

Kinetics and Mechanism of Bacterial Disinfection by Chlorine Dioxide¹

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Survival data are presented for a fecal strain of *Escherichia coli* exposed to three concentrations of chlorine dioxide at four temperatures. Chick's first-order reaction equation is generalized to a pseudo n th-order model. Nonlinear least squares curve-fitting of the survival data to the n th order model was performed on an analogue computer. The data were observed to follow fractional order kinetics with respect to survival concentration, with an apparent activation energy of 12,000 cal/mole. Initial experiments support the thesis that the mechanism of chlorine dioxide kill occurs via disruption of protein synthesis.

Although Joseph Lister is generally considered the initiator of practical disinfection procedures, it was Kronig and Paul's classic paper (8) that first described a quantitative procedure for the study of disinfection. Ten years later, Madsen and Nyman (9), followed by Chick a year later (4), established a mathematical model for chemical disinfection of microbial populations. These two papers suggested the analogy between velocity of microbial disinfection and a unimolecular or first-order reaction that has remained the model for all subsequent investigations.

During the intervening 50 years, refinements in technique have been reported, and hypotheses as to the mechanism of microbial destruction have been postulated. In 1946, Green and Stumpf (5) proposed that chlorine brought about microbial death by inactivating the enzymes needed for oxidation of glucose. Knox et al. (7) suggested the enzyme triase-phosphate dehydrogenase as the prime target of chlorine disinfection. As yet, however, direct biological proof of any of the theories has been unavailable. As Wyss recently noted (10), "The field of disinfection is a leisurely one." The study described herein offers a modified mathematical treatment for chemical disinfection of bacterial populations, as a result of a unique and rapid sampling procedure, organic-free solutions, quantitative spectrophotometric determinations of low doses (fractions of a milligram per liter) of disinfectant, and chemical reactor theory and design.

Additionally, results of biological experiments that complement the mathematical interpretation of the rate curves point to the mechanism of the lethal lesion. The investigation described herein is a direct outgrowth of an earlier report by Benarde et al. (1) which reported substantial kill at orders of magnitude far less than the 30-min end point usually used in disinfection studies. This seemed to indicate that a primary target was affected by ClO_2 . This supposition led to the design of an apparatus that would permit determination of percentage survival after intervals measured in seconds. With proven effectiveness of this apparatus, an investigation of temperature effects on the efficiency of disinfection was undertaken. In this series, the following constants were used: pH , 6.5; cell density, 15,000 per milliliter; and organic-free menstruum. Variables were ClO_2 concentration, temperature, and time of contact.

MATERIALS AND METHODS

The procedure for the preparation of organic-free distilled water (OFD) and glassware, generation of aqueous solutions of pure ClO_2 , stopping the disinfection reaction, washing bacterial cells, and standardizing their density was continued as previously reported (3). To obtain samples at intervals as short as 5 sec, the reaction assembly required considerable modification. The original apparatus consisted of two hypodermic syringes, each with a two-way flap valve assembly, permitting rapid drawing and dispensing of samples from the reaction vessel. It could not be operated fast enough to remove samples at 5-sec intervals. To achieve such rapid sampling, a closed pressurized system was designed to operate within a constant-temperature water bath. Figure 1 is a com-

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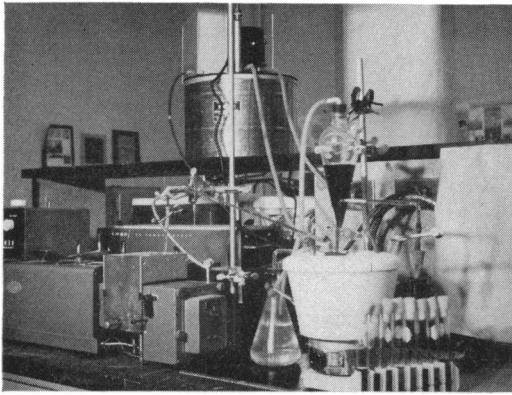


FIG. 1. Complete view of the reaction assembly.

plete view of the apparatus. The water bath containing the reaction vessel and the spectrophotometer that was operated in conjunction with the sampling apparatus are shown. Figure 2 shows the entire assembly ready for use. Figure 3 is a schematic representation of all the essential components of the system. Pressure on the system was generated by adding tap water to a filter flask in a closed system, and was used (i) to force the ClO_2 solution rapidly into the reaction vessel containing the bacterial suspension and (ii) to force samples from the reaction vessel into the spectrophotometer cuvette and into the bacterial sample tubes. Mixing of the bacterial suspension and the ClO_2 solution was rapid and thorough because of the pressure under which the latter was forced into the reaction vessel. Individual sterile units of reaction vessel and separatory funnel were used for each experimental run. The bacterial samples were collected in sterile test tubes containing sterile sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) solution. Bacterial sampling occurred after 5, 10, 15, 20, 25, 30, 60, and 300 sec of contact. The spectrophotometer sample was drawn 3 sec after initial mixing. Changes in ClO_2 concentration were recorded through the 5-min test period.

All glassware, buffered dilution OFD, and ClO_2 solutions were kept at the temperature of the experimental run before and up to the moment of use. The temperature of these materials and of the reaction vessel was maintained within ± 0.1 C. Each experimental run was replicated three times at the following ClO_2 concentrations: 0.25, 0.50, and 0.75 mg/liter. The initial concentration of these ClO_2 solutions was made up in double strength to compensate for the 1:1 dilution obtained by mixing equal volumes of cell suspension and ClO_2 solution. The strength of each solution was verified spectrophotometrically just before the start of each experimental run.

RESULTS

Figure 4 is a plot of survival with time and temperature, when 0.75 mg/liter of ClO_2 was used. It is apparent that the disinfectant efficiency

varies directly with temperature. A similar relationship was observed at 0.50 and 0.25 mg/liter (Fig. 5 and 6). To obtain 99% kill, a concentration of 0.25 mg/liter required 110 sec at 5 C, 74 sec at 10 C, 41 sec at 20 C, and 16 sec at 30 C—almost four times as rapid at 30 C as at 5 C. At the 99.9% level, the time required for disinfection between 5 and 30 C increased to five times as much (21 sec at 30 C versus 150 sec at 5 C).

In the same Q_{10} range, 20 to 30 C, the calculated Q_{10} values (based on the 30% kill level) were 1.67 for 0.5 mg/liter and 1.23 for 0.75 mg/liter. In the 10 to 20 C range, the Q_{10} values were 1.06 for both 0.50 and 0.75 mg/liter. The Q_{10} values varied with dosage and temperature; consequently, we must be cautious in using Q_{10} as an index of relative reaction rates. Increasing the concentration of ClO_2 tended to decrease markedly the time needed for kill. At the 99% level, 0.75 mg/liter required only 14 sec at 30 C versus 60 sec at 5 C. It is also apparent that these plots do not yield straight lines, indicating deviations from simple first-order reactions. Therefore, to ascertain the best reaction order, a more sophisticated treatment is warranted.

From these data, it would be helpful to develop a mathematical model that bioengineers would be able to use in the design of disinfection equipment. In these models, concern is always for the quantification of these nonlinear kinetic parameters. Toward this end, it would be desirable to rely on the classical approaches that correlate complex chemical reaction rate data with temperature and concentration. Chick (4) suggested this type of calculation, which was followed for the past 60 years. However, during the past 10 years, advances in kinetic models have been developed.

Kinetic models. The overall rate of *Escherichia*

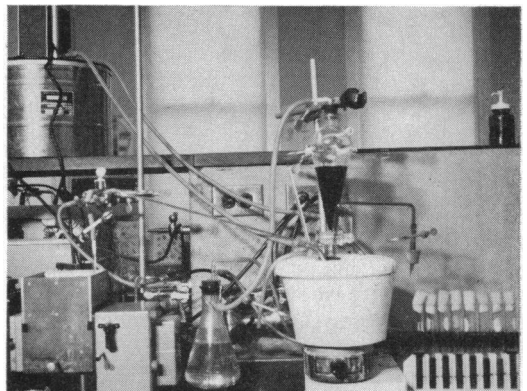


FIG. 2. Reaction assembly ready for use.

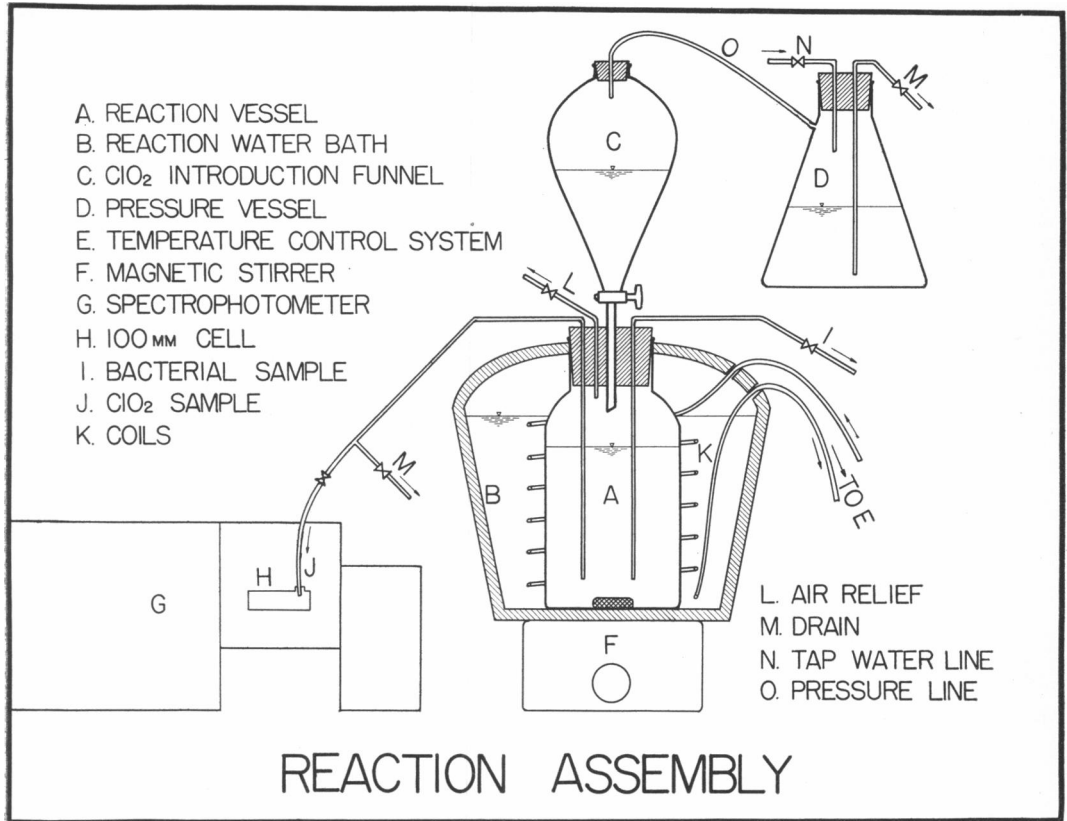


FIG. 3. Schematic representation of the essential components of the reaction assembly.

coli kill with chlorine dioxide is influenced by at least three basic factors, namely, (i) mass transfer of ClO₂ in the liquid to the *E. coli* liquid interface, (ii) chemisorption of the disinfectant at selective active centers on the cell surface, and (iii) surface and intrasurface diffusion of the activated chemisorbed complex with attendant chemical attack on cellular elements. Elucidation of disinfection mechanisms from kinetic data is considerably encumbered by the possibility that several steps may be rate-controlling. It is important, therefore, before kinetic modeling is employed, to determine whether a physical step, such as mass transfer or intrasurface diffusion, or a chemical step, such as chemical attack on cellular elements, is rate-controlling; otherwise, curve-fitting would become a meaningless task.

Since most chemical rate processes, as opposed to purely physical mass transfer or diffusion processes, are strongly temperature-sensitive, determination of rate-controlling steps can be facilitated by observing the effect of increased temperature on the overall rate of kill. In this study, a strong temperature effect was observed. This sug-

gests, as an initial hypothesis, that chemical resistance controls the overall rate of kill. Furthermore, adequate stirring of the reactive mixture eliminated mass transfer in the liquid phase as the rate-controlling step. These phenomenological facts and the hypothesis of a chemical rate-controlling mechanism serve as a convenient point of departure for meaningful kinetic modeling.

If it is assumed that the law of mass action applies to biochemical reactions in the same way it applies to homogeneous liquid-phase reactions, then for a pseudo-unimolecular model the kinetic expression becomes

$$-\frac{1}{V} \frac{dVc}{dt} = kc \quad (1)$$

For the *E. coli* system studied, equation 1 states that the rate of disappearance of *E. coli* cells per unit volume per unit time in the reactor is proportional to the concentration (active mass) of cells present at any time in the system, the system here being the liquid mixture in the reactor at any time. If the volume of the system is nearly constant, which would be the case for batch reactors

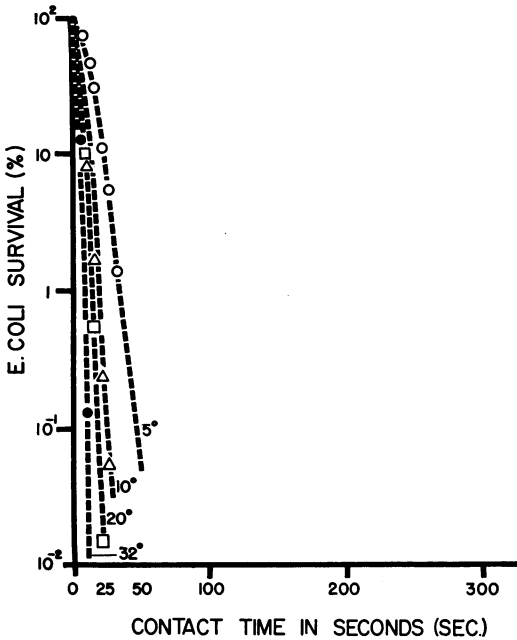


FIG. 4. Effect of temperature on disinfection with 0.75 mg of chlorine dioxide per liter (pH 6.5; 15,000 cells per milliliter).

with no sample removal, equation 1 becomes

$$-\frac{dc}{dt} = kc \tag{2}$$

which is the classical "Chick's Law" expression for disinfection. If the order is other than simple first-order, then equation 2 must be modified accordingly; for example,

$$-\frac{dc}{dt} = kc^n \tag{3}$$

where n = any real number.

Now consider the general case where continuous removal of a sample from the system causes the volume to change according to a volume decrease law, $V = V(t)$. Equation 3 would become

$$-\frac{dVc}{dt} = kc^n V - \frac{dV}{dt} c \tag{4}$$

which reduces to

$$-\frac{dc}{dt} k(c)^n \tag{5}$$

Apparently, the case where volume was constant is identical to the case where volume was variable, providing that concentration variables are used throughout.

According to the "Chickian" philosophy, it is customary to analogue equation 3 as follows:

$$-\frac{dN}{dt} = k'N^n \tag{6}$$

where N = number, not concentration, of surviving cells in system. Proceeding as before for the variable volume case, equation 6 becomes

$$-\frac{dN}{dt} = k'(N)^n - \frac{d \ln V}{dt} \cdot N \tag{7}$$

Equation 7 in no way resembles equation 5, and furthermore k values from the two equations are fundamentally different because of the units and mathematical form of the differential equations. Even if volume were constant, equations 5 and 7 are still different and define different k values. The relationship between the two k parameters for variable volume systems is

$$k' = (N)^{1-n} \frac{d \ln V}{dt} + k(V)^{1-n} \tag{8}$$

It is indeed puzzling why Chick (4), for example, insisted on using N , the number of living cells in the system, in equation 1 as the active mass. Even more puzzling is the fact that many unknowing experimenters, after Chick (4), apparently used c in place of N when calculating k . The conclusion

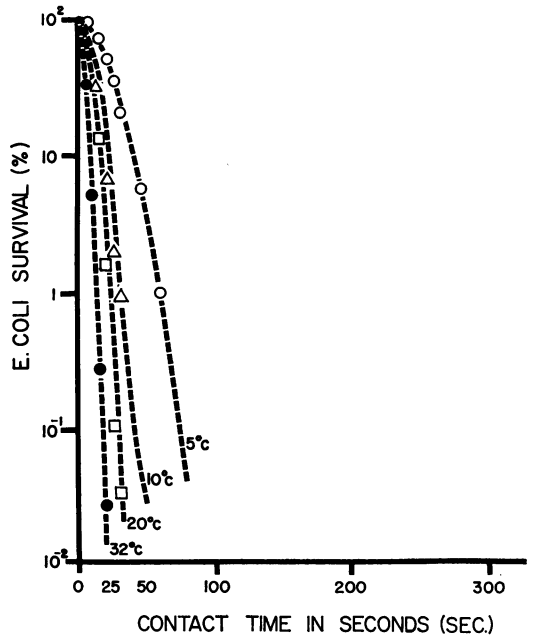


FIG. 5. Effect of temperature on disinfection with 0.50 mg of chlorine dioxide per liter (pH 6.5; 15,000 cells per milliliter).

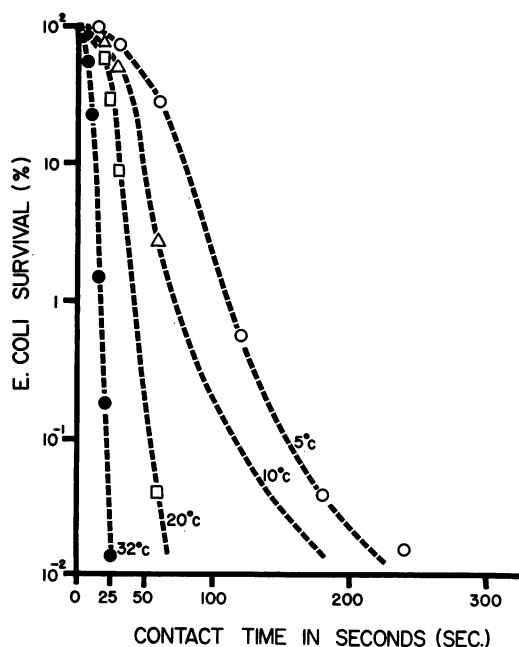


FIG. 6. Effect of temperature on disinfection with 0.25 mg of chlorine dioxide per liter (pH 6.5; 15,000 cells per milliliter).

to be drawn from these observations is that equation 5 represents the least confusing analogue model of the chemical law of mass action. Formulations based on N as opposed to c are not prudent, because $V(t)$ has to be known and taken into account mathematically.

The emphasis in this paper is not on curve-fitting or goodness of fit, but rather on the criteria for developing a rate expression. Equation 3 is one form of the classical law of mass action, whereas equation 13 does not have any such foundation, and is therefore on more tenuous ground than is equation 3.

In curve-fitting, one can always uncover many mathematical rate expressions with equal goodness of fit. Equation 5 should be viewed only as a starting point from which future theorizing can depart.

Parameter estimation. Bear in mind that we are fully aware that our experimental data may not warrant sophisticated statistical analysis, curve-fitting techniques, and detailed kinetic modelling and mechanism elucidation. We are concerned primarily with showing a nonlinear method that can be used with this type of data.

For fixed values of temperature and initial chlorine dioxide concentration, the unknown parameters in equation 5, k and n , can be estimated from a "least squares error" analysis. The

criterion, based on a linearized form, is

$$\min \sum_i^m \left[\ln \left(-\frac{d\bar{c}}{dt} \right)_i - \ln k'' - n \ln \bar{c}_i \right]^2 \quad (9)$$

where $k'' = kc_0^{n-1}$, c_0 = initial cell concentration, and $\bar{c} = \frac{c}{c_0}$. The corresponding "normal equations" are

$$m \cdot \ln k'' + \sum_i^m \ln \bar{c}_i \cdot n = \sum_i^m \ln \left(-\frac{d\bar{c}}{dt} \right)_i \quad (10)$$

and

$$\begin{aligned} \sum_i^m \ln(\bar{c}_i) \cdot \ln k'' + \sum_i^m (\ln \bar{c}_i)^2 \cdot n \\ = \sum_i^m \ln \bar{c}_i \cdot \ln \left(-\frac{d\bar{c}}{dt} \right)_i \end{aligned} \quad (11)$$

Parameter estimation from equations 10 and 11 serves to indicate a starting grid-point from which nonlinear search on an analogue computer can be initiated. Of course, k'' and n could be estimated from a nonlinear "least squares" analysis on a digital computer, but this procedure would require use of nonlinear search techniques, which are, in fact, equivalent in "art" form to the two-step parameter estimation procedure outlined above for the analogue computer.

The scaled analogue computer diagram for equation 5 with $c = \bar{c}c_0$ and $k'' = kc_0^{n-1}$ is given by Fig. 7, and the results are summarized in Table 1 and in Fig. 8 and 9. The goodness-of-fit criterion in Fig. 8 and 9 is based on the minimum-squared error between analogue curve and experimental points. The results indicate that reaction order with respect to time of contact varies with both temperature and ClO_2 concentration. Furthermore, numerical results from the linearized least-squares analysis did not agree well at all with the analogue computer values except at 32 C. The values of n were kept constant at the values computed from 10 and 11 because it was observed that curve-fitting errors were more sensitive to k'' than they were to n . This also provided a basis for comparing k values from two different methods of analysis. Since n was found to be relatively unimportant, an Arrhenius activation energy plot was attempted with the assumption that variance in order of reaction did not invalidate the estimation of the activation energy. The results from this analysis showed that the activation energy was 12,000 cal/mole, independent of ClO_2 concentration.

The kinetic model, equation 5 with parameter values from Table 1, points the way toward future research in the area of ClO_2 disinfection. First of

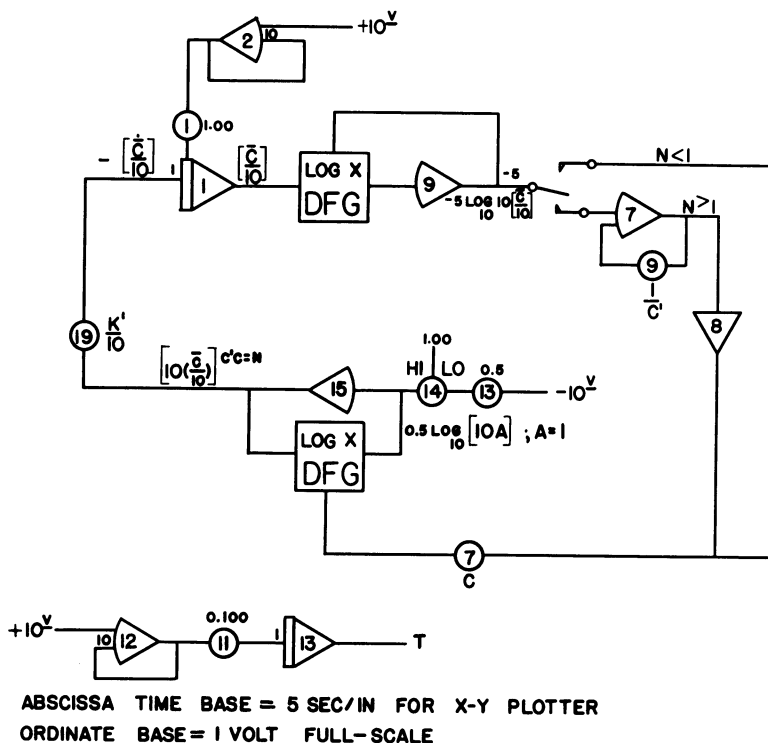


FIG. 7. Scaled analogue computer diagram for equation 5.

all, it was assumed that ClO_2 was the excess reactive component whose concentration change with time of kill did not vary as significantly as the *E. coli* concentration. This condition led to the pseudo n th order formulation of equation 5.

TABLE 1. Summary of curve-fitting from equations 10 and 11 and figs. 8 and 9

ClO_2 concn ^a	Temp	n from equations 10 and 11	k^n from equations 10 and 11	k^n from Fig. 8 and 9 with n from equations 10 and 11
0.5	C			
	32	0.634	0.160	0.118
	20	0.394	0.074	0.048
	10	0.538	0.074	0.028
	5	0.522	0.039	0.013
0.75	32	0.689	0.179	0.175
	20	0.773	0.171	0.078
	10	0.858	0.195	0.039 ^b
	5	0.614	0.071	0.046

^a Data at 0.25 mg/liter were not included, as it did not appear to follow the power law rate expression.

^b Questionable data point not following temperature trend.

Future research should aim at elucidating the effects of including ClO_2 concentration, in the kinetic model, on curve-fitting the data. Furthermore, the order of reaction with respect to initial concentration of *E. coli* cells should be determined to ascertain whether reaction intermediates inhibit

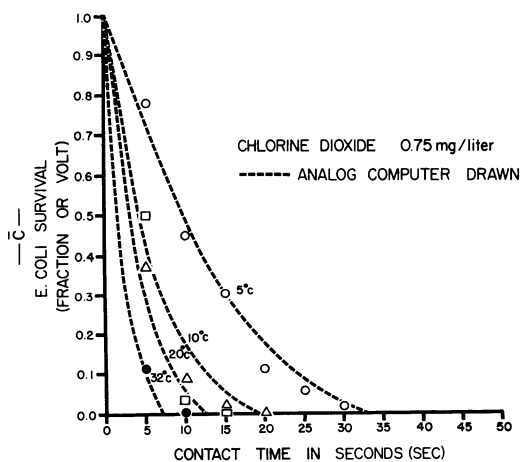


FIG. 8. Effect of temperature on disinfection with 0.75 mg of chlorine dioxide per liter.

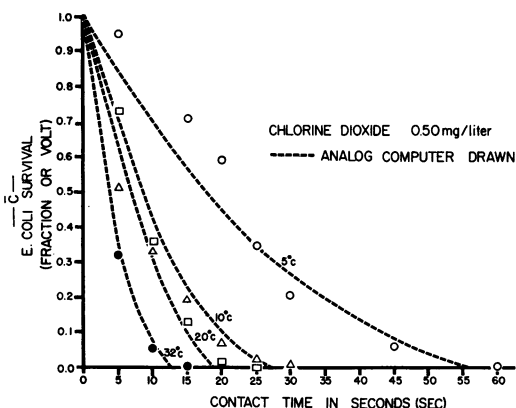


FIG. 9. Effect of temperature on disinfection with 0.50 mg of chlorine dioxide per liter.

the rate of kill or whether an autocatalytic mechanism prevails. The strong temperature effect observed in this study has already pointed out the chemical nature of the rate of kill, as opposed to purely physical controlling steps. An activation energy of 12,000 cal/mole is certainly reasonable if the assumption of variable order of reaction does not affect E . The value of the activation energy reported herein is more reflective of the true activation energy than those commonly reported based on either of the following kinetic equations:

$$-\frac{dN}{dt} = kNt \quad (12)$$

or

$$-\frac{dc}{dt} = kct \quad (13)$$

Biological studies of mechanism. The development of the kinetic model was predicated on an assumption that pseudo n th order kinetics described the rate of reaction and its temperature dependence. This model attempts to explain the overall mechanism. The individual steps, however, which comprise the overall mechanism remain to be elucidated. For this reason, attempts were made to discover the biological pathway taken by ClO_2 that might explain the rate-controlling step.

The velocity of disinfection, shorter by far than previously reported values, and the strong oxidizing character of ClO_2 suggested that chemical disruption of the bacterial cell wall was the likely mechanism. If the cell wall was severely breached, protein and nucleic acid should be released into the menstroom in sufficient quantity to yield characteristic peaks in the 260- and 280- $m\mu$ region

on spectrophotometric analysis. To ascertain the fact of protein and nucleic acid peaks, untreated washed cells were disrupted mechanically in a sonic oscillator. The response was sufficiently large to force the pen off the chart. Upon a 1:10 dilution, a peak was traced at 260 and 280 $m\mu$. At this point, ClO_2 was added to the solution. On further analysis, no changes in tracings occurred, indicating no demonstrable reaction between ClO_2 , protein, or nucleic acid.

A large harvest of cells was suspended in OFD-phosphate buffer (pH 6.5). These cells were centrifuged and washed three times. The washed cells were treated with ClO_2 for 5 min. They were then centrifuged into a pellet, leaving a clear supernatant liquid. A sample of the supernatant solution was placed in a 10-cm cuvette for analysis. The added length of the light path increased sensitivity 10-fold. The absence of absorbance peaks in the 260- and 280- $m\mu$ region indicated that protein and nucleic acid had not been released from the cells. It was concluded that ClO_2 did not produce leakage of cell contents and apparently did not oxidize proteinaceous material to other products. At about this time, we learned of Bringmann's work, reported in 1953 (3), which showed via electron photomicrographs that no visible changes in cell wall integrity occurred to chlorine-treated cells.

Our disinfection curves were then shown to Robert J. Kuchler, Department of Bacteriology, Rutgers University. He suggested that destruction in 5 to 10 sec was about the same time required for protein synthesis, and that the lethal lesion in that interval might well be due to a hit on a primary target, such as protein synthesis. Haggis (6) noted that synthesis of a single hemoglobin molecule required 5 to 10 sec.

To test the hypothesis that protein synthesis might be involved, ^{14}C -labeled phenylalanine was added to Braun's (2) minimal essential medium (MEM) for *E. coli*, containing approximately 10^9 cells, in an ice-water bath. This was immediately placed in a 37 C water bath and allowed to incubate. At intervals, samples were withdrawn and added to hot trichloroacetic acid to precipitate protein. Chlorine dioxide was then added to the system, and samples were again withdrawn at intervals to trichloroacetic acid. Counts of the ^{14}C -containing protein showed wide differences in protein synthesis between ClO_2 - and MEM-treated cells.

To establish firmly this observation, a second trial was carried out. This time, ^{14}C -labeled valine was added to cells in an ice-water bath. To obtain meaningful data on the rate of protein synthesis, with and without ClO_2 treatment, an apparatus was designed that permitted withdrawal of sam-

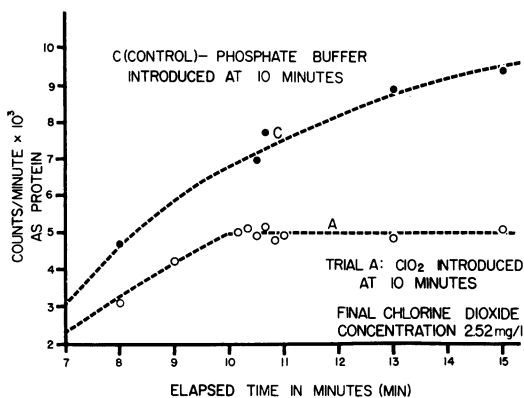


FIG. 10. Effect of chlorine dioxide on protein synthesis.

ples at 10-sec intervals. From the results of this experiment (Fig. 10), it is clear that ClO_2 markedly inhibits protein synthesis, and apparently does so abruptly. A lag phase is not seen. This indicates the lethal lesion to be directly related to protein synthesis, rather than inactivation of an enzyme system in the catabolism of glucose, wherein protein, sufficient to produce a lag phase, would be expected to be synthesized.

Several possibilities present themselves, for example, inhibition of formation of amino acyl adenylate, or inhibition of amino acid activation. These two steps might properly be considered as one possibility, and would be represented as follows: amino acid + adenosine triphosphate → adenosine monophosphate-amino acid, and soluble ribonucleic acid (sRNA) + amino acid (20) → sRNA-amino acid. A second possibility could occur at the ribosome level, represented by the expression sRNA · amino acid + ribosomes $\xrightarrow[\text{enzymes}]{\text{transferase}}$ specific protein. A third possibility may be inactivation of messenger RNA, preventing translation of coded information. A fourth explanation that suggests itself is a structural rather than a functional one: the destruction of the ribosome by ClO_2 . Consideration of Fig. 10, however, tends to rule this out. Destruction of ribosomes should yield a loss in counts as cell contents leak into the surrounding menstroom. Thus, inactivation of a functional process appears most likely.

The key experiment that must now be undertaken to ascertain conclusive direct proof of the site of the lethal lesion requires a cell-free system employing ribosomes obtained from disrupted homogenates of *E. coli*. This experiment is now in progress, and the results will be reported at a later date.

A step in the direction of determining the effect of ClO_2 on cell constituents that are involved in protein synthesis was taken and can be reported at this time. Each of six amino acids (histidine, asparagine, phenylalanine, arginine, proline, and leucine) was treated with ClO_2 for a period of 30 min. Samples were removed and spotted on sheets (8 by 8 inches) of Whatman no. 1 filter paper. The chromatogram was developed in an Eastman sandwich chamber with butanol-acetic acid-water (4:1:5). Amino acids were detected with 0.5% ninhydrin in butanol. An untreated sample of each amino acid was placed on the filter paper alongside the treated samples. From the observed spots of both treated and untreated amino acids, it was evident that size, color, and intensity were quite similar. In addition, R_F values appeared equal. A second set of amino acids was treated with ClO_2 , and samples were removed every 30 sec for 5 min. Because ClO_2 is volatile, none remained on the paper after the spot dried. Again, the R_F values, size, intensity and spot color did not differ with time. From this set of experiments it was concluded that ClO_2 apparently did not react with the amino acids sufficiently to alter their characteristic structures. Consequently, we would not anticipate a ClO_2 -free amino acid reaction within the cell, eliminating this as a possible reason for depression of protein synthesis.

It is our belief that the ability to obtain samples at 5-sec intervals, combined with the organic-free menstroom, enabled us to observe the particularly rapid bacterial destruction, which prompted the series of experiments that pinpointed the mechanism of ClO_2 disinfection. We believe this is the first time this has been demonstrated, and hope that other investigators will undertake studies in the important area of disinfection research.

ACKNOWLEDGMENTS

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