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CHLORINE DIOXIDE REACTIVITY WITH PROTEINS

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Abstract—Studies were conducted to evaluate chlorine dioxide reactivity with proteins and the role of these reactions in the inactivation of the bacterial virus f2 with chlorine dioxide. The effect of chlorine dioxide on the ability of f2 to specifically attach to its host *Escherichia coli* K13 was compared to the inactivation of virus during the initial seconds of contact time. At pH 7.2 and and 5°C, the virus was rapidly inactivated with 0.6 mg l^{-1} of chlorine dioxide. Approximately 2 log units of inactivation were observed within 30 s. The loss of protein specific attachment function nearly paralleled intact virus inactivation with 1.2 log units of attachment inhibition occurring within 30 s. The inactivation of virus and the inhibition of specific attachment both increased with increasing pH and increasing disinfectant concentration.

Inactivation of f2 was hypothesized to occur as the result of chlorine dioxide reacting with discrete chemical moieties in the viral protein. Cysteine, tyrosine and tryptophan reacted with chlorine dioxide within a time frame that could affect viral inactivation. In denatured f2 capsid protein monomers, these amino acids were almost totally degraded within 2 min by chlorine dioxide. Only tyrosine reacted with chlorine dioxide following treatment of the intact virion with disinfectant. Even though the degradation of tyrosine residues occurred at a much slower rate than the rate of virus inactivation, the protein component of f2 virus appeared to be the site of the lethal lesion produced by chlorine dioxide. These tyrosine reactions with chlorine dioxide appeared to alter the virus such that specific attachment was inhibited.

Key words—chlorine dioxide, f2 bacterial virus, disinfection, inactivation, reactivity, proteins

INTRODUCTION

The decline in the incidence of waterborne diseases during the 20th century has been attributed in part to the practice of water chlorination. However, in recent years chlorination has been implicated in the production of low concentrations of chlorinated organic pollutants. These pollutants, generally grouped as trihalomethanes, are believed to pose a public health hazard. In the search for safe alternative disinfectants, considerable interest has focused recently on chlorine dioxide. Chlorine dioxide does not produce trihalomethanes, and has demonstrated viricidal (Cronier, 1977) and bactericidal (Ridenour and Ingols, 1947; Ridernour and Armbrewster, 1949; Benarde *et al.*, 1965) properties similar in magnitude to those of chlorine.

Few investigations have attempted to determine the mechanism of virus inactivation by chlorine dioxide. Alvarez and O'Brien (1982) proposed that the primary target for the inactivation of poliovirus with chlorine dioxide was the viral genome. However, recent studies with f2 bacteriophage (Hauchman *et al.*, 1986), $\phi \times 174$ bacteriophage and poliovirus 1 (Hauchman, 1983) indicated that the nucleic acid remains infectious after treatment of these viruses with chlorine dioxide. Thermodynamic analysis of poliovirus inactivation by Brigano *et al.* (1979) suggested that the disruption of virus protein was the fundamental reaction responsible for poliovirus inactivation.

Although the reactivity of chlorine dioxide with selected amino acids has been documented (Schmidt and Braunsdorf, 1922; Schirle, 1953), little is known about the contribution of the reactions to the inactivation of viruses. The objective of this study was to provide information of the biological function and chemical reactivity of the protein components of f2 virus during disinfection with chlorine dioxide.

METHODS

Procedures for the preparation and measurement of chlorine dioxide solutions, and for the preparation and assay of f2 virus, are described elsewhere (Hauchman *et al.*, 1986).

Labelling of f2 virus

The proteins of f2 were labelled with [^{3}H]leucine. Host bacteria *Escherichia coli* K13 were first passed 6 times on MPTA medium (Vinuela *et al.*, 1967) deficient in leucine. A 200 ml volume of fresh medium was inoculated with *E. coli* from the last passage, and f2 virus was added at a multiplicity of 10 when the culture absorbance reached 0.6 (at 525 nm). Eight minutes after addition of f2 virus, 5 mCi of [^{3}H]leucine were added and incubation was continued until lysis occurred. Bacterial debris were removed by centrifugation. The virus preparation was concentrated and purified by isopycnic centrifugation in cesium chloride, followed by

dialysis against 0.01 M phosphate buffer. Approximately 1% of the label was incorporated into f2 protein.

The RNA of f2 virus was labelled with ^{32}P as previously described (Hauchman *et al.*, 1986).

Attachment assay

Attachment studies were performed by the method of Brinton and Beer (1967) using ^{32}P labelled virus. *E. coli* K13 were mixed with control or chlorine dioxide treated f2 virus for 15 min at 4°C with slight agitation. The suspensions were then passed through a 0.45 μm HA Millipore filter pre-coated with bovine serum albumin. Filters were dried and immersed in a toluene based scintillation cocktail and counted. Nonspecific attachment was evaluated by mixing duplicate virus samples with non-host *E. coli* C, and following the same procedures used for assay of specific attachment.

SDS–acrylamide gel electrophoresis

Viral proteins were subjected to electrophoretic analysis in sodium dodecyl sulfate (SDS) polyacrylamide gels based on the discontinuous gel system of Laemmli (1970). The resolving gel consisted of 10% acrylamide, 0.26% bis-acrylamide, 0.375 M Tris–HCl (pH 8.8), 0.1% SDS and 0.75% ammonium persulfate. Immediately after adding 15 μl of tetramethylethylenediamine (TEMED), the vertical slab gel was prepared, and allowed to cast overnight. The stacking gel mixture consisted of 3.75% acrylamide, 0.1% bis-acrylamide, 0.125 M Tris–HCl (pH 6.8), 0.1% SDS and 0.075% ammonium persulfate. The stacking gel was poured following polymerization of the resolving gel.

Virus protein labelled with [^{3}H]leucine was prepared for electrophoresis by diluting virus samples 1:5 in buffer consisting of 0.0625 M Tris–HCl (pH 6.8), 12.5% glycerol, 1.25% SDS, 0.125% 2-mercaptoethanol and 0.00125% bromphenol blue. A constant current of 20 mA was applied through the stacking gel and 30 mA through the resolving gel. Electrophoresis was carried out in circulated reservoir buffer containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS at pH 8.3. At the termination of the run, the gel was sectioned and slices were assayed for radioactivity by scintillation spectrometry. Low molecular weight standards included in each gel were stained overnight in Coomassie blue R250 (Weber *et al.*, 1972).

Purification and hydrolysis of viral protein

Glacial acetic acid was added to f2 virus suspensions to a final concentration of 66% (Fraenkel-Conrat, 1957). After 1 h in an ice bath, the RNA precipitate was removed by centrifugation. The supernatant containing f2 capsid protein was either freeze-dried or dialyzed against 3 mM acetic acid to remove excess acetic acid. Freeze dried f2 coat protein was hydrolyzed with redistilled hydrochloric acid under nitrogen gas for 24 h at 110°C. The digestion was stopped by freeze drying the contents of the ampules.

Since hydrochloric acid hydrolysis destroys tryptophan, duplicate coat protein samples were hydrolyzed using methanesulfonic acid (Fishbein *et al.*, 1980). Viral protein was added to 4.0 N methanesulfonic acid and digested at 115°C for 24 h. The digestion was halted by addition of sodium hydroxide.

Amino acid analysis of protein hydrolysates

Amino acids from hydrochloric acid protein hydrolysates were eluted with 0.2 N sodium citrate at pH 3.25 for 20 min, 0.2 N sodium citrate at pH 4.25 for 12 min and 1.0 N sodium citrate at pH 7.40 for 35 min. Amino acids from methanesulfonic acid protein hydrolysates were eluted with 0.2 N sodium citrate at pH 3.25 for 20 min, 0.2 N sodium citrate at pH 4.25 for 12 min, and 0.2 N sodium citrate at pH 5.88 for 58 min. Four nanomole amino acid standards were chromatographed with each set of unknowns by ion exchange chromatography. Unknown concentrations ranged from 1 to 10 nmol of individual amino acids depending upon the quantity of sample applied.

Measurement of sulfhydryl groups

Sulfhydryl groups were measured with ^{302}Hg labelled *p*-chloromercuribenzoic acid (PCMB). The procedure of Erwin and Pedersen (1968) was modified to facilitate the separation of reacted from unreacted PCMB. Chlorine dioxide treated and untreated virus and denatured f2 coat protein were mixed with SDS to a final concentration of 0.25% at pH 7.5. The mixture was then heated to 60°C for 15 min. Ten μl of ^{203}Hg labelled PCMB solution were mixed with 2 ml of SDS treated protein, and allowed to react for an additional 15 min. The reaction was stopped by placing the virus/PCMB mixture on a 1 × 12 cm Sephadex G25 column. The eluant consisted of 0.01 M phosphate buffer at pH 7.0 with a flow rate of 3 ml min^{-1}. The column retention time for viral proteins was measured using [^{3}H]leucine labelled f2 coat protein prior to studies involving ^{203}Hg labelled PCMB. The collected fractions were counted on a Packard Auto-Gamma Spectrometer.

Amino acid reactivity

Half-lives for amino acids which reacted with chlorine dioxide were determined spectrophotometrically using a Durrum Model 110 Stopped-Flow Spectrophotometer. Consumption of chlorine dioxide by f2 virus and viral proteins was measured in a similar manner.

Reaction system

All glassware was presoaked in chlorine dioxide solution to avoid extraneous oxidant demand. Experiments were performed in 0.01 M acetate, phosphate or borate buffers. Reagents and buffers were prepared in triple distilled water which demonstrated negligible chlorine dioxide demand.

Inactivation experiments were performed in a trypsinizing flask to increase turbulent mixing. Chlorine dioxide containing solutions were quenched with 0.1 ml of 10% sodium thiosulfate in 0.5 M phosphate buffer (pH 7.0).

RESULTS

The ability of f2 virus to attach to host cells after treatment with 0.6 mg l^{-1} of chlorine dioxide is shown in Fig. 1. At pH 5.0, greater than 1 log unit of viral inactivation was observed within 120 s and attachment function showed a decrease of 0.5 log units. At pH 7.2, nearly 2 log units of viral inactivation was accompanied by a decrease in attachment function of 1.2 log units after 30 s of contact time. At pH 8.7, greater than 3 log units of viral inactivation and loss of attachment function were observed within 15 s of exposure to chlorine dioxide.

Figure 2 demonstrates the consumption of chlorine dioxide by intact f2 virus (left panel) and capsid protein (right panel). Virus and capsid protein were separately mixed with chlorine dioxide in a stopped-flow spectrophotometer which monitored and recorded adsorption at 357 nm, indicative of chlorine dioxide concentration. The intact virus suspension reacted with chlorine dioxide at pH 8.9 but consumed little chlorine dioxide at pH 4.8 or 6.9. Denatured virus capsid proteins extracted from virus suspensions demonstrated similar patterns of chlorine dioxide consumption. These data suggested that chlorine dioxide was consumed by reactions associated with the viral protein, irrespective of the protein conformation.

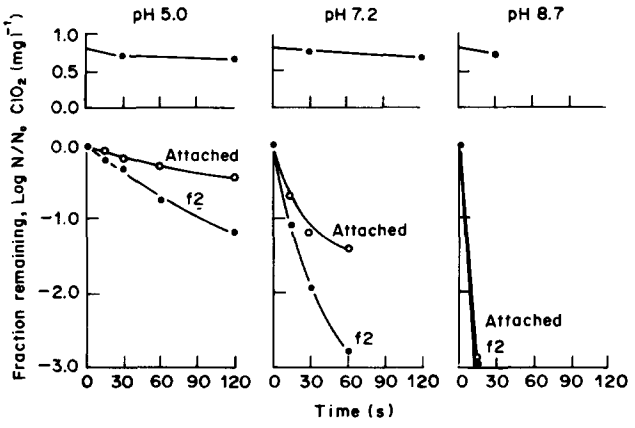

Fig. 1. Inactivation of f2 bacterial virus compared to loss of specific attachment of treated virus. Experiments were performed using 0.6 mg l^{-1} of chlorine dioxide at pH values of 5.0, 7.2 and 8.7 at 5°C.

To determine the extent of chlorine dioxide reactivity with viral proteins, SDS–polyacrylamide gel electrophoresis was used to detect breakage in protein monomers. Analysis of f2 capsid and A proteins by electrophoresis following treatment of virus with chlorine dioxide at pH 8.7 is shown in Fig. 3. Despite up to 4.6 log units of virus inactivation, the capsid of [^{3}H]leucine labelled virus remained intact, as demonstrated by similar migrations of this protein from untreated and treated virus in the gel. An expanded scale for the CPM associated with the A protein is shown on the upper panel of Fig. 8. An alteration in the gel profile of the A protein was observed following 3.0 and 4.6 log units of virus inactivation at pH 8.7. No changes in the gel protein profile were observed when f2 was inactivated at pH values of 5.0 and 7.2.

Because the inactivation of f2 virus by chlorine dioxide appeared to be associated with the protein portion of the virus, chlorine dioxide reactivity with individual amino acids was assessed. Table 1 shows that 6 of 19 amino acids tested consumed at least 10% of the chlorine dioxide mixed with 10-fold excess amino acids at pH 7.0 and room temperature after a 1 h contact time. Methionine consumed only 11% of the chlorine dioxide after 1 h. The reaction was too slow to be given further consideration. Similarly, the relatively long half-lives for chlorine dioxide in solutions containing excess histidine (17.5 min) or proline (> 10 min) indicated that these reactions were not responsible for viral inactivation. Only cysteine, tryptophan and tyrosine reacted in a time frame that could cause inhibition of specific attachment and inactivation of f2 virus.

Table 2 demonstrates the stability of cysteine sulfhydryl groups within f2 virus after treatment with chlorine dioxide. Cysteine sulfhydryl groups were measured relative to their ability to react with radiolabelled PCMB. After treatment of viruses with 100 mg l^{-1} of chlorine dioxide at pH 7.0 and 23°C, the number of CPM associated with bound PCMB decreased slightly from 231,796 to 225,858 despite 9.4 log units of virus inactivation. These data indicate that almost no loss of cysteine residues occurred with the treated viruses. In contrast, denatured f2 capsid protein lost its ability to bind radiolabelled PCMB upon treatment with chlorine dioxide under the identical reaction conditions. The CPM dropped from

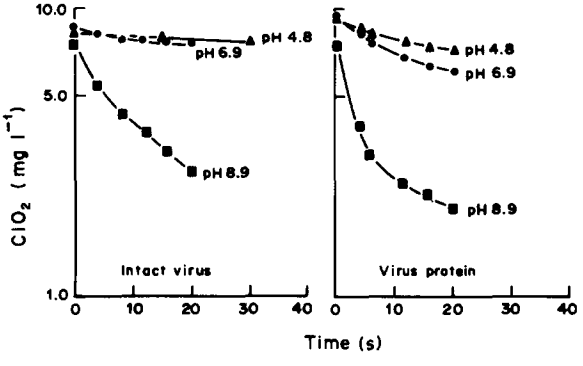

Fig. 2. Consumption of chlorine dioxide by intact f2 virus (left panel) and f2 capsid protein (right panel). Chlorine dioxide concentration was determined spectrophotometrically at 357 nm with a stopped-flow spectrophotometer. The initial virus titer was 3.0×10^{12} pfu ml^{-1} at 23°C. No chlorine dioxide was lost in control experiments at each pH value tested.

Table 1. Reactivity of amino acids with chlorine dioxide after 1 h at room temperature

Substrate	Reactivity	Substrate	Reactivity
Glycine	–	Serine	–
Phenylalanine	–	Cysteine	+
Proline	+	Glutamine	–
Asparagine	–	Leucine	–
Lysine	–	Isoleucine	–
Threonine	–	Tyrosine	+
Valine	–	Tryptophan	+
Alanine	–	Glutamic acid	–
Arginine	–	Methionine	+
Histidine	+		

The amino acid concentrations were 1×10^{-3} M. The chlorine dioxide concentration was 1×10^{-4} M (6.8 mg l^{-1}).

176,968 for control denatured capsid protein to 32,300 for chlorine dioxide treated capsid protein. These data indicate that although cysteine reacts rapidly with chlorine dioxide in pure solutions, cysteine residues may not be accessible for reactions with chlorine dioxide within the capsid of intact f2 virus particles.

The amino acids tryptophan and tyrosine were quantified in f2 protein hydrolysates following disinfection trials. Figure 4 (upper panels) shows chromatograms of hydrolyzed f2 capsid protein before (left) and after (right) intact virus was treated with 50 mg l^{-1} of chlorine dioxide for 2 min at pH 7.0 and 4°C. The initial f2 titer was 4.6×10^{12} pfu ml^{-1}. No virus was recovered after treatment with chlorine dioxide. The lower panels of Fig. 4 show chromatograms of hydrolyzed f2 capsid protein before (left) and after (right) purified capsid was treated with chlorine dioxide. Whereas there was no change in tryptophan residues after treatment of the virions with chlorine dioxide, tryptophan was not detected after treatment of the capsid. This suggested that tryptophan residues were buried within the viral capsid. Destruction of tryptophan residues was observed only when the capsid was disrupted and allowed to react with chlorine dioxide.

A similar analysis was performed to determine the reactivity of tyrosine residues in the viral capsid after treatment with chlorine dioxide. Figure 5 (upper panels) shows chromatograms of hydrolyzed f2 capsid protein before (left) and after (right) intact virus was treated with 50 mg l^{-1} of chlorine dioxide for 2 min at pH 7.0 and 4°C. The initial f2 titer was 5.2×10^{12} pfu ml^{-1}, and no virus was detected after treatment with chlorine dioxide. A comparison of the peaks in each chromatogram indicates that tyrosine residues were only partially accessible for reaction within the virus capsid. The lower panels of Fig. 5

Table 2. Measurement of cysteine residues within virus as indicated by the reaction with ^{203}Hg labelled PCMB

Sample	cpm
Intact f2 control	231,796
Intact f2 treated with 100 mg l^{-1} chlorine dioxide	225,858
f2 Coat protein control	176,968
f2 Coat protein treated with 100 mg l^{-1} chlorine dioxide	32,300

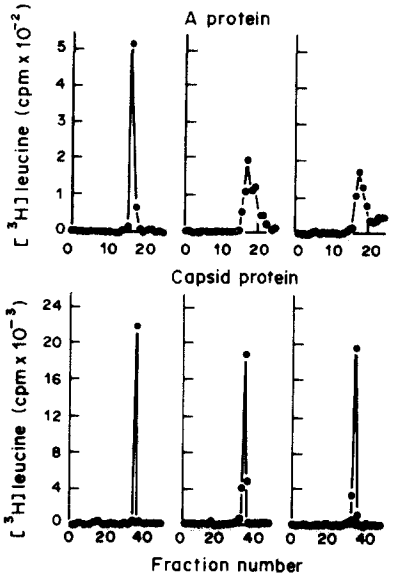

Fig. 3. SDS–polyacrylamide gel electrophoresis of f2 capsid protein and A protein following treatment of virus with 0.9 mg l^{-1} of chlorine dioxide at pH 8.7 and 5°C. The data for the panels on the left were collected at $t = 0$. The center panels represent 3.0 log units of f2 inactivation after 15 s. The panels on the right correspond to 4.6 log units of inactivation after 60 s.

show chromatograms of hydrolyzed f2 capsid before (left) and after (right) purified coat protein was treated with chlorine dioxide. These data suggest that most of the reactive tyrosine residues were buried within the viral capsid. When the capsid protein was disrupted and allowed to react with chlorine dioxide, essentially no tyrosine residues remained. From the

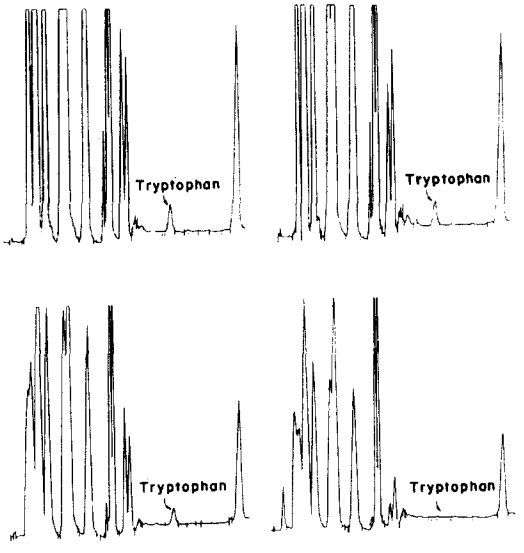

Fig. 4. Tryptophan content of untreated f2 virus (upper left), chlorine dioxide treated virus (upper right), untreated f2 protein (lower left) and chlorine dioxide treated f2 protein (lower right). Chlorine dioxide dose of 50 mg l^{-1} was applied for 2 min at pH 7.0 and 4°C. Proteins were analyzed for tryptophan content by ion exchange chromatography.

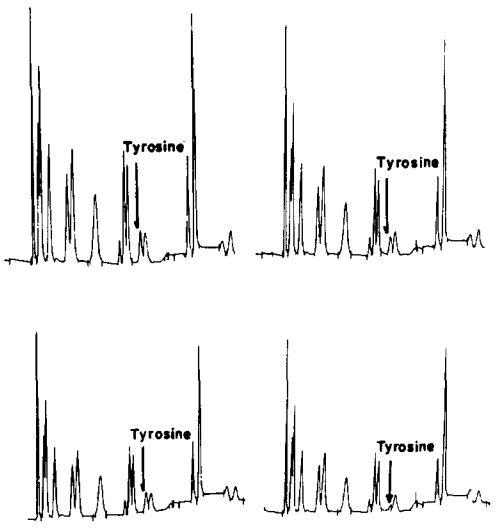

Fig. 5. Tyrosine content of untreated f2 virus (upper left), chlorine dioxide treated virus (upper right), untreated f2 protein (lower left) and chlorine dioxide treated f2 protein (lower right). Chlorine dioxide dose of 50 mg l^{-1} was applied for 2 min at pH 7.0 and 4°C. Proteins were analyzed for tyrosine content by ion exchange chromatography.

chromatographic data, it was calculated that approx. 70% of the tyrosine residues present within intact f2 were buried, and were most likely unavailable for reaction with chlorine dioxide. It is also possible that the tertiary structure of the protein may have altered the reactivity of tyrosine residues through changes in the local environment. Reactive tyrosine residues are shown as a function of chlorine dioxide concentration and contact time in Fig. 6. The loss of tyrosine residues followed a trend similar to the inactivation of f2 virus, but at a much slower rate.

DISCUSSION

Disinfection studies were conducted with f2 bacterial virus. Rates of inactivation and inhibition of specific viral attachment to host cells after treatment with chlorine dioxide increased with increasing pH. Evidence that the capsid remained intact was provided by SDS–polyacrylamide gel electrophoresis of the f2 proteins after inactivation of the virus. The gel profiles of treated and untreated virions were similar, indicating that no major disruption of the coat protein had occurred. The A protein similarly remained intact after treatment of chlorine dioxide at pH 5.0 and 7.2, but an alteration of the gel profile was observed at pH 8.7. Due to the presence of only one monomer of A protein within each f2 virion compared to 180 monomers of capsid protein, an adequate amount of A protein was not obtained for quantitative analytical measurements of amino acid content from control and chlorine dioxide treated virus. Therefore, chlorine dioxide reactions within the A protein cannot be ruled out as factors affecting f2 virus inactivation.

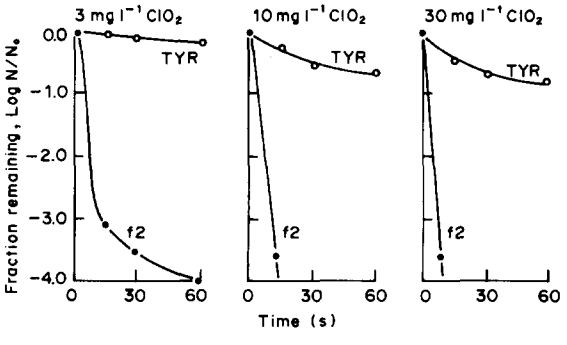

Fig. 6. Inactivation of f2 virus and loss of tyrosine residues as a function of chlorine dioxide concentration and contact time. The reactions were conducted at pH 7.0 and 4°C.

Consumption of chlorine dioxide by f2 virus and f2 capsid protein increased as the pH increased. This observation is consistent with the pH dependent increase in viral inactivation rates, specific attachment inhibition rates, and the reactivity of amino acids in pure solutions. There appeared to be no major destruction of viral protein by chlorine dioxide disinfection of virus. However, the similarity in viral inactivation rates and attachment inhibition rates suggested an amino acid reaction which resulted in an altered capsid structure.

Cysteine was unreactive with chlorine dioxide within the capsid of undenatured virus, as shown by the reactivity of this amino acid with the sulfhydryl reagent PCMB before and after treatment with chlorine dioxide. The resistance of the —SH group of cysteine in f2 protein to oxidation was also demonstrated by Hsu *et al.* (1966), who found that f2 virus survived after treatment of the virion with iodine under conditions in which only the —SH groups were affected. These investigators also observed that treatment of the virus with 1 mM PCMB had little effect on virus survival. The loss in the ability of denatured f2 protein to bind radiolabelled PCMB after treatment with chlorine dioxide in this study suggests that reactive cysteine residues are buried within the virion. This is supported by the observations of Matthews and Cole (1972), whose studies indicated that cysteine residues play a role in stabilizing the internal structure of the f2 virion.

Tryptophan residues within the f2 capsid of intact virus were unreactive when treated with chlorine dioxide. When capsid protein was denatured and then treated with chlorine dioxide, tryptophan residues were destroyed. Like cysteine, tryptophan residues appeared to be highly protected within the internal structure of the virion.

Histidine, which exists only within the A protein of f2 virus, reacted slowly with chlorine dioxide as a free amino acid in solution. Oxidation of histidine residues by chlorine dioxide is not likely to be responsible for f2 inactivation, since histidine reacted so slowly in free solution. However, the increased reactivity of histidine due to changes in the local environment and

tertiary structure of the virion after treatment with chlorine dioxide cannot be ruled out as factors affecting inactivation.

In intact virus, only tyrosine residues reacted with chlorine dioxide. These residues appeared to be primarily buried within the f2 capsid. This is consistent with the findings of Riordan *et al.* (1965), who reported that tyrosine residues were often buried within proteins, as shown by their inability to react with *N*-acetylimidazole. Matthews and Cole (1972) demonstrated that the carboxyl terminal tyrosine residue of f2 bacteriophage was involved in stabilizing the native protein conformation of the viral capsid. The role of this terminal residue in the inactivation of f2 virus with chlorine dioxide warrants further study.

Argetsinger and Gussin (1966) suggested that the A protein might be wrapped inside the coat protein of the phage, where it confers infectivity by determining the overall conformation of the particle. According to Paranchych (1975), the inability of defective particles to adsorb to host bacteria would be explainable on the basis of unfavorable configuration of coat protein subunits in the unstable capsid, rather than the absence of the A protein as a specific attachment organelle. Thus it seems likely that f2 virus was inactivated by chlorine dioxide reacting with tyrosine residues on the capsid protein and/or the A protein, such that viral adsorption to host bacteria was not possible. This hypothesis is supported by the data presented herein, which showed that the attachment function of f2 virus was inhibited by chlorine dioxide treatment, and the only demonstrable change in amino acid composition was the degradation of tyrosine residues.

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