Agosin *et al.* (9) reported that the epimastigotes of *T. cruzi* possess an active drug-detoxifying (hydroxylating) system associated with the microsomal fraction, with properties like those of a monooxygenase system linked to cytochrome P-450. These authors speculated that the ability of *T. cruzi* to hydroxylate drugs to inactive metabolites may partially explain why no chemotherapeutic agents that cure Chagas' disease have been found; however, I, for which hydroxylation is a major route of metabolism in dogs (and mice) (5), is active against *T. cruzi* in mice (2, 5). Furthermore, since a synthetic dihydroxy derivative of this compound (the 6,7-*cis*-diol) exhibits severalfold greater activity than the parent drug (5), the possibility is raised that the enzyme system suggested (9) to detoxify other chemotherapeutic agents actually may enhance the bioactivity of I. Thus, the proposal of Agosin *et al.* (9) is of limited validity.

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# Antimicrobial Activity of *N*-Chloramine Compounds


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**Abstract** □ Cellular mechanisms of action of two representative *N*-chloramines were studied. Both compounds, 3-chloro-4,4-dimethyl-2-oxazolidinone (I) and *N*-chlorosuccinimide (III), inhibited bacterial growth and exerted profound inhibition of bacterial DNA, RNA, and protein synthesis at a concentration of $10^{-5}$ *M*. Enzymes containing sulfhydryl groups generally were significantly inhibited by these chloramines at $10^{-4}$ *M*. Dihydrofolate reductase, which contains no sulfhydryl groups, also was inhibited but at much higher chloramine concentrations ($10^{-2}$ *M*); ribonuclease, which also contains no sulfhydryl groups, was unaffected. All of these inhibitory effects of the chloramines could be prevented if sulfhydryl-containing reagents (mercaptoethanol or dithiothreitol) were added before or together with the chloramine. Once inhibition was produced by the chloramine, it was not reversible by later addition of the sulfhydryl reagents. These results suggest that these chloramines act at sulfhydryl sites as well as at other sites in both cells and purified enzymes.



**Keyphrases** □ *N*-Chloramines—antimicrobial activity, inhibition of bacterial DNA, RNA, and protein synthesis □ Antimicrobial activity—*N*-chloramine-induced inhibition of bacterial DNA, RNA, and protein synthesis □ Enzyme inhibition—*N*-chloramine-induced inhibition of sulfhydryl-containing enzymes in bacteria


Recent studies of the chemical (1–5) and antimicrobial (6–8) properties of *N*-chloramine compounds suggested that many of these compounds may be potentially useful antibacterial agents. Preliminary results indicated that these *N*-chloramines are effective antibacterial agents. It was hypothesized that the bactericidal actions of *N*-chloramines are derived from the direct transfer of positive chlorine ions from the *N*-chloramines to an appropriate cellular receptor (5).

The present study was an attempt to elucidate the cellular processes affected by these agents which might account for their observed antimicrobial properties. In seeking the mechanisms of action of such agents, the determination of the primary targets in terms of overall cellular function is vitally important. This paper reports the effects of two representative *N*-chloramines on overall DNA, RNA, and protein synthesis in bacteria. A more specific action in terms of direct inhibition of cellular enzymes also is discussed.

## EXPERIMENTAL

**Bacterial Cultures**—Bacteria [*Escherichia coli* (ATCC 25922) and *Staphylococcus epidermidis* (ATCC 12228)] were grown and harvested as described previously (9). The bacterial cells then were resuspended in fresh nutrient medium[1] at a density of $3 \times 10^8$ colony-forming units/ml. Bacterial purity and the absence of contamination were established routinely by the Gram stain and by colony characteristics on blood agar plates.

**DNA, RNA, and Protein Synthesis**—All stock solutions of 3-chloro-4,4-dimethyl-2-oxazolidinone (I), 4,4-dimethyl-2-oxazolidinone (II), and *N*-chlorosuccinimide (III) were prepared in 0.1 *M* phosphate buffer (pH 7). The stock solutions were diluted with distilled, deionized water.

The bacterial suspensions were added to test tubes containing the respective agents diluted in water. All determinations were made in duplicate or triplicate along with controls containing an identical volume of bacteria without the agents. The bacteria then were incubated at 37° for 1 hr with various concentrations of the *N*-chloramines. Then 1–2 μCi of the following radioisotopes[2] were added: [*methyl*-$^3$H]thymidine, 90

[1] Bacto nutrient broth 003-03, Difco Laboratories.

[2] New England Nuclear Corp., Boston, Mass.




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Table I—Incorporation of $^3$H-Labeled Precursors into DNA, RNA, and Protein of *E. coli* [a]

| Reaction Time at 37°, hr | Thymidine (DNA) | Uridine (RNA) | Leucine (Protein) |
|---|---|---|---|
| 1 | 9,120 | 6,100 | 3,410 |
| 2 | 20,500 | 12,900 | 7,500 |
| 3 | 31,221 | 18,928 | 11,500 |
| 3, acid blank | 295 | 190 | 300 |
| 3. ice blank | 210 | 220 | 310 |

[a] The values are in counts per minute, which represent the average of triplicate determinations; each value was within 10% of the average shown.

Ci/mmole; [5,6-$^3$H]uridine, 42 Ci/mmole; and [4,5-$^3$H]-L-leucine, 40 Ci/mmole. Incorporation was allowed to proceed for 2 hr at 37°. Five milliliters of ice-cold trichloroacetic acid (10% w/v) containing nonradioactive thymidine, uridine, and leucine ($10^{-4}$ *M*) was added. This addition terminated the incorporation reactions and precipitated DNA, RNA, and protein. The added nonradioactive thymidine, uridine, or leucine helped to displace adsorbed but unincorporated radioactivity and contributed to the low blank values seen in Table I.

Samples were refrigerated for 30 min, and the precipitated material was suspended mechanically using a vortex mixer. This material then was filtered through glass fiber filters[3]. Each filter was washed with 70 ml of 1.3 *N* HCl followed by 40 ml of absolute ethanol. The filters were dried under a heat lamp and placed into liquid scintillation vials. A solubilizing agent[4] (0.5 ml) was added to the vials and left overnight to digest the radioactive film on the filter surface. Scintillation fluid[5] (15 ml) was added, and the vials were mixed gently. Complete solubilization was assured by removing the filters and noting the residual radioactivity remaining on the filter. At least 95% of the radioactivity associated with the filters usually was solubilized by this process.

Radioactivity was counted in the solution phase using a liquid scintillation spectrometer[6]; the counting efficiency was established by the channels ratio method and was verified periodically through the use of a tritiated toluene standard. Toluene-soluble tritium was counted at an efficiency of 45%. Counting of the toluene-insoluble material contained on the filter could be done at 7% efficiency. Further details concerning these procedures may be obtained from the literature (9–12).

Thymidine is a specific precursor of DNA, while uridine and leucine are incorporated into RNA and protein, respectively. The specific nature of these processes was confirmed in this study by both chemical and enzymatic techniques as outlined previously (10, 13, 14).

The effect of the chloramines on the uptake of [$^3$H]thymidine into the acid-soluble pool of *E. coli* was studied. The concentrations of radioactive precursors found in the acid-soluble pool reflect the level of precursor transport into the bacterial cell (15). Acid-soluble radioactive thymidine was measured as follows. Bacteria were incubated at 37° for 60 min in contact with the chloramine ($5 \times 10^{-5}$ *M*) and radioactive thymidine or with thymidine alone (controls). Tubes containing bacteria then were centrifuged and washed three times with ice-cold bacterial nutrient broth containing 0.1 *M* thymidine. Tubes were drained thoroughly, and 5 ml of ice-cold 0.2 *N* perchloric acid was added to the precipitated bacterial pellets. After 15 min, the perchloric acid solutions were collected, and the tubes were rinsed with an additional 2 ml of 0.2 *N* perchloric acid. Radioactivity in the combined 0.2 *N* perchloric acid solutions was determined by liquid scintillation counting.

The effects of I–III on the activities of several enzymes were studied. The following enzymes were obtained commercially[7] and used without further purification: malate dehydrogenase (EC 1.1.37), fumarase (EC 4.2.1.2), and pancreatic ribonuclease (EC 3.1.4.22). Dihydrofolate reductase (EC 1.5.1.3) was purified from a methotrexate-resistant strain of *Lactobacillus casei* (15). Lactate dehydrogenase (EC 1.1.1.27) and creatine phosphokinase (EC 2.7.3.2) were studied using a reconstituted lyophilized bovine enzyme control serum[8].

**Enzyme Assays**—Chloramines were added to buffered enzyme solutions and allowed to incubate at room temperature for 30 min before being assayed. The specific assays employed the following literature methods: malic dehydrogenase (16), lactic dehydrogenase (17), creatine phosphokinase (18, 19), pancreatic ribonuclease (20), dihydrofolate reductase (21), and fumarase (22).

[3] GF/C, Scientific Products.
[4] Protosol, New England Nuclear.
[5] Omnifluor (4 g/liter of toluene), New England Nuclear.
[6] Serle Delta 300.
[7] Sigma Chemical Co.
[8] Worthington Biochemical Corp., Freehold, N.J.

Table II—Inhibitory Actions of I and II on *E. coli* DNA, RNA, and Protein Synthesis

| Concentration, *M* | Inhibition, % | | |
|---|---|---|---|
| | DNA | RNA | Protein |
| $4 \times 10^{-6}$ [a] | 0 | 0 | 0 |
| $8 \times 10^{-6}$ [a] | 14 | 0 | 0 |
| $1.6 \times 10^{-5}$ [a] | 29 | 0 | 0 |
| $3.3 \times 10^{-5}$ [a] | 80 | 5 | 0 |
| $6.6 \times 10^{-5}$ [a] | 89 | 62 | 31 |
| $1.33 \times 10^{-4}$ [a] | 98 | 89 | 88 |
| $2.67 \times 10^{-4}$ [a] | 100 | 98 | 98 |
| $2.67 \times 10^{-3}$ [a] | 100 | 100 | 100 |
| $2.67 \times 10^{-3}$ [b] | 0 | 0 | 0 |

[a] I. [b] II (unchlorinated precursor).

Table III—Inhibitory Actions of I and II on *S. epidermidis* DNA, RNA, and Protein Synthesis

| Concentration, *M* | Inhibition, % | | |
|---|---|---|---|
| | DNA | RNA | Protein |
| $4 \times 10^{-6}$ [a] | 0 | 0 | 0 |
| $8 \times 10^{-6}$ [a] | 16 | 0 | 0 |
| $1.6 \times 10^{-5}$ [a] | 20 | 0 | 0 |
| $3.3 \times 10^{-5}$ [a] | 29 | 8 | 0 |
| $6.6 \times 10^{-5}$ [a] | 32 | 16 | 0 |
| $1.33 \times 10^{-4}$ [a] | 36 | 29 | 22 |
| $2.67 \times 10^{-4}$ [a] | 95 | 63 | 86 |
| $2.67 \times 10^{-3}$ [a] | 100 | 73 | 96 |
| $2.67 \times 10^{-3}$ [b] | 0 | 0 | 0 |

[a] I. [b] II (unchlorinated precursor).

## RESULTS

The data shown in Table I demonstrate increasing tritium incorporation into the DNA, RNA, and protein of *E. coli* for up to 3 hr at 37° in all three processes. The low counts observed by the addition of acid at zero time or by placing the tube in an ice bath to quench the reactions indicate that the washing procedure used is effective in removing adsorbed but unincorporated radioactivity. Variation of the bacterial stock solution from 0.3 to 0.9 ml also showed proportionally increased incorporation of the radioactive precursor when studied for 1 hr of incubation. Similar results were seen using *S. epidermidis*.

The percent inhibitions of DNA, RNA, and protein synthesis by the *N*-chloramines shown in Tables II–V represent the mean of two experiments, each of which was carried out in triplicate. The individual values of these triplicate determinations were within 10% of the mean values indicated. Table II shows progressively increasing inhibitory effects with increasing concentration of 3-chloro-4,4-dimethyl-2-oxazolidinone (I) for all three cellular processes studied using *E. coli*. Similar conclusions are apparent from the data of Table III with *S. epidermidis*.

Tables IV and V show the effects of the chloramine molecule *N*-chlorosuccinimide (III) on the same two bacterial species shown in Tables II and III. The effects of III were similar to those seen using I, namely, progressively increasing inhibition for all three cellular biochemical processes with increasing chloramine concentration. The effects of 4,4-dimethyl-2-oxazolidinone (II), the unchlorinated precursor molecule of I, also were studied, and it was completely inert (Tables II and III). The

Table IV—Inhibitory Actions of III on *S. epidermidis* DNA, RNA, and Protein Synthesis

| III Concentration, *M* | Inhibition, % | | |
|---|---|---|---|
| | DNA | RNA | Protein |
| $4 \times 10^{-6}$ | 0 | 0 | 0 |
| $8 \times 10^{-6}$ | 0 | 0 | 0 |
| $1.6 \times 10^{-5}$ | 0 | 13 | 0 |
| $3.3 \times 10^{-5}$ | 19 | 14 | 4 |
| $6.6 \times 10^{-5}$ | 22 | 17 | 12 |
| $1.33 \times 10^{-4}$ | 37 | 35 | 29 |
| $2.67 \times 10^{-4}$ | 47 | 40 | 30 |
| $2.67 \times 10^{-3}$ | 100 | 80 | 96 |

**Table V—Inhibitory Actions of III on *E. coli* DNA, RNA, and Protein Synthesis**

| III Concentration, *M* | Inhibition, % DNA | RNA | Protein |
|---|---|---|---|
| $4 \times 10^{-6}$ | 0 | 0 | 0 |
| $8 \times 10^{-6}$ | 0 | 0 | 0 |
| $1.6 \times 10^{-5}$ | 4 | 0 | 0 |
| $3.3 \times 10^{-5}$ | 10 | 5 | 8 |
| $6.6 \times 10^{-5}$ | 15 | 12 | 10 |
| $1.33 \times 10^{-4}$ | 32 | 16 | 16 |
| $2.67 \times 10^{-4}$ | 60 | 45 | 40 |
| $2.67 \times 10^{-3}$ | 100 | 100 | 95 |

**Table VI—Effect of Addition of a Sulfhydryl-Containing Compound on the Chloramine Inhibition of Bacterial DNA Synthesis**

| Agent ($2.67 \times 10^{-3}$ *M*) | Dithiothreitol Concentration, *M* | Inhibition of DNA Using *E. coli*, % |
|---|---|---|
| I | 0 | 100 |
| I[a] | $5.4 \times 10^{-3}$ [a] | 0 |
| I[b] | $2.67 \times 10^{-3}$ [b] | 26 |
| I[b] | $5.4 \times 10^{-3}$ [b] | 0 |
| I[c] | $5.4 \times 10^{-3}$ [c] | 0 |
| None | $5.4 \times 10^{-3}$ | 0 |
| II | 0 | 0 |
| III | 0 | 100 |
| III[a] | $2.67 \times 10^{-3}$ [a] | 36 |
| III[b] | $2.67 \times 10^{-3}$ [b] | 4 |
| III[b] | $5.4 \times 10^{-3}$ [b] | 0 |

[a] Mixed together and added to bacteria as a mixture. [b] Dithiothreitol added to bacteria first, followed by chloramine. [c] Compound I added to the bacteria containing tritiated thymidine and incubated at 37° for 1 hr followed by the addition of dithiothreitol. Fresh bacteria were added, and the incorporation of tritiated thymidine was measured 2 hr later.

unchlorinated precursor molecule (II) was studied under exactly the same conditions as the chloramines (I and III).

Table VI shows that addition of dithiothreitol can protect against the inhibition of DNA synthesis in bacteria. This result was seen when I was combined with dithiothreitol and then added to bacteria or, alternatively, when the bacteria were supplemented with dithiothreitol before the addition of I. Similar protective actions of dithiothreitol using III also have been demonstrated. Again, II had no inhibitory action. Addition of dithiothreitol or mercaptoethanol also could protect against the inhibition of RNA and protein synthesis.

The inhibitory actions of I are not due to chemical alteration of the tritiated thymidine, uridine, or leucine by the chloramine in which these precursors are rendered inert. This conclusion was reached since the chloramines, when added to bacteria with tritiated thymidine and incubated for 60 min at 37°, showed *no significant inhibition of incorporation* after fresh bacteria were added and incorporation was allowed to proceed for 2 additional hr (Table VI). Residual chloramine was inactivated by adding dithiothreitol immediately before the addition of fresh bacteria. Experiments using tritiated thymidine, uridine, and leucine with I or III gave similar results under identical experimental conditions. However, partial inactivation of the tritiated precursors at far higher chloramine concentrations ($1 \times 10^{-2}$ *M*) was observed.

The data obtained indicate that while the bacterial synthesis of DNA was significantly inhibited based on a *t* test for paired samples ($p <$ 0.001), the chloramine did not produce statistically significant inhibition of the uptake or transport of tritiated thymidine into the bacterial cells. Bacteria were incubated together with $5 \times 10^{-5}$ *M* I and [$^3$H]thymidine for 1 hr at 37°. The amount of tritiated precursor found in the bacterial acid-soluble pool is a direct reflection of the extent of thymidine transport into the bacterial cell (15). Similar results also were obtained using tritiated uridine and leucine.

These chloramines were effective inhibitors of sulfhydryl-containing enzymes (Table VII). Three general groups of enzymes were compared: (*a*) enzymes containing sulfhydryl groups at their active sites [malic dehydrogenase (23), lactic dehydrogenase (24), and creatine phosphokinase (25)]; (*b*) enzymes containing no sulfhydryl groups at their active sites but having sulfhydryl groups at other sites [fumarase (26)]; and (*c*) enzymes containing no sulfhydryl groups at any site [ribonuclease (27) and dihydrofolate reductase (21)].

The enzymes in the first group are inhibited easily (Table VII). The sulfhydryl group does not have to be present at the active site for inhibition to occur since fumarase also is quite sensitive. Some enzymes containing no sulfhydryl groups (dihydrofolate reductase) also can be inhibited but only at much higher chloramine concentrations ($\geq 10^{-2}$ *M*). However, ribonuclease was not inhibited, even at $10^{-2}$ *M*. All of the chloramine-induced enzymatic inhibitions could be prevented if the chloramines were mixed first with twice the concentration of the sulfhydryl-containing reagent dithiothreitol. The limiting amount of dithiothreitol that was capable of preventing chloramine-induced enzymatic inhibition was not explored. Reactivation of the inhibited enzymes by later addition of sulfhydryl agents after first reacting the enzyme with chloramine was not achieved. The unchlorinated precursor (II) was not effective in inhibiting any of the sulfhydryl-containing enzymes (Table VII).

The effects of increasing I concentrations on the overall growth of *E. coli* also were studied but are not presented in tabular form. Inhibition of bacterial growth was studied by incubating inoculated cultures of *E. coli* at 37° and monitoring the effect on turbidity (absorbance increase) at 450 nm against time at 10-hr intervals. The bacterial growth data represent the mean of triplicate determinations. Varying concentrations of I were added to the bacterial suspensions, and bacterial growth was followed for 4 days. The chloramine appeared to act as a bacteriostatic agent, at concentrations ranging from $5 \times 10^{-5}$ to $4 \times 10^{-4}$ *M*, in that bacterial growth was delayed. At concentrations of $5 \times 10^{-4}$ *M* and higher, no bacterial growth was evident. When the organisms exposed to these higher doses were subcultured, no growth was apparent. These results suggest death of the organisms and show that I exerts bactericidal actions at higher concentrations.

**Chemical Verification**—Bacterial incorporation was allowed to proceed for 3 hr using radioactive thymidine, uridine, or leucine in different tubes. Cold 0.2 *N* perchloric acid was added, and the precipitates were washed twice with 0.2 *N* perchloric acid. The supernates were discarded, and the precipitates were dissolved and incubated in 0.3 *N* KOH for 1 hr at 37°. The material was reprecipitated with 0.7 *N* perchloric acid,

**Table VII—Percent Inhibition of Enzyme Activity by Agent**

| Agent Concentration, *M* | Inhibition, % Malic Dehydrogenase | Lactic Dehydrogenase | Creatine Phosphokinase | Fumarase | Ribonuclease | Dihydrofolate Reductase |
|---|---|---|---|---|---|---|
| $1.33 \times 10^{-5}$ [a] | 0 | 0 | 0 | 50 | 0 | — |
| $2.67 \times 10^{-5}$ [a] | 0 | 0 | 0 | — | 0 | — |
| $5.34 \times 10^{-5}$ [a] | 16 | 10 | 0 | — | 0 | — |
| $1.07 \times 10^{-4}$ [a] | 20 | 15 | 5 | 100 | 0 | — |
| $2.14 \times 10^{-4}$ [a] | 48 | 30 | 15 | — | 0 | — |
| $4.28 \times 10^{-4}$ [a] | 54 | 40 | 30 | 100 | 0 | — |
| $8.56 \times 10^{-4}$ [a] | 62 | 50 | 53 | 100 | 0 | — |
| $1.28 \times 10^{-2}$ [a] | 100 | 100 | 85 | 100 | 0 | — |
| $1.72 \times 10^{-2}$ [a] | 100 | 100 | 100 | 100 | 0 | 50 |
| $1.72 \times 10^{-2}$ [a] Added to dithiothreitol | | | | | | |
| $3.4 \times 10^{-2}$ [a] (SH) | 0 | 0 | 0 | 0 | 0 | 0 |
| $1.72 \times 10^{-2}$ [b] | 0 | 0 | 0 | 0 | 0 | 0 |

[a] I. [b] II (unchlorinated precursor).

collected on filters, and counted as described previously. The potassium hydroxide treatment resulted in >90% breakdown of the radioactive polymer produced with radioactive uridine.

The polymers produced with radioactive thymidine or leucine were not significantly affected by the alkaline treatment. RNA is known to be degraded by alkali, whereas DNA and protein are not affected (10, 13, 14). Heat treatment (70°) of the radioactive polymers with 0.5 *N* perchloric acid for 30 min resulted in the breakdown of the polymer produced using thymidine, while the polymer produced with leucine was unaffected. DNA is known to be degraded under these conditions of heating in the presence of acid (10, 13, 14).

**Enzymatic Verification**—The filters containing the radioactive polymers were immersed in solutions of deoxyribonuclease[7], ribonuclease[7], and a mixture of proteolytic enzymes[7] for 1 hr at 37°. The polymer produced using thymidine was degraded by deoxyribonuclease but was not affected by ribonuclease or the proteolytic enzymes. The polymer produced using uridine was degraded only by ribonuclease, and the polymer resulting from leucine incorporation was degraded only by proteolytic enzymes. These reactions strongly support the specific nature of these incorporation processes. (Further details may be obtained from Ref. 10.)

## DISCUSSION

The data presented here and previous data (1–5) indicate that chloramines exert both bacteriostatic and bactericidal actions. The extent of the antimicrobial actions depends on the ratio of the bacterial concentration to that of chloramine and the length of exposure. These parameters also control the extent of enzyme inhibition. Both the antibacterial effects and the enzymatic inhibitory actions appear to be due to the powerful oxidative action of the chlorine component of the chloramine molecules. Support for this view is contributed by the facts that the unchlorinated precursor molecule is inactive and that the inhibitory effects of I and III can be abolished completely by first mixing the chloramine with the sulfhydryl compounds dithiothreitol or mercaptoethanol.

Thus, the present study indicates that one cellular mechanism for these antibacterial actions might be that these chloramines effectively attack the sulfhydryl groups, which can lead to enzymatic inactivation or denaturation. A natural consequence of such actions would be interference with major cellular processes (not shown previously) such as DNA, RNA, and protein synthesis, which depend on the subtle interaction of many sulfhydryl-containing enzymes. All three processes appear to be inhibited, and the antimicrobial actions apparently are not due to the selective inhibition of any one specific cellular function that is vastly more sensitive than the others. The data in Tables II and III suggest that DNA synthesis is slightly more sensitive to the action of I than is RNA or protein synthesis. The basis of action of these chloramine agents appears to be similar to that proposed for the bactericidal actions of chlorine (28).

The data in Tables II–V suggest that these chloramines inhibit bacterial synthesis of DNA, RNA, and protein. These results have several possible causes:

1. Chemical alteration of the radioactive precursors by the chloramine, resulting in the decreased incorporation into DNA, RNA, or protein. However, the data presented in Table VI show that this is not true. When bacteria, radioactive precursors, and chloramine were first incubated, no inhibition of incorporation was found after inactivating the chloramine by adding dithiothreitol and then adding fresh, viable bacteria. If the chloramines had reacted with the radioactive precursors and rendered them inert, normal incorporation would not have been demonstrated after inactivating the residual chloramine and adding fresh viable bacteria.
2. Chloramine inhibition of the transport of the radioactive precursor into the bacterial cell. However, the data discussed previously do not support inhibition of precursor transport but rather synthesis inhibition.

The data in Table VII demonstrate that enzymes containing sulfhydryl sites are inhibited by I at $10^{-4}$ *M*. Other enzymes without sulfhydryl sites (dihydrofolate reductase) may be inhibited at far higher concentrations ($10^{-2}$ *M*) of chloramine, while another enzyme (ribonuclease) appears to be completely resistant. Attempts were made to protect the sulfhydryl groups of malate dehydrogenase and fumarase by reacting these enzymes with agents that reversibly combine with sulfhydryl groups (29). Both enzymes were incubated with 5,5-dithiobis(2-nitrobenzoic acid) and with *p*-hydroxymercuribenzoate. Chloramine was added later, and the incubation was continued. Dithiothreitol then was added to remove the protective agents and to inactivate any residual chloramine. No protective effects were evident against the actions of chloramine by these reversible sulfhydryl agents. It generally can be concluded that the sulfhydryl groups are attacked by these chloramine agents but that other sites also are attacked since non-sulfhydryl-containing enzymes are inhibited and since the chloramines still are capable of causing enzyme inhibition, even when the sulfhydryl groups are protected. The chloramine-induced inhibitions were not reversed simply by adding sulfhydryl reagents to bacteria or to purified enzymes after first incubating with chloramine.

While some enzymes containing sulfhydryl groups are inhibited by chloramines, this fact may not mean that all sulfhydryl-containing enzymes are inhibited, although they could be. Reversal of antibacterial activity by dithiothreitol also does not prove that a compound reacts by a sulfhydryl inhibition.

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