THE INHIBITION OF SULFHYDRYL ENZYMES AS THE BASIS OF THE BACTERICIDAL ACTION OF CHLORINE[1]

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Current hypotheses of the mode of bactericidal action of chlorine suppose a direct combination of some chemical species of the chlorine with the bacterial protoplasm producing a toxic organic complex (Porter, 1946). Although this view is a great advance over earlier concepts, it still lacks precision in terms of our present knowledge of the intermediary metabolism of cells.

The marked efficiency of chlorine, exerting a bactericidal action in concentrations of 0.2 to 2.0 ppm in water, will at once classify it as a biologically active "trace substance." It may consequently be assumed to exert its effect upon the enzyme systems of the cell (Green, 1941). By investigation of the effect of chlorine on bacteria and on various enzymes, its bactericidal effect has been shown to depend upon the inhibition of certain essential enzyme systems, and the mechanism of this inhibition involves the powerful oxidative action of chlorine on the —SH groups of these enzymes.

METHODS

Active chlorine was estimated by the acid-iodine thiosulfate titration procedure (Calvert, 1943). Chlorine solutions, when not otherwise specified, were obtained by neutralizing and filtering a fresh solution of calcium hypochlorite.

The various bacteria were grown in Roux bottles on meat infusion agar at 37 C for 16 hours, harvested by centrifuging the saline washings, washed twice with 0.4 per cent saline solution, and stored at 0 C. In later experiments *Escherichia coli* were grown in 8-liter quantities of casein digest broth, aerated by a stream of filtered air, then harvested similarly.

Bacterial suspensions of known nitrogen content were exposed to the chlorine solutions at 37 C for 10 minutes (except in experiments on exposure time, table 2) in the presence of 0.2 M phosphate buffer, pH 7.0. The amount of chlorine used is expressed as μg of active chlorine per mg of bacterial nitrogen present. In some experiments (tables 1, 2, and 3) any excess chlorine compound remaining after the exposure was removed by the addition of a slight excess of 0.1 N thiosulfate. The suspension was then washed by dilution with water, centrifuged at high speed, and resuspended in the original volume. No differences in metabolic activity or viability were detected between cells treated in this manner and those diluted directly from the chlorine solution for the experiments. The

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metabolic activity of the treated bacterial suspensions and of untreated controls was then determined by the usual Warburg technique. *E. coli*, for example, consistently showed with glucose a Q_{O_2}: N of 1,000 to 1,200 μl.

The viability of the bacteria was determined by subculture of the same suspension used for the manometric determinations. A loopful of the suspension was streaked onto a Difco agar plate and incubated for 12 hours at 37 C; 0 indicated sterility, $\pm$, trace of growth, +, sparse growth, and ++, prolific growth. In other experiments with *E. coli*, plate counts were made (figures 1, 2). At the conclusion of the manometric run (15 minutes), a suitable dilution of the bacteria from the cups was made with sterile saline and inoculated into pour plates of Difco Endo agar. Counts recorded are the average of duplicates after 24 hours' incubation.

The aldolase-triosephosphoric dehydrogenase system of muscle was prepared by the method of Green, Needham, and Dewan (1937), the *d*-amino acid oxidase by the method of Warburg and Christian (1938), and the glutamic-aspartic transaminase by the method of Green, Leloir, and Nocito (1945). Aldolase from bacteria was prepared by grinding a thick suspension of *E. coli* in a Booth-Green mill, centrifuging off the cell debris, dialyzing against dilute $NaHCO_3$ overnight, and fractionating with ammonium sulfate. It was assayed by the method of Herbert, Gordon, Subrahmanyan, and Green (1940) with the addition of 0.001 M $MnSO_4$ to the reaction.

We wish to thank Dr. Gordon Fair for generous supplies of chlorine and iodine compounds.

DETERMINATION OF THE BACTERICIDAL AMOUNT OF CHLORINE

Only those effects of chlorine produced by concentrations equal to, or less than, the bactericidal concentration are germane to the problem of the mechanism of bactericidal action. Preliminary experiments to determine the bactericidal amount under our conditions revealed that the bactericidal effect of chlorine was not simply related to the concentration of chlorine, but depended largely upon the number of bacteria present.

As shown in table 1, more chlorine is required to kill more bacteria. The bactericidal concentration of chlorine therefore varies with the number of bacteria, but the ratio of chlorine to bacteria is remarkably constant. This is shown more precisely in figures 1 and 2. This dependence of bactericidal action upon the amount of chlorine for a given amount of bacteria proved to be experimentally useful, since comparable results could be obtained under widely different conditions. In particular, it provided a value for the bactericidal amount of chlorine which could be applied equally well to bacteria and to enzyme proteins.

The bactericidal range of chlorine amounts was determined more precisely by plate counts of the surviving bacteria. Figure 1 shows the percentage of death in bacterial populations with increasing amounts of chlorine per mg of bacterial N. The inhibition by chlorine of a certain enzyme function, if it is to be responsible for the bactericidal effect on the bacteria, must occur within this range of chlorine amounts shown in figure 1. Such bactericidal amounts of

chlorine are far below the amount causing cell lysis of *E. coli* or nonspecific protein denaturation.

EFFECT OF CHLORINE

In figure 1 is also given the percentage of inhibition of glucose oxidation with increasing amounts of chlorine per mg of bacterial N, determined in the same experiments as the bactericidal effect. Of the various segments of metabolic

TABLE 1

Variation of minimum effective bactericidal concentration of chlorine with concentration of Escherichia coli

	CONCENTRATION OF E. COLI (MG N/ML)			
	0.078		0.390	
Active chlorine concentration (μg/ml)	13	10	100	66
Growth (subculture)	0	+	0	+
μg Cl/mg N		130		170

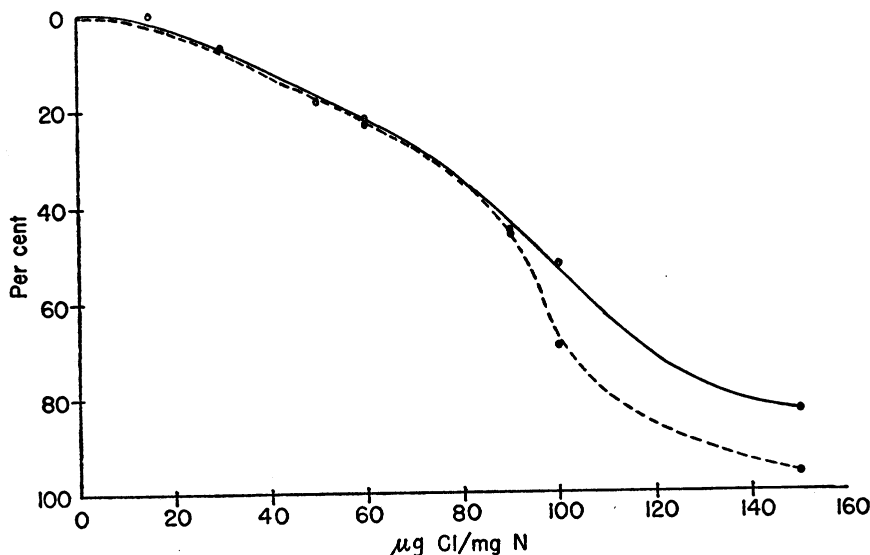

FIG. 1. THE PERCENTAGE OF E. COLI KILLED (●) AND INHIBITION OF GLUCOSE OXIDATION (O) BY CHLORINE

activity susceptible to measurement, glucose breakdown may be accepted as the reaction most generally essential to all cells. It can be seen that death of the cells parallels the inhibition of glucose oxidation, as would be expected if loss of such essential enzymes caused death. The separation of the curves at higher chlorine concentrations suggests that the relatively resistant cells of the population are those that oxidize glucose more rapidly. This inhibition of glucose oxidation equal to the killing effect has been found consistently in over a hundred experiments under different conditions and occurs with bacteria grown

on different media, aerobically and anaerobically, as well as with a variety of bacterial species.

In tables 2 and 3 are given values determined by subculture and manometric experiments showing that sterility of bacterial suspensions occurs only when chlorine has inhibited glucose oxidation fully. In table 2 the effects on glucose oxidation and killing are seen to be parallel for different times of exposure. The effect is complete in 10 minutes under our conditions. In table 3 is shown the

TABLE 2

Inhibition of glucose oxidation in Escherichia coli suspensions and viability after various times of exposure to chlorine

	ACTIVE CHLORINE (μG/Cl/MG N)					
	95			186		
Exposure time (minutes)	0.5	5	10	0.5	5	10
Glucose oxidation (per cent inhibition)	16	41	59	95	100	100
Growth (subculture)	++	++	++	+	0	0

TABLE 3

Effect of various chlorinating agents on oxidation of glucose and viability of bacteria

	COMPOUND					
	Halazone		Hypochlorite		Succinchlorimide	
Proteus vulgaris						
μg chlorine/mg N	214	106	286	143	214	106
Glucose oxidation (per cent inhibition)	100	91	100	92	100	75
Growth (subculture)	0	±	0	±	0	+
Serratia marcescens						
μg chlorine/mg N	214	106	286	100	160	80
Glucose oxidation (per cent inhibition)	100	50	100	40	100	20
Growth (subculture)	0	++	0	++	0	++
Aerobacter aerogenes						
μg chlorine/mg N	214	106	286	143	160	80
Glucose oxidation (per cent inhibition)	100	79	100	95	100	71
Growth (subculture)	0	++	0	±	0	++
Escherichia coli						
μg chlorine/mg N	237	117	286	188	188	34
Glucose oxidation (per cent inhibition)	100	77	100	83	100	52
Growth (subculture)	0	++	0	+	0	++

parallelism of inhibition of glucose oxidation and bacterial death for various chlorine compounds in several bacterial species.

This correlation of glucose oxidation inhibition with cell death is sufficiently general to suggest that chlorine acts by specifically inhibiting one or more of the enzymes of glucose oxidation. Since the integrity of this system is essential for viability, the cell dies. The possibility that the inhibition of glucose oxidation is a result of cell death, rather than a cause, is unlikely. Bacteria killed by

other means, such as acetone or ultrasonics, can still oxidize glucose. Further proof of this view is given in table 4. Iodoacetic acid, known to inhibit glucose breakdown specifically, likewise causes a parallel inhibition of glucose oxidation and cell viability.

CHLORINE AS AN —SH ENZYME INHIBITOR

Although chlorine has not previously been shown to act on —SH groups, its effect on several classical sulfhydryl enzymes shows it to be an exceptionally good inhibitor. The inhibition of papain (table 5), for example, is sufficiently

TABLE 4

Effect of iodoacetic acid on glucose oxidation and growth of Escherichia coli

(Growth determined after 10 hours' incubation of bacteria plus iodoacetate in 3 ml of meat infusion broth, pH 7. Glucose oxidation measured with bacterial suspension after 2 hours' exposure to iodoacetic acid in phosphate buffer, pH 7)

CONCENTRATION OF IODOACETIC ACID	GROWTH	PER CENT INHIBITION OF GLUCOSE OXIDATION
ppm		
1,200	0	100
600	0	100
120	+	54
60	++	0
0	+++	0

TABLE 5

Effect of chlorine concentrations of 1 to 10 ppm on inhibition of papain action (0.26 mg enzyme N/ml)

CONCENTRATION OF CHLORINE		$\frac{1}{\text{CLOTTING TIME}}$	INHIBITION
ppm	*μg/mg N*	*minutes*	*per cent*
0	0	1.5	0
0.7	2.7	1.1	27
1.4	5.4	0.86	43
4.2	16.2	0.43	71
8.4	32.3	0.24	85

great that it has been used for an extremely sensitive assay of free chlorine in water supplies (Green and Stumpf, 1946). In table 6 the effect of different chlorine compounds on the extremely sensitive sulfhydryl system, zymohexase-triosephosphate dehydrogenase from rabbit muscle, described by Herbert, Gordon, Subrahmanyan, and Green (1940), and on *d*-amino acid oxidase and transaminase, identified as sulfhydryl enzymes by Singer and Barron (1945), is compared with the inhibition of glucose oxidation in *E. coli*. Similarly, succinic oxidase is inhibited by low amounts of chlorine, but catalase, a nonsulfhydryl enzyme, is not affected.

Chlorine may, therefore, be classed with that group of sulfhydryl enzyme

inhibitors acting by oxidation. It has the property common in this group of producing an irreversible inhibition of the enzyme. All attempts to reverse the chlorine effect on enzymes or bacteria, after it was once established, by the addition of cysteine or glutathione were unsuccessful.

Consideration of the impressive array of —SH enzymes collected by Barron

TABLE 6

Amount of chlorine for complete inhibition of several animal sulfhydryl enzyme systems compared with glucose oxidation in Escherichia coli

(500 μg/ml of N in all experiments. Figures refer to active chlorine concentration in μg/mg N)

REAGENT	GLUCOSE OXIDATION OF E. COLI	ALDOLASE-TRIOSEPHOSPHATE DEHYDROGENASE	*d*-AMINO ACID OXIDASE	TRANSAMINASE
Hypochlorite	159	27	270	1,360
Halazone	127	22	—	—
Succinchlorimide	82	22	270	1,090

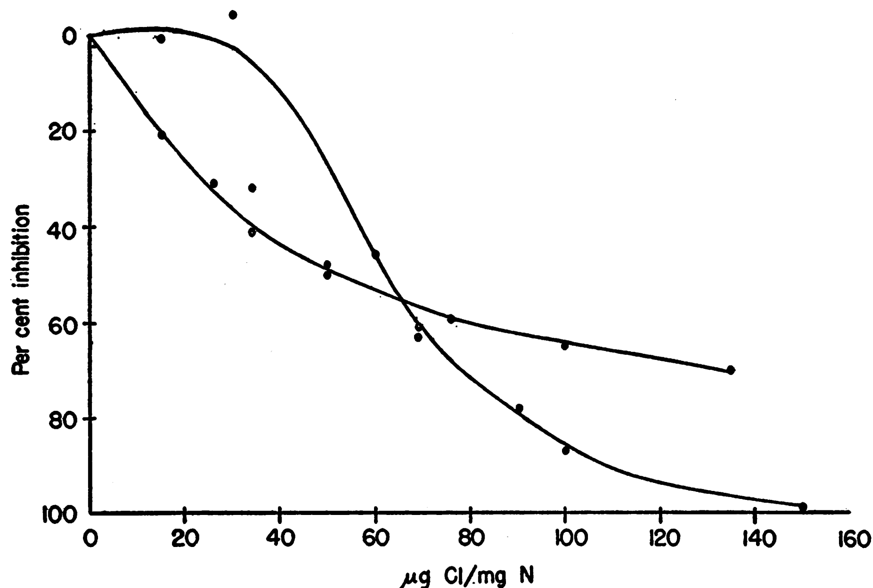

FIG. 2. THE PERCENTAGE OF INHIBITION OF E. COLI GLYCOLYSIS (●) AND OF THE ISOLATED ALDOLASE (○) BY CHLORINE

and Singer (1945) leaves no doubt that such an effective —SH reagent as chlorine could cause cell death by the interruption of essential metabolic systems, probably at several loci, and produce the observed effects on glucose oxidation and viability. It remained only to be demonstrated, therefore, that the inhibition of glucose oxidation by chlorine causing cell death could indeed be referred to a sufficiently sensitive enzyme in the bacteria.

IDENTIFICATION OF ALDOLASE AS A CHLORINE-SENSITIVE ENZYME IN E. COLI

Although it is quite probable that several enzymes may possess the characteristics sensitizing them to the action of chlorine, and that a multiple effect of chlorine occurs in the cell's metabolism, attempts were made to identify at least one such locus by studying different segments of the glucose-oxidizing system.

Figure 2 shows the inhibition by chlorine of glycolysis in *E. coli*. Although the full relationship of the glycolytic to oxidative cycles for *E. coli* has not been worked out, glycolysis occurs similarly in yeast and muscle (Utter and Werkman, 1941) and may be assumed to represent part of the glucose oxidation pathway. An enzyme of the glycolytic system in muscle, the aldolase-triosephosphoric dehydrogenase, is shown in table 6 to be easily sensitive enough to account for the bactericidal effect if a similar enzyme exists in the bacteria.

The aldolase enzyme of *E. coli* has been studied by Utter and Werkman (1941). They concluded from their study that the bacterial enzyme was identical with the analogous enzymes of muscle and yeast. This enzyme was prepared by us (see Methods) and found to be sensitive to the various oxidizing sulfhydryl reagents such as chlorine, ferricyanide, H_2O_2, and iodosobenzoic acid, but not alloxan. It was unaffected by the alkylating or mercaptide-forming sulfhydryl reagents. It differed from other aldolases in its specific requirement for Mn^{++} for full activity. In figure 2 the chlorine inhibition of this partially purified enzyme prepared from *E. coli* is presented along with the inhibition of glycolysis in the intact cells. It can be seen that the aldolase of *E. coli* is sensitive to chlorine in the same degree as glycolysis and glucose oxidation. The failure to obtain 100 per cent inhibition may be a property of the *in vitro* system, which is tested in the presence of cyanide. However, the effect on aldolase can only be considered representative of the effects on other —SH enzymes in the cell, and the sum of these effects on the various —SH enzymes would be expected to approximate the effect on glycolysis shown in figure 2.

DISCUSSION

In the determination of the mode of action of any germicide or chemotherapeutic agent on biological systems, the essential information is the ultimate action of the compound on a specific metabolic process. To this end we have neglected consideration of the chemical species of the active chlorine and of its permeation into the cell. We may assume that hypochlorous acid is the active form, and that penetration occurs as the un-ionized hypochlorous acid or chloramine (Marks, Wyss, and Strandskov, 1945). Similarly, the possibility that amino groups of various peptides are chlorinated in the cell has not been discussed. The specific effect on metabolism by oxidation of sulfhydryl groups of essential enzymes by the chlorine in itself suffices to explain the observed phenomena. Intracellular chlorination of nitrogen compounds, followed by dissociation of chloramines, would eventually produce the same oxidative result. Such combination when involving extracellular organic material would, of course,

increase the amount of chlorine needed for killing (chlorine demand), but this again is not relevant to the problem of mode of action (Chang and Fair, 1941).

The rationale of these experiments has subsequently been useful in elucidating the mode of action of iodine, organic mercurials, and cationic surface-active agents as germicides. It depends upon the determination of the amount of agent necessary to produce the biological effect, in this case the bactericidal effect. This effect must be exerted through some of the enzyme reactions producing the biological phenomenon, and must act on these enzymes in the same amount necessary to produce the effect in the intact cell. Identification of the enzymes sensitive to this amount of agent will show the locus of action, and will frequently give information as to the actual chemical mechanism of the effect on the enzymes by virtue of other known characteristics of these enzymes.

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SUMMARY

Chlorine in bactericidal amounts or less inhibits various sulfhydryl enzymes and other enzymes sensitive to oxidation. Inhibition of essential enzymes in this way causes death of the bacterial cell; inhibition of glucose oxidation is paralleled by the percentage of bacteria killed. The aldolase of *Escherichia coli* has been shown to be one of the essential enzymes of glucose oxidation sufficiently sensitive to chlorine to explain its bactericidal effect.

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