THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

II. The Relation between Constitution and Bactericidal Action in the Substituted Benzylhexamethylenetetraminium Salts.

By WALTER A. JACOBS, Ph.D., MICHAEL HEIDELBERGER, Ph.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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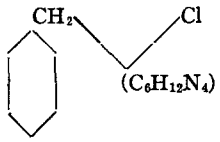
Hexamethylenetetramine, as a tertiary nitrogen compound, reacts readily with benzyl chloride, bromide, or iodide, and their numerous nucleus substituted derivatives to form quaternary salts. The results obtained in the study of the bactericidal properties of such substances are the subject of the present communication. In these compounds, the structure of which may be represented as follows,



it is seen that by means of a CH_2 side-chain the hexamethylenetetramine molecule is linked to a benzene nucleus. By the use of a great variety of substituted benzyl halides it was found possible to prepare for study a variety of hexamethylenetetraminium salts[1] in which the benzene nucleus was varied at will in the character, number, and position of the different atoms and groups introduced. By this procedure the opportunity was afforded of studying the effect of chemical constitution upon bactericidal action in a uniform series of substances.

Because the number of substances involved in the investigations

[1] For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 659; 1915, xxi, 465.

described in these papers was so large, it was found necessary for the practical execution of the bactericidal tests to adopt a scheme which would involve the least amount of work and still furnish a satisfactory measure of the activity of the substances. For this reason the drug dilutions, which as a rule started with 1: 200, were doubled in each successive dilution so that the series 1: 200, 1: 400, 1: 800, 1: 1,600, etc., were the concentrations with which the observations were made. With this scheme it is seen that as the dilutions increase the differences between them become greater, making it possible to regard the figures obtained only as rough approximations to the true values. In the strictest sense account should be taken of the molecular weights of the substances in a direct comparison of their bactericidal properties, but with the dilution scheme here employed this was deemed unnecessary.

In spite of the crudity of our figures it will be seen that certain relationships between the constitution and the bactericidal action are plainly in evidence. The results given below clearly demonstrate that by the addition of hexamethylenetetramine to benzyl chloride a bactericidal substance is obtained, and that by the substitution in the benzene nucleus of different atoms and groups this action may be altered at will, the resulting effect depending upon the number, character, and position of these substituents. In this class of compounds we possess a new group of bactericidal substances in which the hexamethylenetetramine nucleus is directly responsible for their bactericidal character.

EXPERIMENTAL PART.[2]

Technique.—A strain of *Bacillus typhosus* which had been growing on artificial media for several years and which is a good agglutinator was used in testing the germicidal effects of the compounds.

0.5 or 1 per cent solutions of the substance to be tested were made up in physiological salt solution and filtered immediately through a Berkefeld N filter. With sterile salt solution the dilutions of 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 were made and

[2] Some of the tests with *B. typhosus* were performed by Dr. Paul F. Clark, now of the University of Wisconsin, to whom we are greatly indebted for furnishing us with the results of his experiments.

all brought to a temperature of 37°C. To 4 cc. of each dilution, there was added 0.5 cc. of a 24 hour broth culture of *Bacillus typhosus*, and the tubes were put into an incubator or water bath at 37°C. for 4 hours. At the end of this time, one small loopful was taken from each tube and plated in plain agar. It was found necessary to incubate the plates for 48 hours before counting, because some of the colonies did not grow out in 24 hours. Control plates under the same conditions usually contained about 1,000 colonies. Duplicates were run in each case.

Table I presents the bactericidal results obtained with the different preparations tested upon *Bacillus typhosus* by the technique described above. As stated in the introduction, the number of preparations tested and the pressure of other work rendered impossible a detailed study of each substance employed in the tests, so that the figures here given can be regarded only as approximations to the true bactericidal powers of the substances in question. In most instances the figures as given are, if anything, too low. A consideration of the scheme of dilutions employed will show how great the underestimation of the true bactericidal power might be. For example, in the case of the *o*-nitrobenzylhexamethylenetetraminium chloride, given in Table I, the greatest dilution in which this substance killed all the bacteria in 4 hours was 1: 3,200. The next higher dilution tried was 1: 6,400 and this was found ineffective. But if this compound could really kill in a dilution of 1: 5,000 or even 1: 6,000, the value obtained as a result of the scheme of dilution used would be only 1: 3,200. For this reason we must regard the values given only as relative. In spite of this, the alteration in character or position of the substituents in the nucleus was accompanied by changes in the bactericidal action which were too marked to be masked by the dilution scheme employed.

The tests with hexamethylenetetramine itself and the simple aliphatic quaternary salt methylhexamethylenetetraminium iodide showed them to be devoid of action in a dilution of 1: 200. By the substitution in the latter compound of the methyl by the benzyl group the customary influence of the aromatic nucleus was observed. Although not a strong bactericide, the benzyl salt was found to kill all the bacteria present in a dilution of 1: 200. This bactericidal

TABLE I.

Substance.	Killed *B. typhosus* in 4 hrs. at 37°C. in a dilution of 1:
Hexamethylenetetramine	+*
Methylhexamethylenetetraminium iodide	+
Benzylhexamethylenetetraminium chloride	200
o-methylbenzylhexamethylenetetraminium chloride	3,200
m- " "	800
p- " "	800
3, 5-dimethylbenzylhexamethylenetetraminium chloride	400
o-chlorobenzylhexamethylenetetraminium "	1,600
p- " "	800
o-bromobenzylhexamethylenetetraminium "	1,600
p- " "	200
o-iodobenzylhexamethylenetetraminium bromide	1,600
p- " "	1,600
o-cyanobenzylhexamethylenetetraminium chloride	3,200
p- " "	400
o-nitrobenzylhexamethylenetetraminium "	3,200
m- " "	400
p- " "	1,600
2, 4-dinitrobenzylhexamethylenetetraminium "	3,200
o-methoxybenzylhexamethylenetetraminium "	+
p- " "	200
2, 3-dimethoxybenzylhexamethylenetetraminium chloride	+
3, 4- " "	200
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride	200
5-nitro-2-methoxybenzylhexamethylenetetraminium "	400
3-nitro-4- " "	800
2-nitro-3, 4-dimethoxybenzylhexamethylenetetraminium "	3,200
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide	1,600
4-acetoxy-3, 5- " "	1,600
2-acetoxy-3, 5-dimethyl-4, 6-dibromobenzylhexamethylenetetraminium bromide	1,600
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride	+
3-carboxy-4-oxybenzylhexamethylenetetraminium "	1,600
3-carbomethoxy-4-oxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carboxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carbomethoxybenzylhexamethylenetetraminium "	400
o-acetaminobenzylhexamethylenetetraminium chloride	800
p- " "	+
1, 2-xylylenedihexamethylenetetraminium dichloride	12,800
1, 3- " "	6,400
Mesitylylenedihexamethylenetetraminium "	12,800

* + indicates growth after exposure to a dilution of 1: 200.

power was further developed by the introduction into the nucleus of various atoms and groups, resulting in the series of substances given in Table I. A study of these brings out the following relationships.

The methyl, chlorine, bromine, iodine, cyano, and nitro groups were all found to increase the bactericidal power of the parent unsubstituted benzyl compound. This behavior of the alkyl, halogen, and nitro group has been frequently observed with other types of organic bactericides; for instance, in the case of the phenols. However, this effect may by no means be regarded as inevitable, as there are many bactericidal substances the power of which is in no way influenced by the introduction of these groups. Examples of this will be found among other types of hexamethylenetetraminium salts to be described in the following paper.

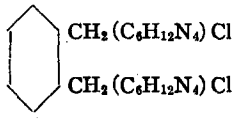
A perusal of the table will show the interesting influence of the position of the substituent upon the bactericidal action. In most cases the *ortho* substituents were found to be more active than either the *meta* or *para* compounds. The regularity of this rule, at least as far as the present series was extended, is striking. The *ortho* methyl, chloro, bromo, nitro, and cyanobenzyl salts were all more active than their other position-isomers.

With this type of hexamethylenetetraminium salt the methoxy group is, on the whole, without influence on the bactericidal effect, if indeed not detrimental. In those compounds which contain the nitro group besides the methoxy group the influence of the former appears to dominate. The 2-nitro-3,4-dimethoxybenzyl derivative was found to equal in effectiveness the 2-nitrobenzylhexamethylenetetraminium chloride. In the same way the 3-nitro-4-methoxy compound was bactericidally about as powerful as the 3-nitrobenzyl salt.

The optimum effect in varying substituents was apparently obtained with the mono- substituted benzyl compounds. In the few cases in which the dimethyl, dibromo, and dinitro derivatives were made, it was found that no advantage as regards the bactericidal value was to be gained, as a rule, by the multiplication of groups. At the same time the greater the number of substituents, particularly in the case of the nitro and halogen compounds, the less was the solubility in water.

In one direction, however, at least when *Bacillus typhosus* was used

as the test object, it was found that a distinct advantage was to be gained by the multiplication of groups; namely, in those compounds in which hexamethylenetetramine was introduced twice into the side-chains. Such salts were obtained by the addition of two molecules of hexamethylenetetramine to ω_1,ω_2-dichloro-*o*-xylene, ω_1,ω_3-dichloro-*m*-xylene, and ω_1,ω_3-dichloromesitylene. These substances possess the following structural formula, in which, of course, the relative positions of the side-chains are different in each case.



These compounds are the strongest bactericides of this group.

Here it should be emphasized that the bactericidal results obtained with this group of substances refer only to their behavior towards *Bacillus typhosus*. As the work developed, other species of bacteria were made the object of an occasional test, but owing to the incompleteness of the results obtained, and to the fact that the technique was varied, it has not been deemed advisable to enlarge on these results in the present paper. It may, however, be said that in general this class of substances was considerably less effective against the streptococcus and meningococcus, but that the results with the gonococcus approached those obtained with *Bacillus typhosus*. This particular group of hexamethylenetetraminium salts cannot, therefore, be regarded as general disinfectants. As a matter of fact, there are but few, if any, organic bactericides which act uniformly against all species or strains of bacteria. A few cases have been selected in Table II to afford a comparison of the effects of several of these salts upon different microorganisms. With the streptococcus, meningococcus, and gonococcus the technique was altered, the time of exposure of the bacteria to the drug being changed to 3 hours and the temperature to 20°. Such a change in technique should, of course, alter the results, but our experience has shown that this rarely exceeded the space of one whole dilution. It is seen from the table that the nature of the substance used determined the effect upon a particular microorganism. A constant relation between the resistances of the various types of organisms is out of the question. The far greater effectiveness

of the two dihexamethylenetetraminium salts against *Bacillus typhosus* is striking. These substances may be classed as "partially specific" for this species.

TABLE II.

Substance.	Killed *B. typhosus* at 37°C. in 4 hrs. in dilution of 1:	Killed streptococcus at 20°C. in 3 hrs. in dilution of 1:	Killed meningococcus at 20°C. in 3 hrs. in dilution of 1:	Killed gonococcus at 20°C. in 3 hrs. in dilution of 1:
Benzylhexamethylenetetraminium chloride	200	+	400	800
p-methylbenzylhexamethylenetetraminium chloride	800	+		
o-bromobenzylhexamethylenetetraminium chloride	1,600	+	+	1,600–3,200
o-cyanobenzylhexamethylenetetraminium chloride	3,200		400	1,600
p-cyanobenzylhexamethylenetetraminium chloride	400		400	800
o-methoxybenzylhexamethylenetetraminium chloride	+*	+	200	800
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride	200	+	1,600	800
o-nitrobenzylhexamethylenetetraminium chloride	3,200	3,200	800	800
m-nitrobenzylhexamethylenetetraminium chloride	400		1,600	1,600
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide	1,600	3,200	800	800
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride	+	1,600	800	800
m-xylylenedihexamethylenetetraminium dichloride	6,400	200	+	1,600
Mesitylylenedihexamethylenetetraminium dichloride	12,800	+	400	400

* + indicates growth after exposure to a dilution of 1:200.

From the consideration of the above observations we feel justified in attributing essentially to the hexamethylenetetraminium group the property of determining the bactericidal character of this class of compounds. For direct comparison with another basic side-chain *p*-nitrobenzylpyridinium chloride was prepared. This was found to be ineffective towards *Bacillus typhosus* even in a concentration of 1:200

after 4 hours' contact. The corresponding hexamethylenetetramine quaternary salt killed *Bacillus typhosus* in a dilution of 1: 1,600 in 4 hours.

The function, however, of determining the extent and character of this bactericidal property must be attributed to the substituting groups and to the positions occupied by them in the benzene nucleus to which the hexamethylenetetramine is linked. Our experience has shown that such groups likewise decide other biological properties of this class of substances. Without stopping here to deal at length with the toxicity experiments it may be said that in general the toxicity of these compounds is determined by such groups. For example, whereas the *o*-nitro benzyl derivative could be given to mice intravenously in amounts up to 500 mg. per kilo, the 2,3-dimethoxybenzyl derivative was found to be fatal in 0.1 of this dose.

SUMMARY.

By the addition of substituted benzyl halides to hexamethylenetetramine, a series of quaternary salts of this base was obtained. These salts represent a new group of organic bactericides. The results obtained in the tests with these substances upon *Bacillus typhosus* have demonstrated the existence of direct relationships between chemical constitution and bactericidal action within the series.

The bactericidal character is directly attributable to the presence of the hexamethylenetetramine nucleus. The degree of the bactericidal action, however, is determined by the position, character, and number of the groups substituted in the benzene nucleus.

By the introduction of the methyl, chlorine, bromine, iodine, cyano, and nitro groups into the benzene nucleus of the parent benzyl hexamethylenetetraminium salt, the bactericidal power of this compound was notably enhanced. The substitution of these groups in the *ortho* position almost invariably resulted in substances which were more active than their *meta* or *para* isomers. The introduction of the methoxy group was without marked effect.

Several substances in which two hexamethylenetetraminium side-chains occurred were found to be the most active of the substances of this series when tested against *Bacillus typhosus*. Comparative tests with other bacterial types demonstrated that these compounds possessed a marked degree of specificity for *Bacillus typhosus*.