# THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

## III. The Relation between Constitution and Bactericidal Action in the Quaternary Salts Obtained from Halogenacetyl Compounds.

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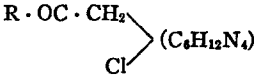
(Received for publication, March 1, 1916.)

In the course of the biological investigations of the substituted benzyl quaternary salts of hexamethylenetetramine discussed in the previous paper, it was found that the further extension of this chemical type was no longer advisable, in spite of the fact that within this group substances had been found which possessed considerable bactericidal power against *Bacillus typhosus*. This was due partly to the inaccessibility or insolubility of the further preparations of this type which seemed theoretically indicated. Our attention was in consequence turned to the problem of finding a new scheme of chemical procedure which would furnish the possibility of new leads and of broader development. This was found in the readiness with which hexamethylenetetramine as a tertiary base reacted with halogenacetyl derivatives to form soluble quaternary salts with the following general structure,




in which R might represent the radical of any aliphatic or aromatic primary or secondary amine R′NH, $R'_2N$, R′R″N, or of any alcohol or hydrocarbon. Because of the general nature of this reaction and the practically limitless number of chemical possibilities offered, it was found possible to furnish the most varied chemical groupings

with the hexamethylenetetramine molecule by the use of the halogenacetyl side-chain.[1]

Without anticipating the detailed discussion of the observations to be found in the experimental part, the following remarks may be taken to sum up the principal results of the work presented in this communication.

As a result of experiments on a large number of drugs of this type, the general statement may be made that hexamethylenetetramine, when combined with halogenacetyl compounds in the form of quaternary salts, gives rise to a new group of organic bactericides. Hexamethylenetetramine may, therefore, be described as a definite bactericidogenic group. The extent of the bactericidal power of these substances is, in a measure, controlled by the general character of the molecule or of the particular groups contained therein. In addition, the employment of several species of microorganisms has served the purpose of bringing out many examples of partial specificity, at least one substance with a high degree of specificity being found for each of the four species of bacteria used.

The fact that this specificity shifted from one group of bacteria to another with the change in the chemical composition of the radical added to the hexamethylenetetramine, demonstrated that, in contradistinction to the bactericidogenic character, the specificity relations were determined, not by the hexamethylenetetramine nucleus, but by the added radicals.

From the facts set forth above it will be seen that the program presented in the introductory paper has been partially fulfilled. Thus it was found possible, by starting with the molecular grouping furnished by hexamethylenetetramine, to add the most varied atomic groupings with the aid of the $-CH_2CO-$ group, which served merely as a connecting link. The chemical differences in these added groups caused the wide variations observed in the bacteriological results.

In these observations, which must be regarded only as a beginning, we thus see that once in the possession of a biologically active or potentially active molecular group, it is possible, without produc-

[1] For the chemistry of these substances and the reference to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

ing profound chemical changes in the molecule itself, to equip it with a reactive side-chain which in turn will react with other molecular groups and which will furnish the necessary biological properties.

In the present paper we wish to present the results of the bactericidal tests performed with these preparations upon *Bacillus typhosus*, streptococcus, meningococcus, and gonococcus. Here, as in the previous communication, the large number of tests which were made necessitated the early adoption of a rough scheme of drug dilutions. For this reason what was said in the former paper regarding the accuracy of the results must be reiterated here. At best the figures given may be considered as rough approximations. In spite of this, however, in many instances pronounced evidence of the influence of constitution on bactericidal action appeared.

## EXPERIMENTAL PART.[2]

*Technique.*—In testing the germicidal efficiency of the compounds to be described below, 0.5 or 1 per cent solutions of each substance, according to the solubility, were made in distilled water and filtered through a Berkefeld N filter. The other dilutions were prepared from this stock solution by the use of sterile distilled water as a diluent.

The series 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800 was employed for the tests. In some cases the sparing solubility of the substance necessitated the omission of the lower dilutions.

5 cc. of each dilution were placed in wide mouthed tubes and the temperature was brought to 20°C. To each of these tubes the bacterial suspensions were added.

In the case of the *Bacillus typhosus* 0.1 cc. of a 24 hour broth culture was added to each of the tubes containing the dilutions of the compound. After 3 hours a standard 4 mm. loop of the mixture was plated in order to determine the number of living organisms. The plates were incubated for 48 hours before counting. Another loopful from the same tube was taken at practically the same time and inoculated into tubes containing 10 cc. of plain broth and the tubes were

[2] We are greatly indebted to Dr. Martha Wollstein and to Dr. Louise Pearce who conducted the tests with the meningococcus and gonococcus, respectively. Their familiarity with the cultural conditions of the two microorganisms was of special value and service.

incubated for 48 hours. It was found that at a certain dilution, using the plating method, there was a very abrupt falling off in the number of colonies in the plates. This point was always marked in the broth tubes by clear-cut differences in appearance of the incubated tubes. The organisms in the lower dilution produced marked turbidity and in the next higher dilution remained absolutely clear. It was apparent that from 50 to 80 organisms were necessary to inoculate a 10 cc. broth tube, so that anything below this number would not show in this culture media. On the other hand, each organism in the plates produced a colony. Having found at the beginning of the work that this point of abrupt decrease in the number of colonies, using the plate method, came always at the same dilution indicated by no growth in the tubes, the plate method was no longer used on account of the large number of drugs tested.

In the streptococcus tests one or two drops of a 24 hour bouillon culture of an ordinary hemolytic strain of streptococcus were added to each dilution tube. After 3 hours at 20°C. a loopful was taken from the bottom of each tube and plated in plain agar or blood agar. A bacterial control was run in distilled water and plated immediately after mixing and also at the end of the incubation period. By such a control it was possible to estimate the percentage of killing when complete killing did not occur. The plates were incubated at 37°C. for 18 hours and the results recorded.

In the case of the meningococcus the tests were made by Dr. Wollstein. Average 24 hour growths of the microorganism on sheep serum agar were washed off with 2 cc. of sterile distilled water. 0.5 cc. of this well mixed suspension was added to each tube containing the compound dilution. This was allowed to stand for 3 hours at 20°C. Then 0.2 cc. of each tube was planted on sheep serum agar slants. These were incubated for 48 hours and the readings taken. Controls accompanied each experiment.

For the gonococcus tests conducted by Dr. Pearce an adult strain of the organism was employed. 24 hour growths on ascitic veal agar were washed off with 3 to 5 cc. of normal salt solution. The exact amount of salt solution depended upon the profuseness of the growth. The object was to obtain a decidedly cloudy but not milky suspension of the bacteria. 0.5 cc. of this suspension was then added to the tubes containing the substance dilutions and allowed to stand at

20°C. for 3 hours. 0.2 cc. was then pipetted from the bottom of the tubes and planted on an ascitic veal agar slant and incubated for 48 hours. The readings were then taken. Controls were run in each experiment.

In all the above experiments the tests were run in duplicate.

TABLE I.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Chloroacetamide | 1,600 | 800–1,600 | 800 | 800–1,600 |
| Chloroacetmethylamide | 1,600 | 800 | 3,200 | 1,600 |
| Chloroacetethylamide | 800 | 800–1,600 | 3,200 | 6,400 |
| Chloroacetdimethylamide | 1,600 | 1,600 | 800 | 1,600 |
| Chloroacetdiethylamide | 1,600 | 1,600 | 1,600 | 1,600 |
| Chloroacetpiperidide | 200 | 800 | | |
| β-iodopropionamide | +* | + | 800 | 800 |

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

The results obtained with the hexamethylenetetramine quaternary salts prepared from chloroacetamide and the chloroacetyl derivatives of the simpler aliphatic amines will be found in Table I. With but few exceptions these substances were found to kill the organisms used for the test in dilutions of at least 1: 1,600 in 3 hours at 20°C. On the whole, but little variation in action, at least of a magnitude which could be detected by the dilution scheme employed, was obtained by the addition of alkyl radicals to the amide nitrogen in the chloroacetamide salt. The exceptions presented by the behavior of the methyl- and ethylamide derivatives toward the meningococcus are worthy of note. With these substances the action was observed to be about four times as great as that of the unsubstituted chloroacetamide salt or its dimethyl derivative. The unusual activity of the compound obtained from chloroacetylethylamine against gonococcus is also noteworthy.

When it is considered that substances of purely aliphatic nature are represented in this series, the bactericidal power observed is quite unusual. Formaldehyde, which is considered one of the most powerful of the aliphatic bactericides, when tested by the same tech-

nique was found to kill *Bacillus typhosus* in a maximum dilution of 1: 1,200. In addition, the molecular weights of these substances are approximately ten times that of formaldehyde, so that if the comparison were strictly drawn the observed figures should be multiplied by ten. On this basis they far exceed formaldehyde in molecular bactericidal power. The activity of the substances of this group as bactericides is attributable entirely to the presence in them of the hexamethylenetetramine molecule.[3]

The comparative results obtained against *Bacillus typhosus* by the same technique with other aromatic substances which have been regarded in the past as strong organic antiseptics are given in Table II. Unfortunately the tests were restricted to *Bacillus typhosus*. Among the substances given in Table I and in those to follow, many will be found which are as active or more active than any given in this table.

TABLE II.

*3 Hours at 20° C.*

| Substance. | *B. typhosus* killed in dilution of 1: |
|---|---|
| Formaldehyde | 1,200 |
| Phenol | +* |
| Lysol | 400 |
| Trichlorophenol | 800 |
| Tribromo-*p*-cresol | 1,600 |
| Tetrabromo-*o*-cresol | 1,600 |
| Tribromo-*m*-xylenol | 1,600 |
| Gentian violet | 3,200 |

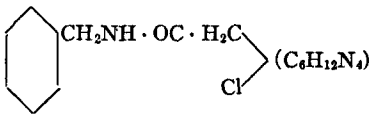
*+ indicates growth after exposure to a dilution of 1:200.

In an attempt to determine what influence might result from the substitution of the halogenacetic acid radical by that of other halogen fatty acids, it was found that the higher $\alpha$-halogen fatty acid

[3] There is evidence that the hexamethylenetetramine molecule does not always persist as such when its quaternary salts are dissolved in water, but undergoes a decomposition which yields methylene derivatives of the corresponding amines. The relation of these substances to the observed effects we shall make the subject of a subsequent communication.

derivatives failed to react with hexamethylenetetramine. The derivatives of β-iodopropionic acid, however, were found to react smoothly with the base to give quaternary salts. The ineffectiveness of the salt obtained from β-iodopropionamide in particular served to indicate the scant promise offered by the further employment of this acid and that the best results would be obtained by the continued use of the halogenacetyl group.

In the logical development of the above substances of purely aliphatic origin, the effect of the introduction of the aromatic nucleus into the alkyl group situated on the amide nitrogen was studied. The opportunity for this was furnished by the quaternary salts obtained from the chloroacetylbenzylamines possessing the following structural formula:




It was thought that here the usual antiseptic influence of the aromatic nucleus would appear, but, as will be seen from the results presented in Table III, this did not conform to the expectations. In interpreting the results, however, the greater molecular weights of these substances should not be neglected. Nevertheless, in those cases in which the observations fell below 1: 800 the chemical structure alone must be held responsible. Owing to the irregular character of the fluctuations observed it is difficult to deduce from this table any general relationships between the chemical constitution and the bactericidal power. In the case of the streptococcus and gonococcus, however, the introduction of the methyl group seemed to enhance the activity. The methoxy derivatives also appeared to be more effective than the corresponding acetoxy compounds. It is possible that a series of salts prepared from the mono- substituted benzylamines would have afforded a less confusing and more comparable group of substances for study. The difficulty of procuring such material and the pressure of other work were obstacles to the further extension of this chemical type.

On turning to the more easily accessible chloroacetylanilines, a series of substances was obtained which afforded ample opportunity

TABLE III.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Chloroacetylbenzylamine | 1,600* | 800 | 1,600 | 800 |
| Chloroacetyl-*o*-methylbenzylamine | 1,600* | 3,200 | 1,600 | 3,200–6,400 |
| *p*-acetaminoiodoacetylbenzylamine | 800* | 200 | 1,600 | 800 |
| 1-methyl-4-acetaminochloroacetylbenzylamine | +† | 800 | 800 | 1,600 |
| 1, 2-diacetoxychloroacetylbenzylamine | 800* | 800–1,600 | 800 | 400 |
| 1, 2-dimethoxychloroacetylbenzylamine | 1,600* | 800 | 400 | 800 |
| 1-acetamino-4-ethoxychloroacetylbenzylamine | 800–1,600 | 800–1,600 | 400 | 800 |
| *β*-acetoxy-*α*-naphthochloroacetylbenzylamine | 800 | 1,600 | 800 | 800 |
| *β*-methoxy-*α*-naphthochloroacetylbenzylamine | 1,600 | 3,200 | 1,600 | 1,600 |
| *m*-carbethoxychloroacetylbenzylamine | 800 | 400 | 400 | 800 |
| *m*-carbamidochloroacetylbenzylamine | + | | 3,200 | 1,600 |
| Diethylaminoethyl ester of *m*-carboxychloroacetylbenzylamine | 200 | 800 | | |

* Tests in these cases were made at 37° C.

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

for ascertaining the influence of the introduction of groups into the benzene nucleus. These substances possessed the following structural formula, in which any group X may occur in the *ortho*, *meta*, or *para* positions:

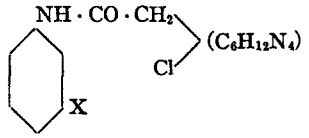



Table IV shows that the linkage of hexamethylenetetramine by means of the chloroacetyl radical with the simpler aromatic amines

TABLE IV.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Chloroacetylaniline | 800 | 1,600 | 1,600 | 3,200 |
| Chloroacetyl-*o*-toluidine | 1,600* | 1,600 | 1,600 | 1,600 |
| Chloroacetyl-*p*- " | 800 | 800 | 1,600 | 3,200 |
| Chloroacetyl-*m*-4-xylidine | 800 | 1,600 | 1,600 | 3,200 |
| Chloroacetyl-*ψ*-cumidine | 800 | 1,600 | 3,200 | 3,200 |
| Chloroacetyl-*α*-naphthylamine | 800 | 800 | 800 | 1,600–3,200 |
| Chloroacetyl-*β*- " | 1,600* | 800 | 6,400 | 6,400 |
| 6-chloroacetylaminoquinoline | 3,200 | 3,200 | 1,600 | 1,600 |
| Chloroacetyl-*o*-chloroaniline | 1,600 | 1,600 | 800 | 3,200 |
| Chloroacetyl-*p*-bromoaniline | 1,600 | 1,600 | 1,600 | 3,200 |
| Chloroacetyl-5-iodo-*o*-toluidine | 1,600 | 800 | 1,600 | 3,200 |
| Chloroacetyl-*m*-nitraniline | 3,200 | 3,200 | 3,200 | 3,200 |
| Chloroacetyl-*m*-nitro-*p*-toluidine | 800 | | | |
| *o*-chloroacetylaminophenol | 1,600 | 800 | 800 | 3,200–6,400 |
| Chloroacetyl-*o*-anisidine | 800 | | 3,200 | 1,600 |
| Chloroacetyl-*p*- " | 3,200 | 1,600 | | |
| *β*-iodopropionyl-*o*- " | +† | 200 | 400 | 400 |
| Chloroacetylmethylaniline | | 1,600 | 800 | 1,600 |
| Chloroacetyldiphenylamine | 400 | 200 | 1,600 | 1,600 |
| *p*-chloroacetylaminobenzoic ethyl ester | 1,600 | 3,200 | 1,600 | 3,200 |
| Chloroacetylnovocaine | | | 1,600 | 3,200–6,400 |
| *o*-chloroacetylaminobenzyl alcohol | 800 | 800–1,600 | 800 | 800 |
| *o*- " benzoate | 800 | 3,200 | 1,600 | |
| *o*-chloroacetylaminophenyl " | 200 | 1,600 | 800 | 3,200 |
| *o*- " *p*-nitrobenzoate | 800 | 1,600–3,200 | 1,600 | 1,600 |
| *m*-chloroacetylaminoacetophenone | | 1,600–3,200 | 800 | 1,600 |

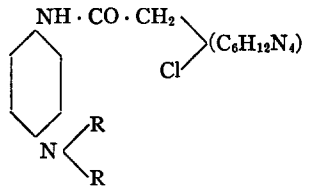
* Tests were made at 37° C.

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

produced a group of substances possessing definite bactericidal properties. Contrary to the results obtained with the substituted benzyl compounds described in the previous communication, it was found that alterations in the benzene nucleus by the usual substituents did not result in sharp differences in the bactericidal effect, at least of a magnitude which could be revealed by the scheme of dilutions employed. Many instances are to be found, however, in which the ac-

tivity of the simplest member, the salt obtained from chloroacetylaniline, has been definitely improved. Among these may be mentioned the substances obtained by the introduction of the methyl, chlorine, bromine, iodine, and nitro groups. Such chemical variations, however, did not always result in an improvement. In many cases the bacteria were killed in dilutions of 1: 3,200 or even 1: 6,400. On the whole, of the microorganisms employed, the gonococcus was the least resistant towards the members of this group. In the absence of indications of a more decided character or of greater regularity there was little assurance of obtaining more powerful bactericides by the further use of these substituents.

The results, however, assumed a different character by the adoption of a new type of variation within this group of substances. This consisted in the use of the dialkylamino group as a substituent in the nucleus of the parent chloroacetylaniline quaternary salt. These substances were prepared by the reaction of the chloroacetylaminodialkyl anilines with hexamethylenetetramine and possessed the following structure:




in which R may be methyl, ethyl, etc. The bactericidal results are given in Table V.

TABLE V.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| *p*-chloroacetylaminodimethylaniline ..... | +† | 800–1,600 | 1,600 | 1,600 |
| *p*-chloroacetylaminodiethylaniline..... | | 3,200–6,400 | 1,600 | 3,200 |
| *p*-chloroacetylaminodipropylaniline* ..... | | 6,400 | 3,200 | 6,400 |
| *p*-chloroacetylaminobenzylethylaniline* .. | | 6,400 | 6,400 | 12,800 |
| *m*-chloroacetylaminodimethylaniline ..... | | 1,600 | 400 | 400 |

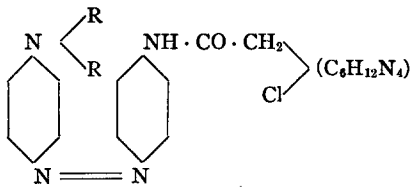
* In these cases, owing to the sparing solubility in water, one mol. of N HCl was used to dissolve the substances.

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

Our attention was first called to the desirability of developing this series by the apparent partial specificity of the dimethyl compound for the streptococcus as compared with the effect observed upon *Bacillus typhosus*. Later its effectiveness against the meningococcus and gonococcus was found to be equally marked. The later members of the group were obtained by replacing the methyl groups by ethyl, propyl, and benzyl. In these tests *Bacillus typhosus* was unfortunately omitted. We are therefore in no position to state whether this organism is more resistant to these substances as a class.

The regularity of the response to this particular chemical alteration is strikingly shown by these results. A progressive improvement occurred in the bactericidal action upon all three species of bacteria upon proceeding from the dimethyl to the diethyl, dipropyl, and finally the benzylethyl derivatives, and this in spite of the constant increase in molecular weight. If the increase with each dilution had not been so great, it is probable that all the columns of the table would have shown the regularity of the gonococcus results. The striking feature of the observations is the magnitude of the effect produced by such slight alterations in a complicated molecule. The inferior results obtained with the *meta*-dimethylamino compound, the last in the table, would indicate that the relative positions occupied by the substituents in the nucleus are modifying factors.

The efficacy of these groups was still further demonstrated by their use in another class of substances obtained by the addition of hexamethylenetetramine to chloroacetylaminoazobenzene derivatives, in which the base was attached by the chloroacetylamino side-chain to one nucleus and the dialkylamino group to the other as presented in the following formula:




In Table VI the salt obtained from chloroacetylaminoazotoluene, which contains no dialkylamino group, is first given as an

TABLE VI.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| *p*-chloroacetylaminoazotoluene | 3,200 | 3,200 | 3,200 | 1,600 |
| *p*-chloroacetylaminobenzeneazo-*p'*-dimethylaniline* | 800 | 12,800 | 3,200 | 3,200 |
| *p*-chloroacetylaminobenzeneazo-*p'*-diethylaniline | +‡ | 12,800 | 1,600 | 800 |
| *p*-chloroacetylaminobenzeneazo-*p'*-dipropylaniline* | | 12,800 | 800 | 800 |
| *p*-chloroacetylaminobenzeneazo-*p'*-benzylethylaniline* | | 12,800 | 1,600 | 800 |
| Benzeneazo-*m*-chloroacetylaminophenol† | | 3,200 | | |

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

† Solution made by the use of 1 mol. N NaOH.

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

object for comparison.[4] The action of this substance upon the different species of bacteria was fairly uniform. The introduction, however, of the dimethylamino group into that position in the molecule farthest removed from the location of the hexamethylenetetramine nucleus resulted in a marked difference. But slight alteration, if any, was observed in the meningococcus, a slight improvement towards the gonococcus, and a considerable reduction in the action upon *Bacillus typhosus*. With the streptococcus, however, the change was profound. The action was increased at least fourfold. The efficacy of this type of chemical modification against the streptococcus was still further confirmed by the replacement of the dimethyl group by the diethyl, dipropyl, and benzylethyl groups. These variations produced compounds which, in spite of the increased molecular weight, exhibited a degree of action of the same order. On the other hand, when tested against the other organisms they were found to be bactericidally less active than the dimethyl compound. We have here an interesting instance of specificity for streptococcus.

[4] The simpler chloroacetylaminoazobenzene derivative could not be conveniently used because of its sparing solubility in water.

Here again the hexamethylenetetramine molecule was found to be a factor in bringing out this effect. This was directly demonstrated in the following manner: *p*-aminobenzeneazodiethylaniline may be considered as the third substance mentioned in the table deprived of hexamethylenetetramine and the $-CH_2CO-$ radical which serves only as a connecting link. This dye was found to kill the streptococcus in a maximum dilution of 1: 3,200, an effect which, though marked, is still but one-fourth of the result obtained with its hexamethylenetetraminium salt.

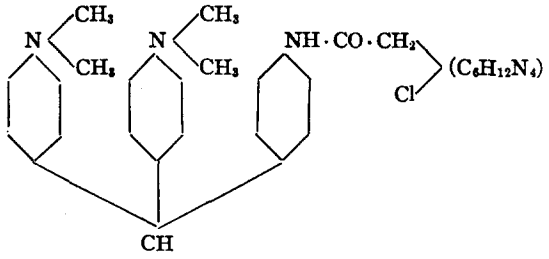
TABLE VII.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| *p*-chloroacetylamino-*p'*,*p''*-tetramethyldiaminotriphenylmethane (*p*-chloroacetylaminoleukomalachite green)* | 400 | 12,800 | 200 | 200 |
| *o*-chloroacetylamino-*p'*,*p''*-tetraethyldiaminotriphenylmethane* | | 12,800 | 6,400 | 1,600 |
| *p*-chloroacetylamino-*p'*,*p''*-tetraethyldiaminotriphenylmethane* | | 51,200 | 1,600 | 1,600 |
| Chloroacetyltriphenylamine | | 3,200–6,400 | 800 | 800 |

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

The group of substances given in Table VII headed by the hexamethylenetetramine quaternary salt of chloroacetyl-*p*-aminoleukomalachite green,

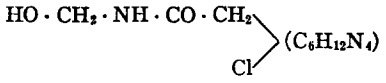



afforded the opportunity of still further testing the value of dialkylamino compounds against the streptococcus. This salt, as well as its homologs, displayed a marked specificity for this microorganism, kill-

ing it in a dilution of at least 1: 12,800, whereas the effect on the other forms was relatively weak. In the next two compounds the methyl groups were changed to ethyl groups, and in one case the chloroacetylamino radical was shifted to the *ortho* position. The dilution of 1: 12,800 was usually the highest dilution employed for the tests in the routine procedure, but fortunately the experiments performed with the third substance were carried further. This preparation was found to kill the streptococcus even in a dilution of 1: 51,200, making it probable that the first and second substances also would have been found to kill above 1: 12,800.

That here also the hexamethylenetetramine molecule is an essential factor was proven as follows. The first substance given in the table when deprived of this base and its connecting group is nothing else than *p*-aminoleukomalachite green. This substance required a concentration of at least 1: 800 to kill the streptococcus in 3 hours. In other words, the hexamethylenetetraminium salt derived from it was at least sixteen times more active.

In the course of the present work our interest was centered for a time in the study of the biological properties of the hexamethylenetetramine quaternary salts obtained from the chloroacetylalcamines. Our attention was attracted first to this group of substances by the powerful bactericidal properties of the simplest representative, that obtained by the addition of hexamethylenetetramine to chloroacetylaminomethanol.[5]



This substance, which possesses the above structural formula, is the first given in Table VIII. It is seen to possess a marked action against all the species used with the exception of the streptococcus. Because of the unusual effectiveness of this product it was hoped that its suitable chemical variation might lead to a series of very active substances.

[5] This substance was first prepared by Einhorn and Göttler (*Ann. d. Chem.*, 1908, ccclxi, 150), who also recognized its antiseptic properties.

TABLE VIII.

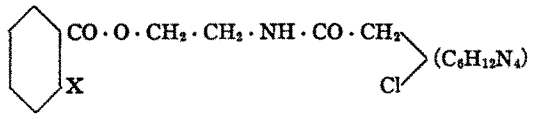
*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Chloroacetylaminomethanol | 3,200 | 400–800 | 6,400 | 6,400 |
| Iodoacetylaminoethanol | | 400 | 1,600 | 800 |
| Chloroacetylaminoisopropanol | | 200 | | |
| δ-chloroacetylamino-*n*-butanol | | 400 | | |
| β-chloroacetylamino-γ- " | | 800 | | |
| γ-chloroacetylamino-β-methyl-β-butanol | | 400 | | |
| α-phenyl-α-oxy-β-chloroacetylaminoethane | | 1,600 | | 1,600 |
| β-phenyl-β-oxy-α-chloroacetylaminopropane | | 800 | | |
| Chloroacetylaminoethyl ether | | 1,600 | | |
| Chloroacetyl-*o*-methylphenoxyethylamine | | 800 | | |

The chemical development of this substance was attempted in two ways: first, by the replacement of its methanol group by the ethanol, propanol, butanol, etc., radicals; and second, by the acylation of the methanol hydroxyl group with various acid radicals. Inthe latter scheme, however, chemical difficulties were encountered which compelled the use of its homologs, in particular the ethanol derivative, as the basis for the study of the effect of acylation.

Table VIII presents the behavior principally towards streptococcus of the substances obtained by the first method of chemical variation. The results show that the first member of the group, the salt obtained from chloroacetylaminomethanol, is the most powerful, so that no bactericidal increase was to be gained by such a chemical procedure.

Quite a different result was obtained by the use of the second scheme of chemical variation, as will be seen in Table IX. Unfortunately the inaccessibility of the acylated methanol derivatives made impossible a direct comparison of the effect of acylation upon the chloroacetylaminomethanol salt itself. The results must therefore be referred to the parent unacylated alcamine compound in question for a strict comparison. The structural formula of this group of substances may be represented as follows, X being any substituting group:




A glance at the table will show that we have here another group of hexamethylenetetraminium salts with strong bactericidal properties surpassing in this respect the parent chloroacetylalcamine compound

TABLE IX.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|
| Chloroacetylaminoethyl benzoate | 800 | 400 | 1,600–3,200 |
| " *o*-methylbenzoate | 1,600 | 3,200 | 1,600 |
| " *p*- " | 3,200 | 1,600 | 800 |
| " β-naphthoate | 3,200 | 6,400 | 6,400 |
| " *o*-nitrobenzoate | 800 | 3,200 | 1,600 |
| " *p*- " | 6,400 | 6,400 | 3,200 |
| " *p*-methoxybenzoate | 3,200 | | |
| " acetylsalicylate | 800 | | |
| " *p*-diethylaminobenzeneazo-*p'*-carboxylate | 6,400–12,800 | 400 | 400 |
| Chloroacetylamino-γ-propyl *p*-nitrobenzoate | 1,600 | | |
| " -γ- " *p*-methoxybenzoate | 1,600–3,200 | 800 | 1,600–3,200 |
| Chloroacetylethylaminoethyl *p*-nitrobenzoate | 3,200 | | |
| Chloroacetylphenylaminoethyl *p*- " | 800 | | 3,200–6,400 |

By the introduction of the simplest aromatic acid, benzoic acid, the bactericidal power of the parent iodoacetylaminoethanol salt was doubled, except for the meningococcus. The use of the substituted benzoic acids, such as the methyl, nitro, and methoxybenzoic, and naphthoic acids, in most cases still further improved the action. In the case of the nitrobenzoates the *para* compound seemed more effective than its *ortho* isomer. With the methylbenzoyl derivatives the *para* compound was also more active towards the streptococcus than its *ortho* isomer. With meningococcus and gonococcus the reverse was the case. The specificity of the *p*-diethylaminobenzeneazo-*p'*-carboxylate for streptococcus was to be expected from the results already discussed in connection with Table VI. This substance possesses the

diethylamino group. With the few acids studied, the best results were obtained with the *p*-nitrobenzoyl and β-naphthoyl compounds. The results yielded by the use of other alcamines, such as aminopropanol, ethylaminoethanol, etc., would seem to indicate that the optimum effect is to be obtained with the aminoethanol series.

In this group of substances but a few representatives were made and tested. By the use of numerous other acids a much broader series might be developed for study with the possibility of obtaining more active preparations. However, the observations obtained with this small group of substances serve to demonstrate again to what extent the bactericidal effect may be altered by relatively small changes in the molecule. Here, as in the case of the benzylhexamethylenetetraminium salts discussed in the previous communication, the degree of action is determined by the character and position of the substituents in the benzene nucleus. The main source of the bactericidal effect, however, is still the hexamethylenetetramine molecule.

TABLE X.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Chloroacetylurea | 3,200 | 800–1,600 | 1,600 | 1,600 |
| α-chloroacetyl-β-methylurea | 800 | 400–800 | 1,600 | 1,600 |
| α-chloroacetyl-β-benzylurea | 400 | 400 | 800 | 1,600 |
| Chloroacetylurethane | 400 | | 1,600 | 800 |

Still another type of hexamethylenetetramine quaternary salt included in the investigations was that represented by the compound obtained by the reaction of chloroacetylurea with hexamethylenetetramine. In Table X it is seen that for a purely aliphatic substance it exhibited a strong bactericidal power. It was hoped that by turning to the substituted ureas this action might be improved. The experience with the methyl and benzyl compounds, however, showed only a diminution of the activity.

Up to this point the substances which have been the subject of discussion were all quaternary salts obtained from halogenacetylamino compounds. Two other types of substances were included in the

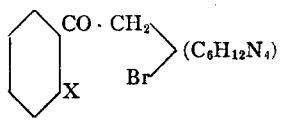
TABLE XI.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Chloroacetone | 1,600* | | | |
| ω-bromoacetophenone | 1,600* | 3,200 | 1,600 | 1,600 |
| *p*-methyl-ω-bromoacetophenone | 1,600 | 800 | 800 | 1,600 |
| *p*-ethyl-ω- " | 800 | | | |
| 1, 2-dimethyl-ω- " | 3,200 | 3,200 | 3,200 | 6,400 |
| 1, 3-dimethyl-ω- " | 1,600 | | 800 | 1,600 |
| *m*-nitro-ω- " | | 1,600 | | |
| *p*-methoxy-ω- " | 800 | 800 | 3,200 | 6,400 |
| *p*-ethoxy-ω- " | 800 | 1,600 | 1,600 | 1,600–3,200 |
| *p*-acetamino-ω- " | 800 | 3,200 | 1,600 | 3,200 |
| 3-acetamino-4-methyl-ω- " | | | 800 | 800 |
| 3-acetamino-4-tolyl ω-iodoethyl ketone | 800 | 1,600–3,200 | 12,800 | 12,800 |
| 1, 2-diacetoxy-ω-iodoacetophenone | 400 | 1,600 | 800 | 800 |
| β-[ω-bromoaceto]-quinaldine | 200 | 3,200 | 3,200 | 3,200 |

* Tests were made at 37° C.

study in which hexamethylenetetramine was joined by means of the halogenacetyl group first to hydrocarbons and then to alcohols. The first of these groups, which was prepared by the addition of halogen ketones to hexamethylenetetramine, may be represented by the following formula:




The results of the experiments made with these substances are contained in Table XI. The bactericidogenic property of hexamethylenetetramine was again demonstrated. The first member, the salt obtained from chloroacetone, was found to kill *Bacillus typhosus* in a dilution of 1: 1,600, which is again striking for an aliphatic substance. Among the aromatic representatives the majority killed one or another of the species tested in dilutions of 1: 1,600 or more. The behavior of the 1,2-dimethyl-ω-bromoacetophenone and *p*-methoxy-ω-bromoacetophenone derivatives toward the gonococcus and the action of the salt obtained from 3-acetamino-4-tolyl ω-iodoethyl

ketone on the gonococcus and the meningococcus are worthy of note. It is seen that the chemical constitution of the compounds determines in a degree the bactericidal power, but any definite regularity is far from apparent. As in many instances to be seen in the other tables, the result of a particular chemical variation upon the bactericidal power varies according to the organism used for the test. An interesting instance of the influence of the relative positions occupied by substituents in the benzene nucleus upon the bactericidal effect is shown by the differing action of the 1,2- and the 1,3-dimethyl-ω-bromoacetophenone salts towards the meningococcus and the gonococcus. The former substance is four times more active than the latter.

The results yielded by the salts obtained from halogenacetyl esters are given in Table XII. The ease of saponification of this chemical

TABLE XII.

*3 Hours at 20° C.*

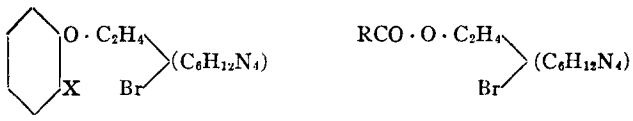
| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Ethyl bromoacetate | 400 | 1,600 | 800 | 800 |
| Phenyl " | 800 | 3,200 | 1,600 | 3,200 |
| Bornyl " | 3,200 | | | |
| Menthyl " | 800 | 1,600–3,200 | 1,600 | 1,600 |
| Ethyl β-iodopropionate | +* | 200 | 200 | 400 |
| Anisoylglycol chloroacetate | 1,600 | 1,600 | 1,600 | 1,600 |
| *p*-nitrobenzoylaminoisopropyl chloroacetate | 1,600 | 1,600 | | 1,600 |

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

type limited its more extended development. The table demonstrates the bactericidogenic properties of hexamethylenetetramine in this combination also. The relatively low bactericidal power of the β-iodopropionyl derivative is also in line with the results obtained with other derivatives of this acid.

In the course of the work still other connecting groups than the halogenacetyl radical were used in order to combine hexamethylenetetramine in the form of quaternary salts with other molecular group-

ings. Bromoethyl alcohol by virtue of its alcoholic hydroxyl group may combine with acids to form bromoethyl esters or may be considered the mother-substance of the bromoethyl ethers. These bromoethyl derivatives react readily with hexamethylenetetramine, giving the two following classes of salts:




The results obtained with the first of these, the bromoethyl ether salts, are given in Table XIII. It is to be observed that this type was, on the whole, most active against the meningococcus and the

TABLE XIII.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Phenyl bromoethyl ether | 200 | + | 400 | 800 |
| *o*-methylphenyl bromoethyl ether | +* | 400 | 1,600 | 1,600–3,200 |
| *m*- " " " | 400 | | 1,600 | 1,600 |
| *p*- " " " | 400 | | 400 | 800 |
| α-naphthyl bromoethyl ether | + | 800 | 3,200 | 12,800 |
| β- " " " | 800 | 1,600–3,200 | 1,600 | 3,200–6,400 |
| *p*-bromophenyl " " | 200 | + | 3,200 | 1,600 |
| Tribromo-*p*-cresyl " " | | 3,200 | 800 | 800 |
| *o*-acetaminophenyl " " | | | 400 | 400 |
| *p*- " " " | | | 200 | 200 |

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

gonococcus. The partial specificity of the α- and β-naphthol bromoethyl ether salts for the gonococcus is especially noteworthy. The α-compound, which killed the gonococcus in a dilution of 1:12,800, was ineffective against *Bacillus typhosus* in a concentration of 1: 200. These instances, together with the other substances mentioned in the table which were found to kill one or the other microorganism in dilutions of 1: 1,600 or 1: 3,200, still further indicate how general in character is the bactericidogenic property of the hexamethylenetetramine molecule

TABLE XIV.

*3 Hours at 20° C.*

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in dilution of 1: | Streptococcus killed in dilution of 1: | Meningococcus killed in dilution of 1: | Gonococcus killed in dilution of 1: |
|---|---|---|---|---|
| Bromoethyl acetate | +* | | 800 | 400 |
| " benzoate | 200 | 400 | 400 | 800 |
| " *p*-nitrobenzoate | + | | 800 | 800 |
| Bromoethylphthalimide | + | + | 400 | 800 |

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

In the case of the bromoethyl ester salts (Table XIV) the introduction of the hexamethylenetetramine molecule is seen to be considerably less effective. However, here again the best results were obtained with the meningococcus and the gonococcus. The last substance in the table is not an ester but a bromoethylamino compound. This also was most active against the meningococcus and gonococcus. It would seem from both this series and the previous group of substances that there is something in the chemical nature of the salts obtained from bromoethyl compounds which renders them most active against these two species of bacteria. Although not as marked, this may be considered analogous to the specific effect of the dialkylamino group upon the streptococcus.

It is highly probable that the further development of any of the leads which have been indicated in these papers might eventually furnish more active preparations which would be of chemotherapeutic value.

In conclusion we wish to present the results obtained in a few experiments on the effect of serum and protein on the bactericidal action of several of the compounds mentioned in the preceding tables. It so happened that in these serum-compatibility tests the technique employed was that described in the preceding paper. For this reason the parallel observations made with solutions of the compounds in physiological salt solution are a dilution higher than those to be found in the preceding tables. Table XV presents the results of these tests.

TABLE XV.

| Hexamethylenetetramine quaternary salt of | *B. typhosus* killed in 4 hrs. at 37° in a dilution of 1: | |
|---|---|---|
| | In physiological salt solution. | In horse serum. |
| Chloroacetyl-*o*-toluidine | 3,200 | 3,200 |
| Chloroacetylaminomethanol | 6,400 | 3,200 |
| ω-bromoacetophenone | 1,600 | 800 |
| 1, 2-diacetoxychloroacetylbenzylamine | 1,600 | 800 |

It is seen that in the case of the salt obtained from chloroacetyl-*o*-toluidine the action was not inhibited by serum. In the other cases the observed effect was reduced by half in the presence of serum. It is possible that in these cases the apparent inhibition was accentuated by the dilution scheme employed, and that in reality but little relative inhibition occurred. With a few other compounds of this class tested by a different technique a much greater relative inhibition of the bactericidal action was observed. From these experiments we may at any rate conclude that the bactericidogenic hexamethylenetetramine portion of the molecule does not in itself cause serum-incompatibility. The source of this must be sought in the remainder of the molecule.

TABLE XVI.

| Hexamethylenetetramine quaternary salt of | Gonococcus killed in 2 hrs. at 20° in a dilution of 1: | |
|---|---|---|
| | In aqueous solution. | In 5 per cent sodium caseinate solution. |
| Chloroacetyl-β-naphthylamine | 3,200 | 1,600 |
| *p*-methoxy-ω-bromoacetophenone | 6,400 | 6,400 |
| Chloroacetylnovocaine | 6,400 | 6,400 |
| α-naphthyl bromoethyl ether | 12,800 | 6,400 |
| Choloroacetylaminoethyl-*p*-nitrobenzoate | 3,200 | 3,200 |
| 3-acetamino-4-tolyl ω-iodoethyl ketone | 12,800 | 1,600 |

In Table XVI will be found the results of a series of tests in which the substances were dissolved in a 5 per cent sodium caseinate solution. The gonococcus was here used and the technique employed was the same as that described in the other gonococcus tests. In only one

case, the last given in the table, was any marked inhibition to be observed. With the remaining substances mentioned relatively little or no inhibition was observed.

## SUMMARY.

The extension of the study of the quaternary salts of hexamethylenetetramine to those obtained by the addition of this base to the most varied types of substances containing aliphatically bound halogen has demonstrated that the introduction of the hexamethylenetetramine nucleus in this manner results in the production of bactericidal substances or enhances the bactericidal action if already present.

In particular it was found possible by the use of the halogenacetyl group, $XCH_2CO$, as a connecting link, to furnish primary and secondary aliphatic and aromatic amines, alcohols, and hydrocarbons of the most varied character with the hexamethylenetetramine molecule and to study the relation between chemical constitution and bactericidal action in the series of substances so prepared. Because of the variety of chemical types studied, the results are too involved for a detailed summary here.

Many of the substances were found to be very powerful bactericides, and in a number of instances derivatives of purely aliphatic nature were found to possess an unusual bactericidal power.

*Bacillus typhosus*, streptococcus, meningococcus, and gonococcus were the microorganisms used for the tests, and striking instances of partial specificity were observed. This specificity was found to favor not one species alone, but instances were found in which each of the types of bacilli was shown to be especially susceptible to one or another of the particular types of compound employed. The source of this partial specificity is to be sought not in the hexamethylenetetramine nucleus itself but in the molecule to which it is attached.

The action of some of the substances was tested in the presence of serum or protein and was found to be not at all or only slightly inhibited. In other cases marked inhibition occurred. The factors controlling the serum- or protein-compatibility of these substances are likewise to be sought in that portion of the molecule other than the hexamethylenetetramine.