VESSEL GROUP				
		A	B	C	D	E
	ml					
Interval	Culture	2.0	2.0	2.0	2.0	2.0
	Antibiotic	0	0.5	0.5	0.5	0.5
	Water	0.5	0.5	0	0	0
	Side arm: Cysteine	0.5	0	0.5	0.5	0.5
Control vessels tipped		21.9	0	7.1	0.4	3.3
1st Per.		70.0	8.0	28.1	10.7	21.3
2nd Per.		88.7	1.2	21.4	11.9	24.3
3rd Per.		97.7	.6	18.8	17.1	31.3
4th Per.		135.4	0	23.8	31.6	49.4
5th Per.		89.9	0	36.0	56.9	43.4
6th Per.		83.2	.6	41.0	64.4	52.4
7th Per.		91.4	1.8	54.8	92.6	68.9
8th Per.		99.1	3.1	84.1	95.9	88.1

Values are averages of 3 vessels.

Culture: a 2-hr culture of *Salmonella paratyphi* A in beef extract broth.

Antibiotic: a 0.06 mg per ml solution of the thiolsulfinate in distilled water.

Cysteine solution in groups A and C, 5.6 mg per ml; in group D, 0.56 mg per ml; and group E, 0.28 mg per ml.

of group A (culture control) having sterile distilled water in place of the antibacterial agent. The side arms of groups A, C, D, and E contained 0.5 ml of cysteine solutions of the following concentrations: A and C, 5.6 mg per ml; D, 0.56 mg per ml; E, 0.26 mg per ml. The side arms of group B (bacteriostasis control) contained a similar volume of sterile distilled water. The cysteine hydrochloride solutions were prepared and sterilized by filtration through sintered glass filters just prior to use, and were then neutralized with solid sodium bicarbonate. After observations had been made to show oxygen uptake in the culture controls and lack of consumption in the bacteriostasis control and the experimental groups, the cysteine was tipped into the vessels and oxygen uptake again measured. To conserve space the results are expressed for 20-minute ob-

servation periods, although the determinations were made every 10 minutes. It is at once obvious that the control culture was respiring and that the concentration of the thiolsulfinate present in the test vessels had inhibited oxygen uptake. Upon the addition of the cysteine there was prompt oxygen uptake by the cultures to which it had been added. That this is not due to mere dilution is shown by the fact that the bacteriostasis control was diluted to the same extent, with no resumption of respiration. At the end of the experiment all vessels except group B (bacteriostasis control) were quite turbid. The speed with which oxygen uptake occurred shows that the resumption of respiration is

TABLE 2

Lack of reversal of the bacteriostatic action of the active principle of Asarum canadense on Staphylococcus aureus

	CONTENTS OF VESSELS	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20-MINUTE INTERVAL			
		VESSEL GROUP			
		A	B	C	D
	ml				
Interval	Culture	2.0	2.0	2.0	2.0
	Antibiotic	0	0.5	0.5	0.5
	Buffer	1.0	0.5	0	0
	Side arm: Cysteine	0	0.5	0.5	0.5
Control vessels tipped		14.0	4.9	7.0	8.3
1st Per.		50.0	8.7	9.1	8.6
2nd Per.		57.8	7.4	7.0	6.3
3rd Per.		81.9	5.7	5.7	5.4
4th Per.		83.7	5.2	5.3	3.8
5th Per.		85.8	5.2	5.3	3.3

Values are averages of 4 vessels in groups A and B and 3 vessels in C and D.
Culture: 3½-hour culture *Staphylococcus aureus* 209.
Antibiotic: a 0.06 mg per ml solution of *Asarum canadense*.
Buffer: a M/2 phosphate buffer pH 6.6.
Cysteine solution in group C vessels, 0.94 mg per ml in buffer similar to the above; that in group D, 0.094 mg per ml in similar buffer.

due to the reversal of bacteriostasis and not to the respiration of a few resistant organisms.

Data typical of those antibiotics the bacteriostatic action of which could not be reversed are presented in table 2, in which results using the active principle of *Asarum canadense* and *Staphylococcus aureus* are recorded. (Similar data were obtained with penicillin, streptomycin, and pyocyanin with *S. aureus*, and with phenyl mercuric acetate and *S. paratyphi*.) With the *A. canadense* antibiotic, no resumption of oxygen uptake was observed. The culture medium in the bacteriostasis control group vessels and that in the experimental groups showed no difference in turbidity at the end of the experiment, whereas the medium of the culture control was very turbid. This antibiotic resembles penicillin in

several respects. The *A. canadense* antibiotic is active in low concentrations against gram-positive organisms, contains sulfur, and is inactivated by alkali and by cysteine (Cavallito and Bailey, 1944). The selective action shown by penicillin on "young" cells, however, is not exhibited by this antibiotic. This is well illustrated in table 3. The test organism in this experiment was *Bacillus subtilis*. The "young" cells were a 4-hour culture prepared by inoculating 50 ml of sterile beef extract broth with 0.1 ml of a 20-hour broth culture of the test organism, whereas the "old" cells were a similar culture incubated 24 hours. The antibiotic, dissolved in 10 per cent ethanol, was placed in the side arms of the experimental vessels; in the controls an equal volume (0.5 ml) of 10 per cent ethanol

TABLE 3

Effect of age of culture on bacteriostasis of Bacillus subtilis by the active principle of Asarum canadense

	CONTENTS OF VESSELS	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20-MINUTE INTERVAL			
		VESSEL GROUP			
		A	B	C	D
	ml				
Interval	Culture, 24 hr	2.5	0	2.5	0
	Culture, 4 hr	0	2.5	0	2.5
	Ethanol	0.5	0.5	0	0
	Side arm: Antibiotic	0	0	0.5	0.5
Control vessels tipped		28.7	18.2	22.2	19.2
1st Per.		50.3	59.5	64.2	37.4
2nd Per.		54.4	105.9	91.9	52.4
3rd Per.		71.6	126.4	64.5	31.6
4th Per.		60.9	75.7	41.2	23.6
5th Per.		94.9	134.9	19.4	11.5

Values are averages; groups A and B, 3 vessels each, groups C and D, 4 vessels.

Ethanol: 10% solution. Concentration of active principle of *Asarum canadense*, 0.15 mg per ml in 10% ethanol.

was used. When oxygen uptake was shown in all vessels, the contents of the side arms were tipped into the reaction chambers and oxygen uptake was again determined. The active principle of *Asarum canadense* is equally effective in stopping the oxygen uptake of "old" and "young" actively growing cells. At the end of the experiment the control vessels were very turbid, whereas the experimental vessels were no more turbid than at the beginning of the experiment.

The antibiotics that produced a bacteriostasis that could be reversed with cysteine (thiolsulfinate, mercuric chloride, and gliotoxin) were tested for reversal with the following thiol compounds: glycylcysteine, N-acetylcysteine, and sodium thioglycolate. Bacteriostasis produced by mercuric chloride was reversed by all of these compounds; bacteriostasis by the thiolsulfinate by all but sodium thioglycolate. Unexpectedly, only N-acetylcysteine was effective with gliotoxin. Presentation of all the data on these compounds with the three anti-

biotics would be repetitious; as examples of the effectiveness and lack of it, the data obtained with the thiolsulfinate are presented in table 4. The rate of reversal is more rapid with cysteine than with the other thiols tested. S-methylcysteine, propanethiol, and β(-dimethylamino)-ethanethiol were without effect in reversing the bacteriostasis of *S. paratyphi* by mercuric chloride.

TABLE 4

Action of glycylcysteine, sodium thioglycolate, and N-acetylcysteine on the bacteriostatic action of thiolsulfinate on Salmonella paratyphi A

	CONTENTS OF VESSELS	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20-MINUTE INTERVAL				
		VESSEL GROUP				
		A	B	C	D	E
	ml					
Interval	Culture	2.0	2.0	2.0	2.0	2.0
	Buffer	0.5	0.5	0.5	0.5	0.5
	Side arm: Buffer	0.5	0.5	0	0	0
	Glycylcysteine	0	0	0.5	0	0
	Na-thioglycolate	0	0	0	0.5	0
	N-acetylcysteine	0	0	0	0	0.5
Control vessels tipped		3.1	1.2	1.7	2.9	1.9
1st Per.		12.1	1.5	4.5	1.0	2.0
2nd Per.		18.8	1.8	1.7	2.9	6.0
3rd Per.		35.5	2.2	5.4	0.5	6.6
4th Per.		58.0	2.6	5.4	4.5	5.4
5th Per.		74.2	0.7	9.0	0.6	3.5
6th Per.		93.2	3.6	11.1	2.4	14.0
7th Per.		131.8	4.0	19.2	4.9	19.7
8th Per.		116.2	7.3	26.8	4.8	34.1
9th Per.		115.2	5.4	42.5	8.8	46.4
10th Per.		115.9	7.1	53.3	6.4	71.6

Three flasks in all groups except B, which had 2; values are averages. Culture: 2-hr culture *S. paratyphi* A in nutrient broth. Buffer: M/2 phosphate pH 6.6. Antibiotic: thiolsulfinate, 0.1 mg per ml; glycylcysteine, 0.43 mg per ml; sodium thioglycolate, 0.46 mg per ml; N-acetylcysteine, 0.65 mg per ml.

DISCUSSION

On the basis of previously reported work from these laboratories and the data here presented, it is possible to classify antibiotics (using the term in its broadest sense) on the basis of their reactions with thiol compounds. A large number of the antibiotics and the heavy metals are inactivated by thiol, but gramicidin, tyrocidin, streptothricin, and aspergillic acids are not. The antibiotics susceptible to thiol inactivation can further be divided into those causing a bacteriostasis that can be reversed by cysteine and those with which cysteine is without effect on the bacteriostasis. The antibiotics characterized by a thiol-reversible bacteriostasis apparently show little specificity, reacting with most

types of —SH groups. Those with which reversal does not occur are specific, capable of reacting with a specific combination of groups (—SH and adjacent —NH_2) in proteins. As an example of specificity, it has been observed in our laboratory that whereas urease is not inhibited by penicillin, the enzyme is readily inactivated by the thiolsulfinate, a nonspecific thiol reagent.

An adequate explanation for the lack of reversibility of bacteriostasis caused by penicillin, streptomycin, and the active principle of *Asarum canadense* is difficult to give. This is largely the result of our lack of knowledge of the chemistry of these antibiotics.

We believe that when a sulfhydryl-reactive antibiotic acts upon an organism to bring about at least bacteriostasis, this effect may be accomplished by the reaction of the antibiotic with biologically essential —SH groups. It is thought that in reversible bacteriostasis the antibiotic reacts only with —SH groups, whereas in irreversible bacteriostasis both —SH and neighboring —NH_2 groups of the protein may be involved. The indirect evidence for this view is to be found in the work of Cavallito and Haskell (1945), who showed that unsaturated lactones reacted in such a manner with aminothiols. Reaction with amino groups probably does not occur with gliotoxin, mercuric chloride, and the thiolsulfinate.

That spacial relations are important in the various reactions of antibiotics is indicated by the fact that whereas β(-dimethylamino)-ethanethiol is very effective in destroying the antibacterial action of penicillin, separation of the thiol and amino groups by one more C atom eliminates the inactivating action. It is entirely possible that within complex proteins, the "geographical position" of the sulfhydryl and amino groups is such that the full reaction, —SH reaction and binding of the protein NH_2, does not occur. The inability of cysteine to reverse the bacteriostasis of some antibiotics may be due to the combination of antibiotic and protein being so arranged that the cysteine —SH is blocked away from the inactivated enzyme —SH and is prevented thereby from regenerating the latter.

If the action of penicillin is truly bactericidal, as claimed by Chain and Duthie (1945), Bigger (1944), Eriksen (1946), Garrod (1945), and Hobby and Dawson (1944), the inability to reverse the bacteriostasis of penicillin and similar antibiotics is readily explained. Against this explanation is the fact that the concentrations of the antibiotics used in these experiments were bacteriostatic.

The observation that mercuric chloride bacteriostasis can be overcome by thiol compounds is not new. It is of fundamental importance, however, that two antibiotics react in the same manner as does this inorganic antibacterial agent. This points to a similar and basic mode of action of the three antibacterial agents. The concept of antibacterial action may be expressed graphically by the following scheme:

$$\begin{array}{ccccc} PSH + An & \overset{(1)}{\rightleftharpoons} & PSH \cdot An & \xrightarrow{(2)} & PSH + An' \\ (4)\downarrow \text{cysteine} & & (3)\downarrow & & \\ \text{cysteine} \cdot An & & P'SH + An' & & \end{array}$$

where PSH is the —SH active enzyme, An is the active antibacterial agent, PSH · An is the enzyme-antibiotic reaction product, P'SH is the enzyme that has been dissociated from the antibiotic but altered by the reaction so as to be inert, and An' is the altered antibacterial agent. A bactericidal system could be one in which the reaction between enzyme and antibacterial agent is irreversible (reaction 1) or in which the antibiotic has altered the enzyme so it is inert and is itself altered (reaction 3). Bacteriostasis may be explained by assuming that reaction (1) is reversible, the concentration of antibiotic forcing the reaction to the right, thus leading to suspension of growth; or that reaction (2) occurs.

The resumption of respiration and growth resulting from the treatment of a bacteriostatic system with thiol compounds may be explained in at least two ways. The cysteine may remove the antibacterial agent from the equilibrium reaction (reaction 4). The reversal of mercuric chloride bacteriostasis may be an example of this type of reaction. The thiol compound may displace the antibiotic fragment from the enzyme-antibiotic reaction product with the resulting formation of an inactive, altered antibiotic fragment and the native enzyme (reaction 2). The reaction of the thiolsulfinate and cysteine is believed to be an example of this type.

SUMMARY

Bacteriostasis by mercuric chloride, allyl 2-propene-1-thiolsulfinate, and gliotoxin can be reversed by the addition of cysteine to the bacteriostatic system. Bacteriostasis caused by penicillin, streptomycin, the active principle of *Asarum canadense*, or pyocyanin was not reversed by cysteine. In addition to cysteine, glycylcysteine, N-acetylcysteine, and sodium thioglycolate were effective reversing compounds for mercuric chloride; the thiolsulfinate was reversed by all except sodium thioglycolate, whereas only N-acetylcysteine had any effect on the gliotoxin bacteriostasis. Thiol-reactive antibiotics may be classified on the basis of thiol reversibility of the bacteriostasis produced.

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