QUANTITATIVE STUDY OF THE STERILIZATION OF BACTERIA BY MERCURIC COMPOUNDS

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(With 4 Figures in the Text)

The problem of disinfection, whether by physical or chemical means, has hitherto been analysed almost exclusively in terms of 'intensity', i.e. concentrations, temperatures, densities of radiation, etc., capable of exerting a lethal effect within a reasonably short time. For this reason quantitative studies of disinfection have usually taken the form of kinetic analyses of the process. Recent developments consist mainly in a refinement of this kinetic method (Jordan & Jacobs, 1944*a*, *b*). It is undeniable that for the current problem of disinfection, which requires that pathogenic organisms should be destroyed quickly, the intensity factor, and the kinetics of sterilization dependent on it, represent the right angle of approach. There is, however, one case at least in which kinetics matter very little—the case of vaccine suspensions. Here the object is not to kill quickly but to kill correctly, i.e. with the least possible damage to the chemical groupings responsible for the antigenic behaviour of micro-organisms. Obviously an exact knowledge of the chemical mechanism of sterilization will provide the surest basis for attaining this object. Sterilization by chemicals may occur through two different mechanisms. It is either due to the mass action of a large excess of the sterilizing agent or to a quantitative reaction between it and specific cell receptors. The action of acids or of bases, possibly that of phenol, may be quoted among the first. Direct evidence proving that the second mechanism also exists has not hitherto been available, although a casual observation made by Rainsford (1939) suggests that such a relationship exists in regard to the sterilization of *B. typhosum* by silver. A similar observation made by us while working with sodium merthiolate provided the first suggestion for our study, and it is the purpose of the present work to demonstrate that in the sterilization of bacteria by mercuric compounds there is a quantitative relationship between the absolute amount of antiseptic and the mass of organisms sterilizable by it.

The antiseptic action of mercury has already been attributed by several authors to a blocking of —SH groups (Rapkine, 1931; Fildes, 1940). In these experiments, however, the amount of antiseptic was in huge excess over the quantity sufficient for the saturation of all the —SH groups of the substrate. Contrasting with these conditions, which are common to bacteriostasis experiments and to all kinetic studies of disinfection, in our experiments the emphasis was placed wholly on the adjustment of the absolute quantity of mercurial antiseptic to the absolute quantity of the substrate.

PLAN OF THE INVESTIGATION

Admitting as a working hypothesis that a mercurial antiseptic acts by blocking —SH radicals, it follows that a certain amount of it, no matter what the concentration, should be able to sterilize not more than a certain number of organisms. This quantity of antiseptic should, moreover, be predictable by titration of the thiol groups in the bacterial suspension. Our first experiments were performed with sodium merthiolate ($C_2H_5.HgS.C_6H_4.COONa$) because in this compound containing 'masked' mercury, one molecule of the antiseptic may be assumed to react with each thiol group. Later the investigation was extended to other mercury compounds when it was found that ionized compounds could react with two or four thiol groups per molecule, depending on the nature of the compound (see Table 2).

Many trials were necessary before deciding on the type of experiments which would clearly reveal the existence of the postulated quantitative relationship. It was found that the best criterion was the time required to produce sterility in a bacterial suspension, in relation to the proportion of antiseptic, in a series of mixtures.

Many variables affect the fate of a mixture of bacteria with an antiseptic, namely, the temperature, reaction of the medium, saline concentration, etc. But even when all these are controlled and fixed at suitable values, the shape of the survival-time curve depends on a series of factors which must be discussed before the results can be properly understood. If in order to kill a bacterial cell with [Hg] complete saturation of all its —SH groups were required, the exact weight of antiseptic

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capable of sterilizing a given bacterial population would be definable with the rigour of a chemical equation. A slightly smaller weight would leave in the suspension a few cells indefinitely viable. Representing the condition by histogram, of which the ordinate gives the number of days of survival, and the abscissa the log of the number of equivalents of antiseptic per thiol equivalent of the bacterial suspension, the graph would have the shape shown in Fig. 1A. In reality things are not so simple. First of all it is probable that a prolonged blocking of a fraction only of the bacterial —SH groups may suffice to cause death, which introduces the possibility of random variations in the degree of saturation of individual cells. This factor, combined with the slowness of the union between antiseptic and cell receptors, the possibility of exchange between

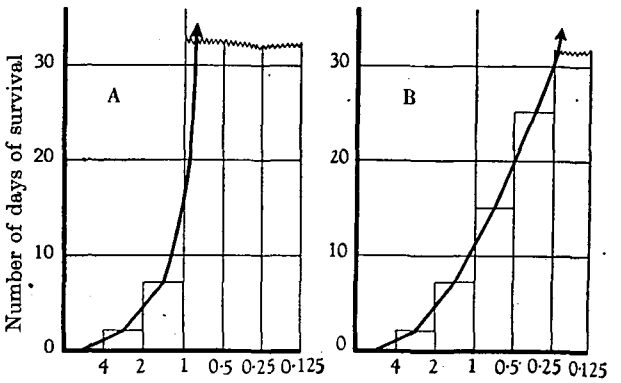

Fig. 1.

more and less saturated cells, and finally the effect of spontaneous mortality, which may be abnormally high amongst slightly injured cells, must inevitably cause the experimental curves to assume a more or less flattened shape. Fig. 1B reproduces one of these curves, in which besides the histogram the frequency polygon which represents it is also drawn.

In the face of these numerous causes of variation, not all of them susceptible of exact statistical analysis, it is suggested that as a sufficient demonstration of the existence of the postulated quantitative relationship the fulfilment of the two following conditions may be required. These are, first, that it may be possible to predict, from the thiol titration, the order of magnitude of the amount of antiseptic capable of sterilizing, after long contact, a given bacterial suspension; and secondly, that the survival time should be independent of the concentration of the antiseptic-bacteria mixture. This second condition will express itself in a rough parallelism of the curves obtained with the same antiseptic at various degrees of dilution of the several mixtures of bacteria and antiseptic.

MATERIAL AND METHODS

Bacterial suspensions. Experiments were performed with *B. typhosum* (Ty 2) and *Staphylococcus aureus* (local strain), but although the results agreed on the whole, only those obtained with *B. typhosum* will be described here, because in the case of *Staphylococcus*, the slower penetration of the antiseptic and the tendency of the live bacterial suspensions to autolyse made the results less regular and clear-cut. The bacteria were grown in an aerated liquid medium, centrifuged, and the packed sediment suspended in four volumes of distilled water. From this 1 : 5 suspension, called 'concentrated suspension',, the necessary dilutions were prepared. In the case of *B. typhosum* the suspension may be stored for a week or two in the ice chest without appreciable change in the thiol titre or in the viability of the organisms.

Thiol titration. For this, the technique of Kuhn, Birkofer & Quackenbusch (1939) was followed, namely, titration with 0·004N iodine in 70 % acetic acid, which gives a sharp end-point and is readily applicable to bacteria. In the two species investigated the thiol titre of the packed sediment varied between 0·018 and 0·022N with a probable error of the titration of $\pm 3\%$. This value is probably fairly constant for most bacterial species, and may be used as a reference in judging how much mercurial antiseptic is needed for the sterilization of heavy vaccine suspensions.

Cell count. This was estimated by measuring the opacity with an Evelyn photoelectric colorimeter calibrated against directly counted suspensions of *B. typhosum*. In Table 1 the data concerning one of the suspensions used in this study are given as an illustration of the results obtained. The last figure shows that the saturation of the thiol groups may proceed by insensible gradations.

Calculation of the equivalent of antiseptic. On account of the method of titration employed the equivalent is conveniently expressed in terms of ml. of 1/1000 solution of antiseptic per ml. of the 0·004N iodine used in titrating the concentrated 1 : 5 suspension, as shown in Table 2. The thiol equivalent is related to the molecular weight in the case of compounds such as merthiolate which do not contain ionized mercury, and to the appropriate fraction of the molecular weight when mercury ions are formed. In Table 2 the thiol equivalent per molecule of various mercuric compounds is given.

Sterilization experiments. Mixtures of the bacterial suspensions with multiples and submultiples of the supposed equivalent of antiseptic were prepared, based on the results shown in Tables 1 and 2.

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The number of thiol equivalents per molecule of the compounds was estimated empirically by comparing the behaviour of a series of mixtures prepared with different compounds. As stated above, the speed of sterilization was greatly affected by the temperature and composition of the medium. In the case of *B. typhosum*, the most consistent results were obtained using distilled water for the dilutions and keeping the mixtures in the ice chest.

Survival tests. The presence of viable organisms was detected by inoculation into 0·1 % NaS broth or agar, in which the effect of any excess antiseptic is inhibited. The first test was performed after 1 day of contact (a 6 hr. test, used at first, was found unnecessary) and on each successive day thereafter, until the results began to stabilize. Then the survival tests were spaced at intervals of several days. become negative, whilst the broth test was still positive. Growth in broth might appear only by the second or third day, and only after 4 days' incubation was the test regarded as surely negative. It happened sometimes that the broth test became negative and later on gave a positive result again. This is an understandable effect due to random sampling from the dwindling number of viable cells. For the purpose of graphic presentation the end-point in such cases was taken not at the date of the last positive test but 1 day after the last of the consecutively positive ones.

RESULTS

Each experiment yielded a mass of data, and a typical example is given, in a slightly abridged

Table 1

No. of cells per ml. of 1 : 5 suspension	278×10^9
No. of cells per ml. of packed sediment	1380×10^9
Volume of 1000×10^9 cells	0·73 ml.
Volume of a single cell	$0{\cdot}73\,\mu^3$
Thiol titre of the packed sediment	$0{\cdot}022\,N$
Thiol equivalent of single cell	$1{\cdot}6 \times 10^{-17}$ g.equivs.
Number of real —SH radicals per cell	10^8

Table 2. *Volumes of* 1/1000 *solutions of various mercurial antiseptics equivalent respectively to* 1 *ml. of standard iodine and* 1 *ml. of centrifuged bacteria* (*average values*)

Antiseptic	Molecular weight	Probable no. of thiol equiv./mol. of antiseptic	Normality of 1/1000 solution	ml. of 1 : 1000 solution equiv. to 1 ml. of 0·004 *N* iodine	ml. of 1 : 1000 solution equiv. to 1 ml. of packed organisms (0·02 *N*)
Na merthiolate	404	1	$2{\cdot}5 \times 10^{-3}$	1·60	8·0
$HgCl_2$	271	2	$7{\cdot}4 \times 10^{-3}$	0·54	2·7
$Hg(CN)_2$	252	2	$7{\cdot}9 \times 10^{-3}$	0·51	2·5
$Hg(CN)_2HgO$	468	4	$8{\cdot}5 \times 10^{-3}$	0·47	2·3

For the agar test, each plate was divided into four to eight sectors and a loopful of each mixture spread on a separate sector. The results were very striking because the reduction in the number of viable cells could be observed directly. So long as there was a large viable population the whole area touched by the loop became covered by a confluent growth visible after a few hours. When the viable cells were fewer, isolated colonies appeared even in the zone of heaviest inoculation. Still later, a few slowly developing colonies were found, and finally the result became negative. In Table 3 these gradations of the intensity of growth are expressed respectively by the signs ∞, +, $\pm$ and −.

For the broth test a loopful was used from the heavier suspensions and, from the more dilute ones, a few drops drawn with a Pasteur pipette. When the sterilization was well advanced the plate test might

form, in Table 3. The results are also shown graphically in Fig. 2, and are represented by a group of frequency polygons in which a semi-logarithmic plot has been used. It is evident that when the amount of merthiolate used was based on the thiol titration of the bacteria, sterilization was effected after sufficient time of contact had been allowed. The actual time required varied very considerably according to the relative concentrations of merthiolate and bacteria, but it is clear that it has been possible to predict from the thiol titration of the bacteria the order of magnitude of the amount of merthiolate required for sterilization. Further, there would seem to be no doubt that, apart from minor and expected irregularities, the sterilization time was unaffected by dilution of the mixture of antiseptic and bacteria, the relative concentrations thereby remaining unchanged.

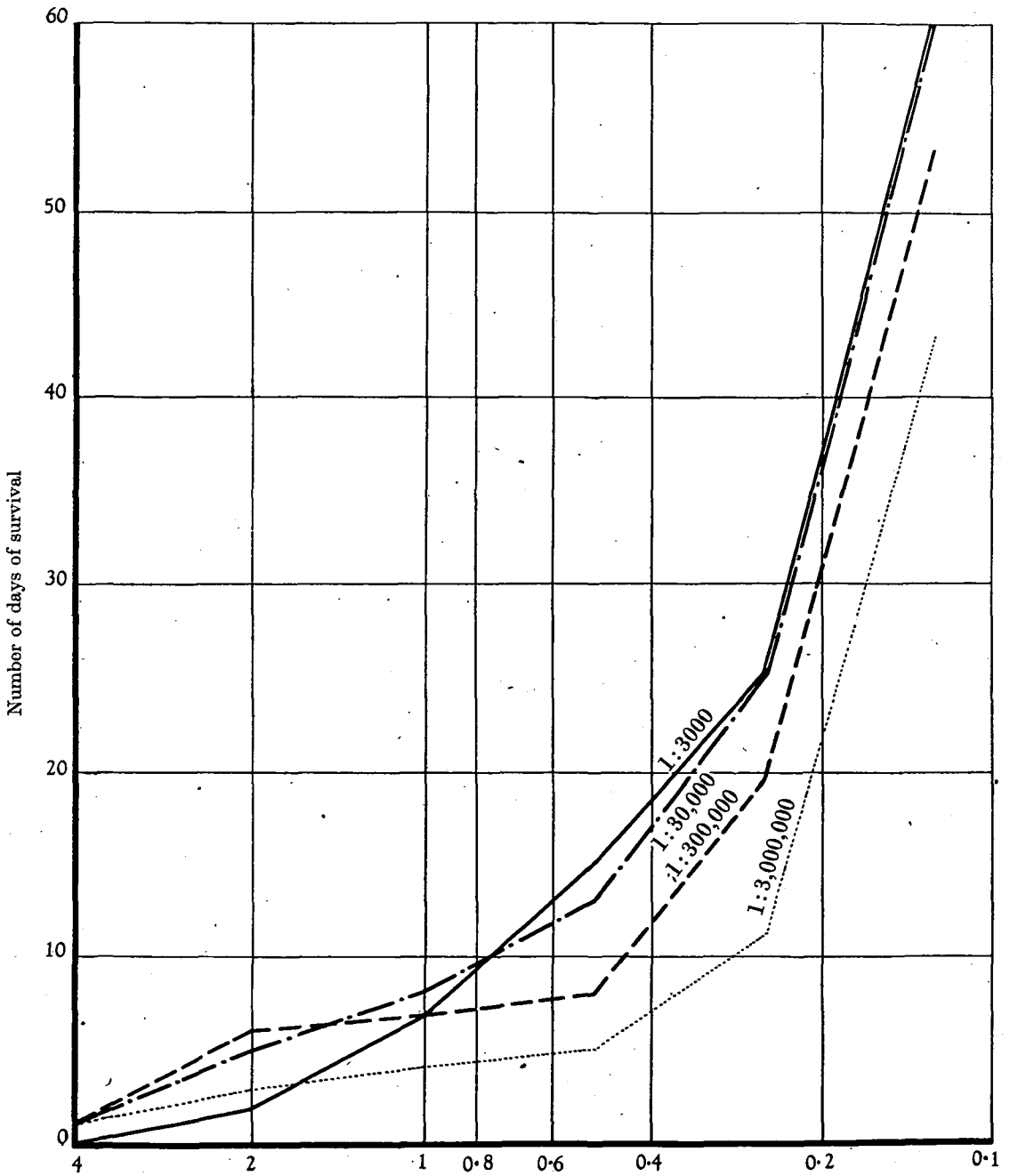

Fig. 2.

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Table 3. *The results of survival tests, in broth and on agar plates, of mixtures of* B. typhosum *suspensions with various proportions of sodium merthiolate, on selected successive dates*

Temperature 2–4° C. Thiol titre of the concentrated suspension $0{\cdot}84 \times 0{\cdot}004$ *N*. 1·4 ml. of 1/1000 merthiolate solution equivalent to 1 ml. of concentrated suspension.

Approximate concentration of bacteria-antiseptic mixture ...	$0{\cdot}3 \times 10^{-3}$						$0{\cdot}3 \times 10^{-4}$						$0{\cdot}3 \times 10^{-5}$						$0{\cdot}3 \times 10^{-6}$					
[Hg]/—SH equivalents ...	4	2	1	½	¼	⅛	4	2	1	½	¼	⅛	4	2	1	½	¼	⅛	4	2	1	½	¼	⅛
Result of tests, days elapsed after admixture																								
Survival tests in broth																								
1	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
3	−	−	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+
4	.	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	−	−	+	+	+
5	.	−	+	+	+	+	.	−	+	+	+	+	.	+	+	+	+	+	.	−	−	+	+	+
7	.	−	+	+	+	+	.	−	+	+	+	+	.	−	+	+	+	+	.	−	−	+	+	+
9	.	.	−	+	+	+	.	.	−	+	+	+	.	−	−	−	+	+	.	.	.	−	+	+
13	.	.	.	+	+	+	.	.	.	+	+	+	.	.	.	.	+	+	.	.	.	.	−	+
17	.	.	.	−	+	+	.	.	.	−	+	+	.	.	.	.	+	+	.	.	.	.	.	+
21	.	.	.	.	+	+	.	.	.	.	+	+	.	.	.	.	+	+	.	.	.	.	.	+
25	.	.	.	.	+	+	.	.	.	.	+	+	.	.	.	.	−	+	.	.	.	.	.	+
34	.	.	.	.	−	+	.	.	.	.	−	+	.	.	.	.	.	+	.	.	.	.	.	+
50	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	.	−
75	.	.	.	.	.	−	.	.	.	.	.	−	.	.	.	.	.	−	.	.	.	.	.	.
Survival tests on agar plates																								
1	−	−	∞	∞	∞	∞	±	+	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	+	+	∞	∞	∞	∞
13	.	.	−	+	∞	∞	.	.	.	±	∞	∞	.	.	.	−	∞	∞	.	.	.	.	−	∞
21	.	.	.	−	∞	∞	.	.	.	−	±	∞	.	.	.	.	+	∞	.	.	.	.	−	∞
34	.	.	.	.	−	∞	.	.	.	.	−	∞	.	.	.	.	−	∞	.	.	.	.	.	−

In Fig. 3 the results of experiments with other mercurial antiseptics are shown. In all cases the concentration of the antiseptic in the equivalent bacteria-antiseptic mixture was fixed at 1 : 10,000. It is apparent that not only has it been possible to predict for mercuric chloride, cyanide and oxycyanide, as well as for merthiolate, the order of magnitude of the effective sterilizing concentration, but that broadly speaking all four antiseptics were equally effective when the amount used was expressed in terms of equivalents per thiol equivalent of the bacteria. On the other hand, Fig. 4 shows that if the amounts of antiseptic were calculated on the basis of molecules of antiseptic per thiol equivalent, then mercuric oxycyanide was the most potent, sodium merthiolate the least, with mercuric chloride and cyanide intermediate and equal. Comparing these results with the data in Table 2, the evidence is very strong that the mercurial antiseptics act by combining with thiol groups in the bacteria; that a quantitative relationship exists, and that the relative effectiveness of different substances depends on the number of thiol equivalents per molecule of the antiseptic.

In similarly planned experiments we found that the antiseptic action of merthiolate was accelerated by the presence of sodium chloride and delayed by glucose or sucrose. The well-known effect of temperature was very apparent in the experimental conditions of this work. At 37°, even the suspensions mixed with one-quarter or one-eighth of the thiol equivalent were completely sterilized within a day or two.

Some information is available regarding the distribution of mercury between the solution and the bacterial sediment. It was found that in sterilized mixtures which originally contained less than the thiol equivalent of mercuric oxycyanide, all the mercury was bound by the bacterial sediment, whilst if more than one equivalent had been present, the supernatant fluid gave a positive test for mercury with hydrogen sulphide.

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DISCUSSION

From Table 3 and Fig. 2 it is clear that the two conditions postulated as necessary for the demonstration of the existence of a quantitative relationship between the amount of antiseptic and the amount of substrate were both fulfilled. Figs. 3 and 4 also confirm that it is possible to predict the order of magnitude of the quantity of antiseptic required for the sterilization of a given suspension, and show that the degree of precision is sufficient to establish a distinction between the various mercuric compounds of differing thiol-combining powers.

Paradoxically, the effect of dilution, if any, would be apparently in favour of the most dilute antiseptic as appears from Table 3. Actually, as Rapkine (1936) has already stated, the binding of —SH is a reaction of the second order, and therefore its speed must decrease with increasing dilution. In our experiments this slowing up with dilution was only noticeable in the first few days. Later, as the probability of fishing a viable cell in the survival tests is much greater from the heavier suspensions, the effect of the reaction kinetics becomes outweighed by this sampling effect.

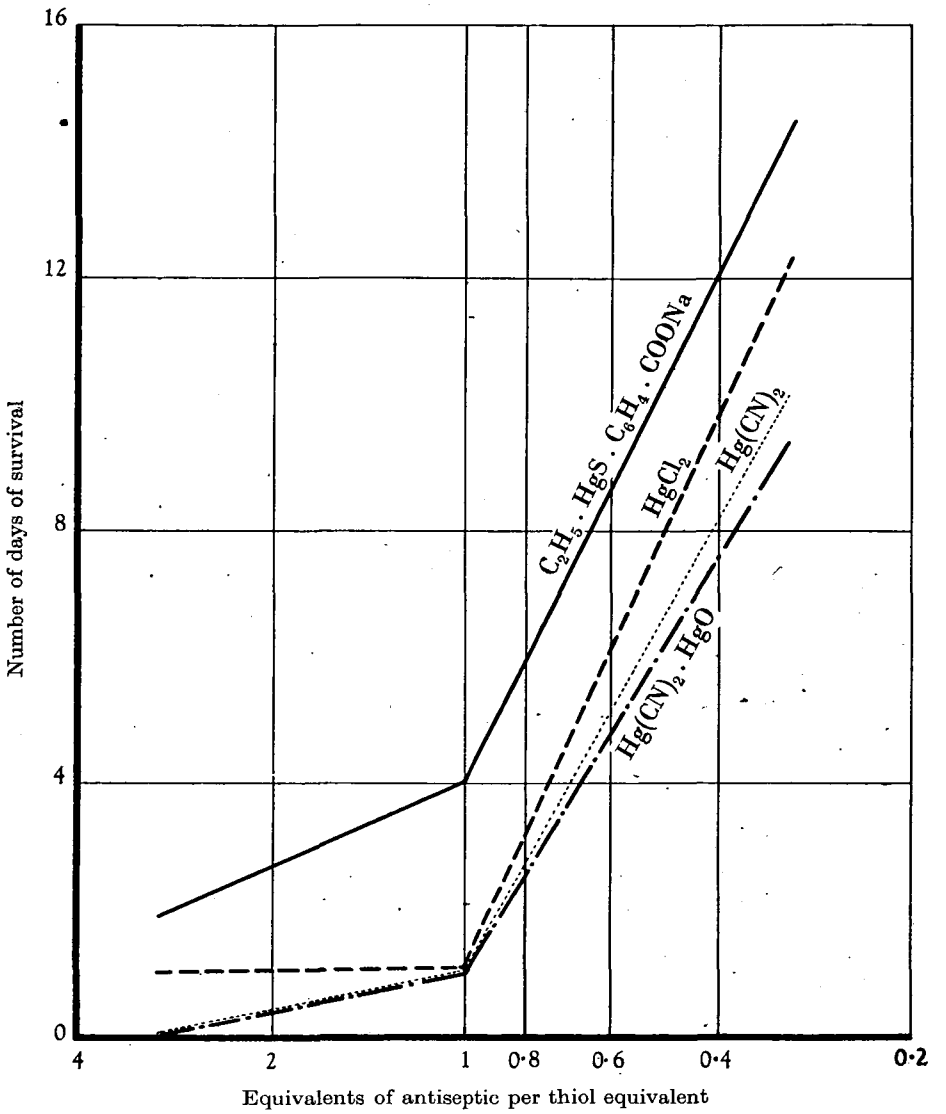

Fig. 3.

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As a matter of interest it may be pointed out that, as shown in Fig. 2, it is sufficient to block for a sufficient time one-quarter only of the thiol groups in the bacteria in order to effect complete sterilization. Another fact that stands out, and is most clearly illustrated by the + and ± results of the survival tests on agar, is that a few individuals may survive many days in conditions which have already killed a population of millions of cells.

further example, the limit of 20×10^9 *B. typhosum* cells per ml. determined by Rainsford (1939) for the sterilizing capacity of 0·002 % silver (as $AgCl_2$) may be quoted. According to Table 1 the thiol titre of such suspension should be 3×10^{-4} *N*, while 0·002 % Ag is 5×10^{-4} *N*. As the mechanism of sterilization by silver and mercury is supposed to be similar, Rainsford's observation clearly falls into line with the present data.

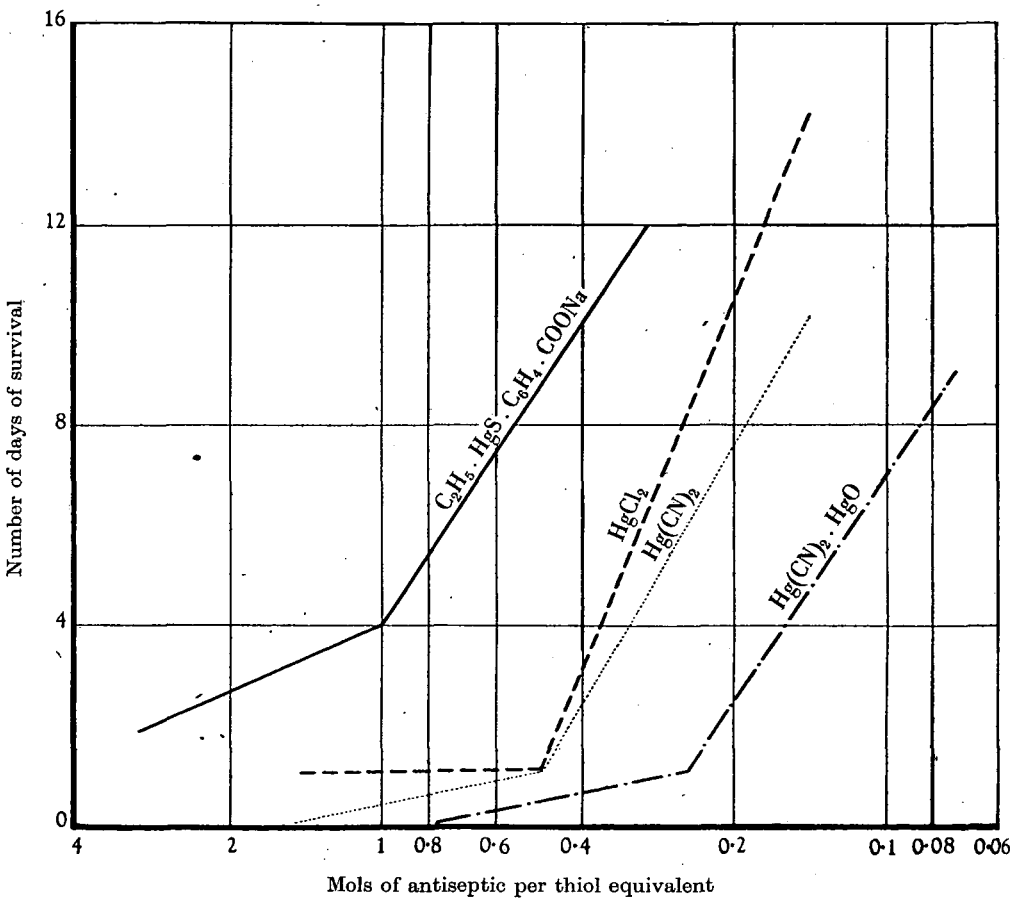

Fig. 4.

Our whole series of experiments shows how fallacious is the exclusive consideration of antiseptics in terms of concentration. The effect of concentration is dominant when the mass of substrate is negligible in regard to that of the antiseptic; but, as soon as both masses are of the same order of magnitude, the quantity factor acquires the greater importance. This happens in the case of the oligodynamic action of extremely dilute antiseptics and, at the other extreme, in the case of the very heavy bacterial suspensions we have dealt with. As a

From the practical standpoint the main consequence of our work is the indication of the possibility of studying systematically the problem of preservatives for vaccines and other biological products. Preservatives must naturally satisfy the conditions of being germicidal, non-toxic and non-irritating at the concentrations adopted, but insufficient attention has been paid to the possibility of secondary reactions occurring between preservative and substrate. The work of Felix & Bhatnagar (1935) has shown how harmful formol and phenol

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are to the labile Vi antigen of *B. typhosum*. It is therefore suggested that the choice of preservatives should be based upon an exact knowledge of the mechanism of sterilization. In this regard sodium merthiolate seems to be one of the best available. It seems to react exclusively with the —SH group, and once a substrate has been saturated by it, any excess remains indefinitely available for the sterilization of accidental contaminants. Its main inconveniences are that its cost is relatively high and that it is not available everywhere. Our experiments show that for some purposes it may probably be replaced by an alkaline solution of mercuric oxycyanide, which, when sufficiently dilute, is not irritating, does not precipitate proteins, and shares with merthiolate the property of reacting specifically with —SH groups but not with the cell receptors responsible for the antigenic behaviour of *B. typhosum*, and which are so easily affected by formalin or phenol.

The type of investigation on the mercurial antiseptics reported above ought to be extended to other substances because there are probably as many possible mechanisms of sterilization as there are functional groups essential for the life of a cell. Their systematic investigation may not only advance the theoretical and practical knowledge of sterilization itself, but also provide more general information concerning the nature of the chemical groupings essential for the life and antigenic specificity of micro-organisms.

SUMMARY

In the present work it is suggested that the sterilization of bacterial suspensions by mercurial antiseptics is a reflexion of a possible stoichiometrical relation between the mercuric compound and the thiol receptors in the bacterial cells.

This has been verified experimentally by studying the times of survival of suspensions of *B. typhosum* mixed with multiples and submultiples of the quantity of antiseptic theoretically equivalent to the thiol content of the suspension, as estimated by iodometric titration.

The considerable differences observed in the time of survival of suspensions mixed respectively with more and with less than the thiol equivalent of antiseptic, and the absence of any marked effect of dilution of the mixture, show that there is a quantitative relationship between the amounts of substrate and the quantity of antiseptic capable of sterilizing it.

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