RELATIONSHIP OF THIOL STRUCTURES TO REACTION WITH ANTIBIOTICS

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(Received for publication, March 12, 1946)

A large number of the antibiotics have demonstrated chemical reactivity toward compounds containing sulfhydryl groups (1, 2). There have also been observed marked differences in reactivity of individual antibiotics toward various types of sulfhydryl-containing compounds. In the present investigation, the effect of structure of thiol compounds on their ability to react with certain antibacterial agents is interpreted in terms of the possible relationship of structure of thiol groups in proteins with factors of specificity and degree of activity of antibiotics. The importance of sulfhydryl groups to enzyme activity has been demonstrated frequently (3–9), although the exact functions of the group are still in doubt.

The antibiotics studied were penicillin G, streptomycin, gliotoxin, pyocyanine, and the active principles of Allium sativum (C_{6}H_{13}S_{2}O), Arctium minus (C_{15}H_{26}O_{5}), and Asarum canadense (principle A) (10). This is a chemically heterogeneous group with examples of antibiotics derived from bacteria, molds, Actinomyces, and higher plants, but all react with certain sulfhydryl compounds. Some of these antibiotics previously (2) were discussed briefly with respect to inactivation by a number of thiols and it was postulated that this class of antibacterial agents acted by combining with essential sulfhydryl groups of enzymes and that probable factors involved were diffusibility of the antibiotic throughout the microbial cell structure, degree of adsorption of the antibiotic by enzymes, and ability of the antibiotic to react with sulfhydryl groups of the enzymes.

Penicillin has shown a high degree of specificity in its reaction with thiols. Cysteine, its esters, and β-amino- and β-dimethylaminomethanethiols are good penicillin inactivators; homocysteine, N-acetylcysteine, and thioglycolate are poor (2). Cysteine and aminoethanethiol are equally effective; however, dl-β,β-dimethylcysteine (dl-penicillamine) (11) is not effective, showing interference from β-carbon substituents other than hydrogen. On the basis of present information the ideal thiol type of inactivator for penicillin should possess a basic amino group (primary through tertiary) on the carbon adjacent to the carbon carrying the sulfhydryl group, with substituents on the nitrogen smaller than ethyl (methyl or hydrogen). When using this information in predicting the possible
reactivity of penicillin toward enzyme —SH groups, one is limited to the
consideration of only such thiol structures derived from known amino acids
which might exist in protein molecules. The low reactivity of N-acetylcysteine as compared with that of cysteine and its esters may indicate that
the cysteine unit in proteins would show greatest reactivity toward penicillin when its amino group is not acylated.

It was felt desirable to prepare sulfhydryl-containing peptides for com-
parison of reactivity toward the antibiotics, since cysteine esters and
acetylcysteine are not true models of structures present in proteins. The
two most simple dipeptides of cysteine, glycyl-l-cysteine and l-cysteinyl-
glycine, were prepared. Cysteine, N-acetylcysteine, glutathione, thio-
glycolate, and S-methylcysteine were included for comparison of reactivity.

EXPERIMENTAL

Glycyl-l-cysteine Hydrochloride—The dipeptide salt was prepared by
applying the procedure described by Greenstein (12) for the preparation
of diglycyl-l-cystine. The residue from the sodium-liquid ammonia
reduction was dissolved in water and acidified with hydrochloric acid,
and a solution of mercuric chloride and sodium acetate was added to
precipitate the mercaptide. The precipitate was washed thoroughly
with water, suspended in water, and treated with hydrogen sulfide to
liberate the dipeptide. After removal of excess hydrogen sulfide by
distillation under reduced pressure, the solution was treated with a dilute
solution of mercuric chloride and the precipitate again thoroughly washed
with water. The dipeptide was again liberated by treatment of the
mercaptide with hydrogen sulfide, the precipitate filtered off, and the
solution concentrated under reduced pressure. The concentrate was made
strongly acid with hydrochloric acid and was allowed to evaporate to
dryness over solid sodium hydroxide in a vacuum desiccator. The hygro-
scopic, amorphous glycyl-l-cysteine hydrochloride (m.p., decomposition,
>90°) was finally dried over phosphorus pentoxide. Grote's test for
sulphydryl groups (13) was positive. The rotation was $[\alpha]_D^{25} = +2.5^\circ$
(20 mg. per cc. of water); rotation of l-cysteine hydrochloride in the same
molar concentration (13.6 mg. per cc. of water) was $[\alpha]_D^{25} = +8.1^\circ$.

$C_9H_{11}O_2N_3SCl$. Calculated. N 13.05, ionic Cl 16.52
Found. " 12.85, " " 17.20

l-Cysteinylglycine Hydrochloride—This dipeptide salt was prepared by
the reduction procedure of Loring and du Vigneaud (14) for N,N'-dicarbo-
benzoxy-l-cystinylglycine. The hydrochloride was isolated by the
procedure described for the previous preparation. The amorphous
hygroscopic salt with a melting point, with decomposition, of >70° gave
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a positive Grote’s test for sulfhydryl groups. The rotation was \([\alpha]_D^{25} = +21.5^\circ\) (20 mg. per cc. of water).

Found. N 12.93, ionic Cl 17.15

**Reaction of Thiols with Antibiotics**—The reactions were carried out in potassium phosphate buffers at pH 6, 7, and 8 at 25°. The rate of reaction of the thiols with the antibiotics (except pyocyanine) was measured by observing loss of activity of the antibiotic when tested by the routine cylinder plate method (against *Staphylococcus aureus*). The pyocyanine reaction was observed visually by the change in color from blue to green to colorless produced by the thiols. The thiol and antibiotic solutions were mixed and samples were removed for testing at intervals up to 24 hours. The thiol concentration in the reaction mixture was 5 mg. per cc. for cysteine and a corresponding molar concentration of the other thiols.

**Table I**

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Approximate time required to inactivate</th>
<th>Penicillin G</th>
<th>Streptomycin</th>
<th>Asarum A</th>
<th>Pyocyanine*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs.</td>
<td>hrs.</td>
<td>hrs.</td>
<td>hrs.</td>
<td></td>
</tr>
<tr>
<td>l-Cysteine</td>
<td>&lt;1</td>
<td>&lt;0.25</td>
<td>4</td>
<td>0.25-0.5</td>
<td></td>
</tr>
<tr>
<td>l-Cysteinylglycine</td>
<td>&lt;1</td>
<td>&lt;0.25</td>
<td>4</td>
<td>0.25-0.5</td>
<td></td>
</tr>
<tr>
<td>Glycyl-l-cysteine</td>
<td>18</td>
<td>3</td>
<td>24</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>72</td>
<td>5</td>
<td>48</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>N-Acetylcysteine</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;48</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Thioglycolate</td>
<td>&gt;&gt;100</td>
<td>&gt;100</td>
<td>&gt;48</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* Reactivity measured by rate of color change.

was used. The concentrations of the antibiotics per cc. of reaction mixture were as follows: penicillin G and gliotoxin, 0.1 mg.; streptomycin, 200 γ; C_{18}H_{20}O_6 (*Arctium*), pyocyanine, and C_{6}H_{10}S_{2}O (*Allium*), 1.0 mg.; and Asarum A, 0.05 mg. The streptomycin hydrochloride assayed approximately 800 γ per mg.; the other antibiotics were crystalline preparations, except pure liquid C_{6}H_{10}S_{2}O (*Allium*).

In no instance did S-methylcysteine react in 24 hours with any of the antibiotics. Gliotoxin, C_{6}H_{10}S_{2}O (*Allium*), and C_{18}H_{20}O_6 (*Arctium*) reacted with all six of the sulfhydryl compounds within a few minutes reaction time. The approximate reaction time for complete inactivation of the other antibiotics is given in Table I. Penicillin and Asarum A showed an increase in rate of reaction with thiols with increase in pH from 6 to 7 to 8; the other antibiotics showed no appreciable differences with change of pH.
It is evident that in those instances in which an antibiotic shows specificity in its reaction with sulphydryl groups the sulphydryl on a non-acylated cysteinyl residue is the most reactive. The generally greater reactivity of glycyleysteine than acetylcysteine indicates that the free amino group in the former has some activating action but not nearly so great as that of a free amino group in the same cysteine residue.

On the basis of specificity and speed of reaction with thiols, the antibiotics may be divided as follows: Group I, those such as gliotoxin, C$_6$H$_{10}$S$_3$O (*Allium*), and C$_{18}$H$_{36}$O$_6$ (*Arctium*), which are rapidly reactive toward most sulphydryl compounds; Group II, intermediate ones such as pyocyanine, which react with most sulphydryl compounds, but with some differences in rate; and Group III, antibiotics such as penicillin, streptomycin, and *Asarum A*, which react slowly, with more specific sulphydryl types and which show, with increase in concentration of the antibiotic, a marked increase in rate of reaction and lower molar ratios of thiol to antibiotic required for inactivation.

**DISCUSSION**

If we accept the proposal that this group of antibiotics inhibits growth by reacting with essential —SH groups in bacterial cells, two explanations for antibiotic action which are possible on the basis of the data are (a) the compounds may react with essential —SH groups of bacterial enzymes; (b) the compounds may react with the —SH groups in cysteinyl residues, as these are joined at the end of a growing polypeptide chain during protein anabolism. In this manner, antibiotics might block further growth of the protein along that chain by producing cysteine "dead ends."

Either or both of the mechanisms may be involved in inhibition of growth; however, with Group III antibiotics, reaction with enzyme — SH groups would be expected to take place readily only when the —SH is part of a cysteine residue attached at the end of a polypeptide chain, less readily with cysteine —SH in which free basic groups were in a neighboring amino acid of the chain, and least of all with a cysteine residue containing no neighboring basic amino groups. Group I antibiotics could react readily with almost any —SH group with which they can come in contact, the latter factor depending on diffusibility and adsorptive properties of the antibiotic. The higher antibacterial activity and specificity of a slowly —SH-reactive antibiotic such as penicillin as compared with rapidly reactive C$_6$H$_{10}$S$_2$O (*Allium*) might be explained partly on the basis of greater adsorbability of penicillin on enzymes and partly by "loss" of C$_6$H$_{10}$S$_2$O (*Allium*) by reaction with non-essential —SH groups of structural proteins, whereas penicillin would be "lost" primarily to essential —SH.
Penicillin, *Asarum* A, streptomycin, and pyocyanine react with cysteine at appreciable rates only in fairly high concentrations of reactants, whereas $C_6H_{13}S_2O$ (*Allium*), gliotoxin, and $C_{18}H_{20}O_6$ (*Arctium*) react readily even in low concentrations even though the former group includes some of the most active antibiotics. In order to account for this, it has been postulated that the more potent antibiotics are readily adsorbed in the vicinity of enzyme sulfhydryl groups. Differential adsorbability on bacterial enzymes of normal bacterial metabolites and certain bacteriostatic agents has been used by numerous investigators to explain the inhibiting action of such agents. In the inhibition of succinic dehydrogenase by malonate, Potter and DuBois (15) postulate specifically that the group blocked by the adsorbed interfering agent is the sulfhydryl and, in the case of quinonoidal compounds, a chemical reaction was proposed involving $\text{--SH}$ and the quinone. There may be at least three types of inhibitors which can block $\text{--SH}$ groups. (a) One type may be adsorbed by groups (such as $\text{--NH}_2$ or $\text{--COOH}$) in the vicinity of the $\text{--SH}$ in such a manner as to block the $\text{--SH}$ physically. (b) A second type may involve adsorption in the vicinity of the $\text{--SH}$ groups, followed by a chemical reaction with the group (as possibly the quinones, penicillin, streptomycin, pyocyanine, and *Asarum* A). With unsaturated lactones, the possibility of a secondary reaction with amino groups also exists (16). (c) The third type may react rapidly with $\text{--SH}$ with little or no selective adsorption prior to the reaction (possibly $C_6H_{13}S_2O$ (*Allium*), $C_{18}H_{20}O_6$ (*Arctium*), $\text{HgCl}_2$, and inorganic oxidizing agents).

The same forces in enzymes which attract the normally involved metabolite to the active $\text{--SH}$ groups may be the forces attracting the inhibitor or antibiotic.

**SUMMARY**

The reactivity of six sulfhydryl compounds including glycyl-$l$-cysteine and $l$-cysteinyglycine toward seven antibiotics has been investigated. Gliotoxin and the active principles of *Allium sativum* and of *Arctium minus* show little specificity in reactivity toward thiols, whereas penicillin, streptomycin, and the antibiotic from *Asarum canadense* react much more readily with sulfhydryl compounds containing basic amino groups in the vicinity of the $\text{--SH}$. Pyocyanine has intermediate properties. The possible significance of the observations to the mechanism of interference of antibiotics with biologically essential $\text{--SH}$ groups is discussed.

We are indebted to Miss Mary Bond and Mr. W. F. Warner for the routine cylinder plate tests, to Dr. A. R. Surrey for the synthetic pyocyanine, and to Dr. Sydney Archer for the synthetic $dl$-penicillamine. All are connected with these laboratories.
THIOL REACTION TO ANTIBIOTICS

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*J. Biol. Chem. 1946, 164:29-34.*

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