A NOTE ON THE REACTION OF MERCURIC CHLORIDE WITH BACTERIAL -SH GROUPS

By K. J. Steel*

From the Department of Pharmaceutics, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

Received October 14, 1959

A linear relation is obtained when the logarithm of the number of organisms (*E. coli*) in inocula is plotted against the logarithm of the quantity of mercuric chloride theoretically necessary to produce bacteriostasis, expressed as a percentage of that practically required. No direct relation exists between the number of organisms and the amount of mercuric chloride necessary to produce bacteriostasis. Mercuric chloride is non-specific in its reaction with bacterial sulphydryl groups. Cells of *E. coli* contain about 10^8 –SH groups per cell.

Cook and Steel[1] showed that the amount of mercuric chloride necessary to produce bacteriostasis of *Escherichia coli* increased with an increase in the number of organisms in the inoculum. It was not possible to obtain a relation between these two variables, although in some cases a semi-logarithmic relation was apparent. Using *Aerobacter aerogenes*, Poole and Hinshelwood[2] demonstrated that for a given inoculum size there was a critical concentration of mercuric chloride above which no growth occurred.

It is now recognised that the antibacterial action of mercury compounds is due to their interference with essential sulphydryl groups of the organism. Attempts were made to compare the amounts of mercuric chloride which theoretically combine with the sulphydryl groups of bacteria, and the amounts which are practically necessary for bacteriostasis.

It is possible to calculate the amount of mercuric chloride utilised in producing bacteriostasis if the following assumptions are made: (i) mercuric chloride acts by combining with sulphydryl groups, 1 molecule of mercuric chloride combining with 2 sulphydryl groups; (ii) each cell of *E. coli* contains 10^8 sulphydryl groups. McCalla[3] calculated there were 10^8 cation adsorption sites on a cell of this organism; Loureiro and Lito[4] reported *Salmonella typhi* and *Staphylococcus aureus* to contain this number of sulphydryl groups per cell and believed this value was applicable to other organisms.

Experimental

Materials. The test organism, *Escherichia coli* type I, the media, peptone agar and peptone water, and the dropping needles were as previously described[5].

Methods. The liquid dilution method for determining the bacteriostatic value of mercuric chloride was carried out as previously described[5] using inocula containing varying number of organisms. Viable counts were made by the surface-viable technique.

* Present address: National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W.9.

From the bacteriostatic concentrations of mercuric chloride and the numbers of organisms in the inocula, data such as that shown in Table I were calculated. The value of $6{\cdot}02 \times 10^{23}$ was used as Avogadro's Number. By plotting the logarithms of the numbers of organisms in the inocula against the logarithms of the percentages of mercuric chloride utilised (Fig. 1) an approximately linear relation was obtained.

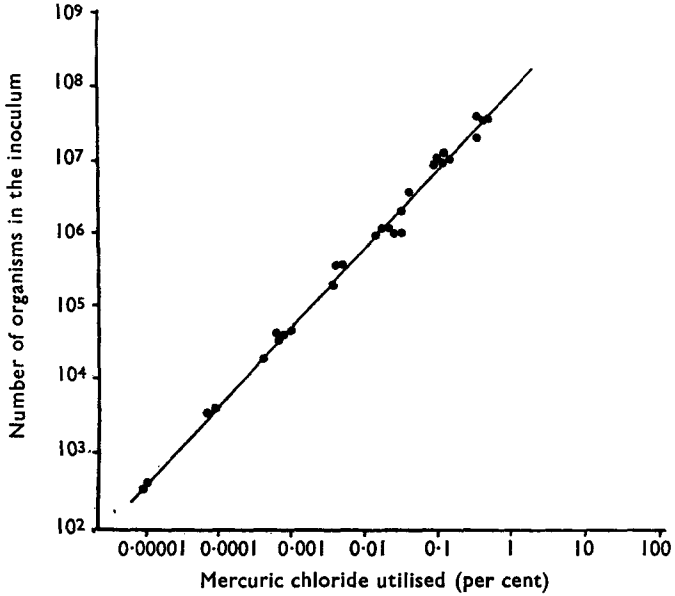

FIG. 1. Graph relating the inoculum size of *E. coli* with the theoretical amount of mercuric chloride utilised for bacteriostasis.

TABLE I

CALCULATION OF THE PERCENTAGE OF MERCURIC CHLORIDE "UTILISED" IN PRODUCING BACTERIOSTASIS

Total number of organisms in the inoculum	Total number of –SH groups in the inoculum	Equivalent number of molecules of $HgCl_2$ required	B'static concn. of $HgCl_2$ in micromoles	Total number of $HgCl_2$ molecules ($\times 10^{17}$)	Excess molecules of $HgCl_2$ ($\times 10^{17}$)	Per cent of $HgCl_2$ theoretically utilised
$3{\cdot}62 \times 10^{7}$	$3{\cdot}62 \times 10^{15}$	$1{\cdot}81 \times 10^{15}$	0·800	4·816	4·7979	0·376
$9{\cdot}25 \times 10^{5}$	$9{\cdot}25 \times 10^{13}$	$4{\cdot}62 \times 10^{13}$	0·525	3·1605	3·16004	0·0146
$4{\cdot}1 \times 10^{2}$	$4{\cdot}1 \times 10^{10}$	$2{\cdot}05 \times 10^{10}$	0·310	1·8662	1·86619	0·000011

Attempts to confirm the quoted sulphydryl content of cells of the test organism were made. Washed aqueous suspensions of *E. coli* were prepared from 24-hour slope cultures on peptone agar. The viable count of a portion of the suspension was determined and the remainder was used for the sulphydryl estimations. By titration of the suspension with potassium iodate in acid conditions, the mean value obtained for the sulphydryl content was $1{\cdot}67 \times 10^{7}$ –SH radicals per cell. Similar values were obtained when the suspension was titrated with mercuric chloride

solution, using diphenylcarbazone as the indicator. Further determinations were made using 0·001M sodium *p*-chloromercuribenzoate and diphenylcarbazone as the indicator. This method gave a mean value, with standard deviation, of $1{\cdot}14 \pm 0{\cdot}28 \times 10^8$ -SH radicals per cell.

Discussion

Figure 1 shows that the amount of mercuric chloride combining with the -SH groups of the bacteria is very small in comparison with the total amount of bacteriostat in the system. From these results it is assumed that most of the mercuric chloride present in these systems probably enters into combination with constituents of the medium or other molecules of the cells. However, it might be argued that a constant amount of the mercuric salt would combine with the media constituents. Such results do not necessarily indicate that the action of mercuric chloride is not on sulphydryl groups but rather that mercuric chloride is not a specific sulphydryl reactant. This view was held by Haarman[6] who considered mercuric chloride combined with some -COOH and $-NH_2$ groups of proteins, but no definite conclusions can be reached in this matter until further work on the availability of sulphydryl, amino and possibly other groupings in the medium, which could combine with the mercuric chloride, has been carried out. Extension of these investigations to more specific mercuric compounds might provide more valuable information.

The much higher values obtained for the sulphydryl content of cells of *E. coli* when *p*-chloromercuribenzoate was used suggest that many of the sulphydryl groups present in the cells were not capable of reacting with mercuric chloride or of being oxidised by potassium iodate under the conditions used. This may be due to stereochemical factors such that they were masked by other groups, hidden in the folding and co-ordination of the peptide chains or were in close proximity to groups capable of combining with the reactants. Further possibilities are the failure of reactions to occur, or be detected, with the low concentrations of reactants and the poor sensitivity of the indicators. The advantages of using *p*-chloromercuribenzoate for the determination of sulphydryl groups has been discussed by Olcott and Fraenkel-Conrat[7].

References

1. Cook and Steel, *J. Pharm. Pharmacol.*, 1959, **11**, 729.
2. Poole and Hinshelwood, *J. chem. Soc.*, 1940, 1565.
3. McCalla, *J. Bact.*, 1940, **40**, 23.
4. Loureiro and Lito, *J. Hyg., Camb.*, 1946, **44**, 463.
5. Cook and Steel, *J. Pharm. Pharmacol.*, 1959, **11**, 666.
6. Haarman, *Biochem. Z.*, 1943, **314**, 18.
7. Olcott and Fraenkel-Conrat, *Chem. Rev.*, 1947, **41**, 151.

THE PHENOLIC ACIDS OF URINE—A STUDY OF METHYLATION

BY S. L. TOMPSETT

From the Biochemical Laboratory, Northern General Hospital, Edinburgh, Scotland

Received September 22, 1959

Normal human urine contains methanol largely as methoxylated compounds; a small quantity of free methanol is also present. The methoxylated compounds exist partly as ether-soluble compounds, for example methylated phenolic acids, and partly as methoxylated compounds not extracted by ether from acid solution. The latter may be related chemically to quercitrin. Feeding experiments suggest that 3,4-dihydroxyphenolic substances are largely methylated while with other phenolic substances methylation is minimal.

THE present communication is an extension of previous studies.[1,2]. It has been shown that, in man tannic and 3,4-dihydroxybenzoic acids are largely methylated to 4-hydroxy-3-methoxy derivatives. The present paper is concerned with an examination of the nature, in very general terms, of methoxy compounds in human urine and also of the extent to which phenols and phenolic acids, other than those with an *ortho* dihydroxy structure, are methylated.

METHODS

The general principles of the techniques employed have been described[2]. The following fractions have been determined.

(*a*) *Free methanol.* 10 ml. of urine diluted to 15 ml. with water was heated to boiling in a 100 ml. R.B. flask attached to a water cooled condenser and 10 ml. of distillate collected.

(*b*) *Methanol liberated by the action of hot strong sulphuric acid on methoxylated compounds present in untreated urine.* Into a 100 ml. R.B. flask attached to a water cooled condenser (all glass equipment) were introduced 10 ml. of urine and 5 ml. of concentrated sulphuric acid. The mixture was heated to boiling and the distillate collected. Heating was continued until the sulphuric acid reached the fuming stage after which the mixture was allowed to cool. After the addition of 5 ml. of water, the mixture was again heated, the sulphuric acid being allowed to reach the fuming stage. This part of the procedure was repeated, so that three distillates in all were collected. A knife point of sodium bicarbonate was added to the combined distillates. The mixture was heated in apparatus similar to that described above and 10 ml. of distillate collected.

(*c*) *Methanol liberated by the action of hot strong sulphuric acid on the acidic fraction of urine.* 10 ml. of urine and 1 ml. of 10N hydrochloric acid in a test tube were heated in a boiling water bath for 1 hour. After cooling, the urine was extracted three times with 40 ml. quantities of redistilled ether. The combined ether extracts were evaporated to dryness.

10 ml. of water and 5 ml. of concentrated sulphuric acid were added to the residue, the procedure as described in (*b*) being then carried out.

Methanol was determined in the distillate[2].

Results and Discussion

The results obtained from the examination of 10 urines are shown in Table I.

TABLE I

FREE "METHANOL" AND METHOXYLATED COMPOUNDS IN HUMAN URINE AS MG. OF METHANOL/DAY

	Free "methanol"	Methoxy compounds		
		Untreated urine (*b*)	Phenolic and acidic fraction (*c*)	
1 ..	6·4	33·2	20·0	(100)
2 ..	9·1	26·6	15·9	(80)
3 ..	1·3	63·0	25·0	(125)
4 ..	4·6	28·4	18·6	(93)
5 ..	4·8	38·6	28·4	(142)
6 ..	7·2	18·8	11·6	(58)
7 ..	8·2	46·8	31·8	(159)
8 ..	4·6	23·6	18·4	(92)
9 ..	3·8	38·4	31·2	(156)
10 ..	4·2	28·6	18·2	(91)

The figures in brackets refer to the vanillic acid (4-hydroxy-3-methoxybenzoic acid) equivalents

Normal human urine contains a small amount of free methanol. The values are about the same as those obtained by Leaf and Zatman[3]. The origin of urinary methanol is obscure but it may be derived from the *in vivo* hydrolysis of methoxy compounds. Leaf and Zatman found that

TABLE II

THE URINARY EXCRETION OF METHOXY COMPOUNDS (MG. OF METHANOL/8 HOURS) AFTER THE ORAL INGESTION OF 1 G. OF SOME PHENOLIC AND RELATED COMPOUNDS

Compound	Methoxy compounds	
	Phenolic and acidic fraction	
	After compound	Control
Salicylic acid	10·3	5·1
Salicylic acid	9·8	4·8
Salicylic acid	8·7	4·6
m-Hydroxybenzoic acid	11·2	6·6
m-Hydroxybenzoic acid	10·4	6·3
m-Hydroxybenzoic acid	8·6	5·8
p-Hydroxybenzoic acid	12·4	5·5
p-Hydroxybenzoic acid	11·8	5·6
p-Hydroxybenzoic acid	8·6	4·9
3,4-Dihydroxybenzoic acid	24·8	4·9
3,4-Dihydroxybenzoic acid	29·8	5·1
3,4-Dihydroxybenzoic acid	26·8	4·6

the percentage recovery from urine of orally administered methanol was extremely low, which suggests that appreciable quantities of methanol may be metabolised. Normal urine appears to contain methylated phenolic acids and also methoxylated substances which are not readily extracted by ether from acid solution. Although the metabolites of adrenaline

are methoxylated compounds[4,5], the methoxylated compounds encountered in urine are probably mainly of dietary origin. The ether insoluble compounds are probably related chemically to quercitrin.

The urinary excretion of methoxylated compounds after the oral administration of some phenolic substances was studied. The night urine (8 hours) was used to minimise the effect of dietary intake. The results are shown in Table II. Methylation of *o*-, *m*- and *p*-hydroxybenzoic acids and resorcinol does appear to take place but not on the same scale as occurs with the 3,4-dihydroxyphenolic acids.

Substances containing a catecholic structure, for example, tannic acid, are widely consumed by man, and as a result are assumed to be non-toxic. There are, however, reports of liver damage[1], and hepatoma[6] by tannic acid. The position is therefore anomalous.

References

1. Tompsett, *J. Pharm. Pharmacol.*, 1958, **10**, 157.
2. Tompsett, *ibid.*, 1959, **11**, 32.
3. Lead and Zatman, *Brit. J. industr. Med.*, 1952, **9**, 19.
4. Armstrong, McMillan and Shaw, *Biochem. Biophys. Acta.*, 1957, **25**, 422.
5. Kirschner, Goodall and Rosen, *Proc. Soc. exp. Biol., N.Y.*, 1958, **98**, 627.
6. Korpassy and Mosanyi, *Brit. J. Cancer*, 1950, **4**, 411.