Myeloperoxidase-Catalyzed Incorporation of Amines into Proteins: Role of Hypochlorous Acid and Dichloramines[†]

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ABSTRACT: Myeloperoxidase-catalyzed oxidation of chloride (Cl⁻) to hypochlorous acid (HOCl) resulted in formation of mono- and dichloramine derivatives (RNHCl and RNCl₂) of primary amines. The RNCl₂ derivatives could undergo a reaction that resulted in incorporation of the R moiety into proteins. The probable mechanism was attack of RNCl₂ or an intermediate formed in the decomposition of RNCl₂ on histidine, tyrosine, and cystine residues and on lysine residues at high pH. Incorporation of radioactivity from labeled amines into stable, high molecular weight derivatives of proteins was measured by acid or acetone precipitation and by gel chromatography and electrophoresis. Whereas formation of RNCl₂ was favored at low pH, the subsequent incorporation reaction was favored at high pH. Up to several hours were required for the maximum amount of incorporation, which was less than 10% of the label in RNCl₂. For the amines tested, incorporation was in the order histamine > 1,2-diaminoethane > putrescine > taurine > lysine > glucosamine > leucine > methylamine. Initiation of the reaction required HOCl, and oxidized forms of bromide, iodide, or thiocyanate did not substitute. Inhibitors of incorporation fell into three classes. First, ammonia or amines competed with the labeled amine for reaction with HOCl, so that larger amounts of HOCl were required. Second, readily oxidized substances such as sulf-hydryl or diketo compounds or thioethers (methionine) reduced RNCl₂. Third, certain compounds competed with protein as the acceptor for the incorporation reaction. The amount required to block incorporation into protein depended on protein concentration. Among these inhibitors were imidazole compounds (histidine), phenols (tyrosine), and disulfides (glutathione disulfide, GSSG). Low yields of derivatives of histidine, tyrosine, and GSSG were detected by thin-layer chromatography. Acid-precipitable derivatives were obtained by reacting RNCl₂ with polyhistidine or polytyrosine, and to a lesser extent with polylysine at high pH, but not with other poly(amino acids). Precipitable derivatives were also obtained by incubating MPO-containing extracts from leukocyte granules with hydrogen peroxide, Cl⁻, and labeled amines. The extracts were found to have a high content of substances with primary amino groups, which competed for incorporation. The results account for oxidative incorporation of amines into proteins in leukocytes and provide evidence that HOCl and nitrogen–chlorine (N–Cl) derivatives are formed in these cells. The characteristics of the incorporation reaction suggest that it would not contribute significantly to the antimicrobial activity of myeloperoxidase (MPO). Nevertheless, the reaction may provide a sensitive method for studying MPO action in vivo.

Myeloperoxidase (MPO) and other antimicrobial enzymes and proteins are stored in granules of polymorphonuclear leukocytes and monocytes. The granule contents are released into the intracellular, membrane-bounded vesicle that is formed during ingestion (phagocytosis) of a microorganism by these cells. Fusion of the granules with the phagocytic vesicle forms the phagolysosome, where MPO catalyzes the oxidation of halide ions to yield products that oxidize and halogenate microbial components, contributing to the microbicidal activity of the leukocytes (Stossal, 1974; Klebanoff, 1975; Babior, 1978; Badwey & Karnovsky, 1980).

In studies on the purified enzyme, MPO was shown to catalyze the oxidation of chloride (Cl⁻) by hydrogen peroxide (H₂O₂) to yield hypochlorous acid (HOCl) or hypochlorite ion (OCl⁻) (Zgliczynski et al., 1971; Agner, 1972; Harrison & Schultz, 1976; Harrison, 1976; Morrison & Schonbaum, 1976). These agents are in acid–base equilibrium, and HOCl (pK_A = 7.5) is the predominant form at acid or neutral pH. If HOCl is formed in leukocytes, this product will be short-lived in that HOCl reacts rapidly with readily oxidized substances such as sulfhydryl or thioether compounds and also reacts with a wide variety of nitrogen-containing compounds to form derivatives containing the nitrogen–chlorine (N–Cl)

bond (Nelson, 1979). These N–Cl derivatives retain the two oxidizing equivalents of HOCl and therefore can act as antimicrobial oxidizing agents. However, the N–Cl derivatives differ from HOCl in stability, solubility, and oxidation potential. For this reason, the antimicrobial activity of the MPO–H₂O₂–Cl⁻ system can be altered in vitro by adding ammonium ion (NH₄⁺), amines, and other nitrogenous compounds (Thomas, 1979b). These substances react with HOCl to form N–Cl derivatives that can be more effective or less effective than HOCl as antimicrobial agents.

If HOCl is formed within phagolysosomes, it may react with leukocyte granule components to form N–Cl derivatives with antimicrobial activity. Therefore, the granule components could mediate the antimicrobial action of the MPO–H₂O₂–Cl⁻ system. Alternatively, the granule components may act as a trap for any HOCl that has failed to react with microbial components. In this way, the granule components could prevent damage to the leukocyte and surrounding tissues by preventing the diffusion of HOCl out of the phagolysosome (Thomas, 1979a).

It would be difficult to directly measure N–Cl derivatives within leukocytes, in that these agents react with many biological materials. Attempts to extract these agents would probably result in reduction by leukocyte cytoplasmic components. Therefore, indirect methods may be required to demonstrate formation of HOCl and N–Cl derivatives and to study their role in leukocyte antimicrobial activity.

Recently, it was reported that incubation of radioactively labeled glucosamine with leukocytes resulted in incorporation of label into leukocyte proteins and that this incorporation was

related to MPO activity rather than to glycoprotein synthesis (Bearman et al., 1980). If N-Cl derivatives of glucosamine are intermediates in this reaction, then the labeled products provide evidence that HOCl and N-Cl derivatives are formed, at least when an exogenous amine is present.

The interaction of radioactively labeled amines with the MPO system was studied in vitro, to determine whether MPO can catalyze the incorporation of amines into proteins and whether N-Cl derivatives of the amines are intermediates in the reaction. Also, we sought to determine (1) the specificity of the incorporation reaction with regard to the donor amine and acceptor protein, (2) the rate and pH dependence of incorporation, and (3) the quantitative relation between formation of N-Cl derivatives and incorporation.

Materials and Methods

All chemicals were of reagent grade. MPO purified from human leukemic leukocytes was provided by Drs. M. Morrison and J. Naskalski. The concentration of MPO was calculated by assuming a millimolar extinction coefficient of 89 at 430 nm (Ehrenberg & Agner, 1958). Lysozyme was from Worthington, and poly(L-lysine) hydrochloride (M_r 306000) was from Schwarz/Mann. RNase,[1] lactoferrin, bovine serum albumin, poly(L-histidine) (M_r 16000), poly(L-tyrosine) (M_r 44000), poly(L-tryptophan) (M_r 5000–15000), poly(L-arginine) (M_r 60000), poly(L-glutamic acid) (M_r 8100), poly(L-serine) (M_r 5200), poly(L-asparagine) (M_r 8000), poly(L-O-acetyltyrosine) (M_r 7000), fluorescamine, and Nbs_2 were from Sigma Chemical Co.

[2-³H(N)]Taurine (23 Ci/mmol), [1,2-¹⁴C]taurine (56 Ci/mol), [1,4-¹⁴C]putrescine (102 Ci/mol), L-[U-¹⁴C]leucine (294 Ci/mol), L-[4,5-³H(N)]lysine (68 Ci/mmol), and [¹⁴C]methylamine (54 Ci/mol) were from New England Nuclear. D-[U-¹⁴C]Glucosamine (286 Ci/mol), 2,4-diamino[U-¹⁴C]ethane (25 Ci/mol), and [ring-2-¹⁴C]histamine (60 Ci/mol) were from Amersham/Searle. The labeled amines were diluted to the desired specific activity with the unlabeled amines as the Cl⁻ or sulfate salts. A 1 mM solution of Nbs_2 in 0.1 M Tris-HCl, pH 7, was reduced to Nbs with excess sodium borohydride, shaken at 37 °C for 15 min, and then stored overnight under nitrogen to permit decomposition of excess borohydride. The Nbs concentration was calculated from the molar extinction coefficient of 13600 at 412 nm (Ellman, 1959). NaOCl from Fisher Chemical Co. was diluted into 0.01 M NaOH. H_2O_2 (30%) from Fisher Chemical Co. was diluted in water and the concentration determined from the molar extinction coefficient of 72 at 230 nm (George, 1953).

Granule Extracts. Leukocyte granule extracts were prepared by a combination and modification of several methods in which cells are disrupted in isotonic sucrose, the crude granule fraction is obtained by differential centrifugation, and the granule contents are extracted at low pH (Cohn & Hirsch, 1960; Leffell & Spitznagel, 1972; Olsson & Venge, 1974; Weiss et al., 1978; Modrzakowski et al., 1979). Leukocytes from patients with chronic myelocytic leukemia were obtained by leukophoresis, and erythrocytes allowed to settle out over a period of several hours. Leukocytes were washed by centrifugation at 1500g in 1 volume of 0.15 M NaCl–6.5 mM phosphate, pH 7.4, and suspended in 2 volumes of 0.34 M sucrose–3 mM EDTA, pH 7. Cells were disrupted by soni-

cation at 4 °C at the lowest power that resulted in 70–80% disruption within 1 min, as evaluated by microscopy. Unbroken cells and nuclei were removed by centrifugation at 200g for 10 min at 4 °C, and then the supernatant fraction was centrifuged at 16000g for 30 min at 4 °C. The pellet was suspended in sucrose–EDTA, frozen in liquid N_2, and stored at −80 °C. Portions were thawed at 37 °C, made 0.2 M in NaCl, and adjusted to pH 4 with 1 M H_3PO_4. After 15 min at 4 °C, the suspension was centrifuged at 20000g for 30 min at 4 °C. The supernatant fraction was dialyzed (M_r 3000 limit) against 0.12 M NaCl, adjusted to pH 4 with 1 mM H_3PO_4, to obtain the granule extract. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Primary amines were determined by reacting samples buffered at pH 7 with 0.5 volume of 0.3% (w/v) fluorescamine in acetone, followed by dilution with 30 volumes of water–acetone (2:1), and measuring fluorescence with excitation at 390 nm and emission at 475 nm (deSilva & Strojny, 1975).

Incorporation of Radioactivity. Total incorporation into proteins or polyaminoacids was determined by acid precipitation. Reaction mixtures were diluted with 10 volumes of cold 5% (w/v) trichloroacetic acid and filtered through 0.45-μm nitrocellulose filters (Millipore Corp.), and the filters washed with 5% trichloroacetic acid. Filters were dissolved in scintillation fluid (Bray, 1960), and radioactivity was determined in a liquid scintillation spectrometer.

Labeled proteins were separated by chromatography of reaction mixtures (1 mL) on 62 × 1.6 cm columns of Sephadex G-25 or G-100 (Pharmacia Fine Chemicals) equilibrated and eluted with 0.1 M NaCl–20 mM phosphate, pH 7. Fractions of about 2 mL were collected and the volume was determined by weighing. Portions were taken for determination of total label and acid-precipitable label. Protein was determined by the dye-binding method (Bradford, 1976) with bovine plasma γ-globulin as the standard and reagents from Bio-Rad Laboratories.

Reduced and denatured polypeptides were separated according to apparent molecular weight by electrophoresis in the presence of sodium dodecyl sulfate by the method of Laemmli (1970). Reaction mixtures were precipitated with 5 volumes of 20% trichloroacetic acid, resuspended in water, and solubilized in 1.5 volumes of 40 mM Tris-HCl, pH 6.8, containing 2.1 M urea, 2.5% (w/v) sodium dodecyl sulfate, and 3.5% (v/v) 2-mercaptoethanol. Portions were electrophoresed on gels formed with a 7.5–15% gradient of acrylamide, with 0.1% linear polyacrylamide (Gallard-Schlesinger Corp.). Polypeptides were detected by staining with Coomassie Brilliant Blue (Fairbanks et al., 1971), and labeled polypeptides were detected by fluorography (Bonner & Lasky, 1974).

[¹⁴C]Taurine was separated from low molecular weight oxidation and incorporation products by thin-layer chromatography on silica gel G plates (Analtech) in 1-butanol–acetic acid–water (4:1:1). labeled compounds were visualized by autoradiography and scraped from the plates, and radioactivity was determined as described above.

Determination of HOCl and N-Cl Derivatives. An excess of the Nbs solution was added to the sample adjusted to pH 7 with 0.1 M phosphate buffer, and absorbance at 412 nm was measured. It was necessary to add an immediate, large excess of the sulfhydryl compound (Nbs) to favor oxidation to the disulfide (Nbs_2) over the competing reactions (Silverstein & Hager, 1974). Concentrations of Cl atoms of valence +1, Cl(+1), were calculated from half the difference between the amount of Nbs added and the amount remaining (Aune &

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[1] Abbreviations: RNase, bovine pancreatic ribonuclease A; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; fluorescamine, 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione.
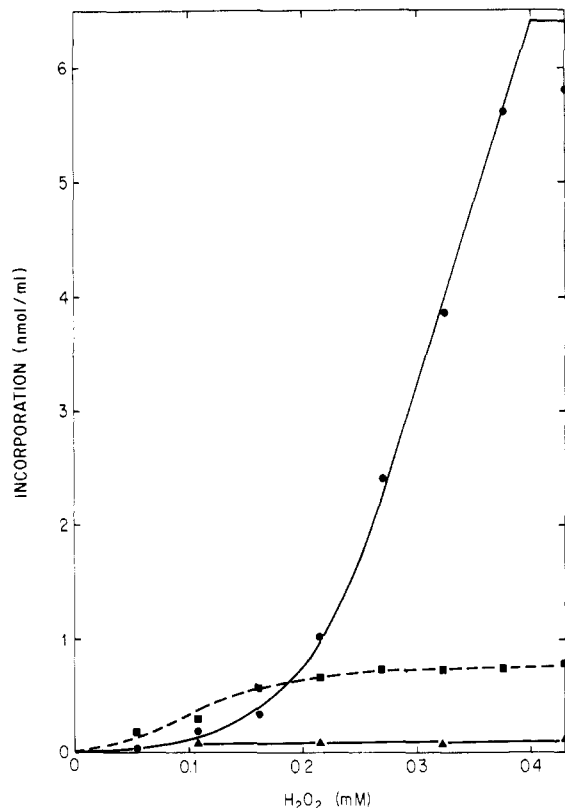
FIGURE 1: MPO-catalyzed incorporation of [³H]taurine into RNase. Reaction mixtures (0.1 mL) containing 80 nM MPO, 0.2 M NaCl, 50 mM phosphate, pH 5, and [³H]taurine were supplemented with varying amounts of H₂O₂, incubated 15 min at 25 °C, mixed with 0.1 mL of 1 mM RNase in 0.5 M phosphate at pH 8 (●) or pH 5 (■), and incubated 90 min at 37 °C. The final concentration of [³H]taurine was 0.2 mM and final concentrations of H₂O₂ are shown. Alternatively, H₂O₂ was added last to 0.2-mL mixtures of all the components at pH 5 (▲) and incubated 90 min at 37 °C.

Thomas, 1977; Thomas, 1979a). Each Cl(+1) contains 2 oxidizing equiv and so is able to oxidize two Nbs to Nbs₂. There is one Cl(+1) moiety in Cl₂, HOCl, OCl⁻, or RNHCl, and two Cl(+1) in RNCl₂. The Cl(+1) moiety is not to be confused with free chloronium ion, Cl⁺.

Results

Activation of Donor Amines. Figure 1 shows incorporation of label from [³H]taurine into acid-precipitable derivatives of RNase, with the MPO–H₂O₂–Cl⁻ system. The greatest amount of incorporation was obtained by carrying out the oxidation of Cl⁻ to HOCl at pH 5 in the presence of [³H]-taurine, then adding RNase at pH 8, and continuing the incubation for 1 h or more. Incorporation was highest at a H₂O₂/[³H]taurine ratio of 2, but the maximum amount of incorporation was only 3% of the label in [³H]taurine. In other experiments, similar results were obtained with [¹⁴C]taurine.

Figure 1 also shows that less incorporation was obtained by adding RNAse at pH 5. Also, a very small amount of incorporation was obtained by adding H₂O₂ to a mixture of MPO, Cl⁻, [³H]taurine, and RNase. Other orders of addition, such as adding H₂O₂ to MPO, Cl⁻, and RNase and then adding ³[H]taurine, were ineffective.

Similar results were obtained with the MPO–H₂O₂–Cl⁻ system, or with nonenzymatically prepared HOCl, when expressed as a function of the amount of added H₂O₂ or HOCl. These results indicated that at pH 5, the MPO system produced 1 mol of HOCl/mol of H₂O₂. Figure 2 shows incorporation of label from [³H]taurine into RNase at three
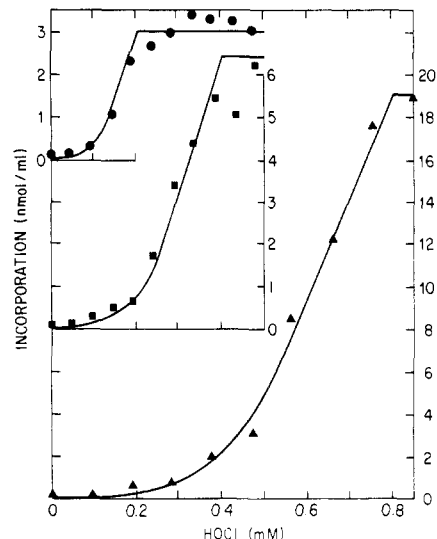
FIGURE 2: Effect of [³H]taurine concentration on incorporation. Reaction mixtures (75 μL) containing [³H]taurine in 0.12 M NaCl–50 mM phosphate, pH 7, were supplemented with varying amounts of HOCl, incubated 5 min at 25 °C, diluted with 25 μL of 2 mM RNase, and incubated 1 h at 37 °C. Final concentrations of [³H]taurine were 0.1 (●), 0.2 (■), and 0.4 mM (▲). Final concentrations of HOCl are shown.

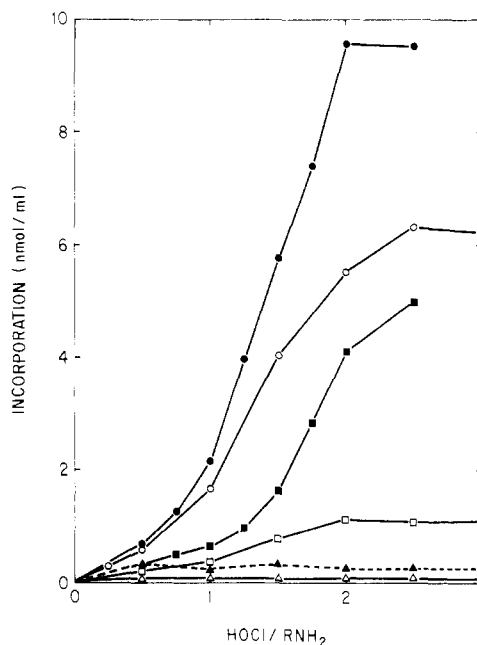FIGURE 3: Incorporation of amines. Reaction mixtures (75 μL) containing 0.12 M NaCl–50 mM phosphate, pH 7, and labeled amines were supplemented with HOCl at the indicated ratios of HOCl to primary amino groups, incubated 5 min at 25 °C, diluted with 25 μL of 2 mM RNase, and incubated 1 h at 37 °C. Final concentrations were 0.2 mM [¹⁴C]histamine (●), 0.1 mM 1,2-diamino[¹⁴C]ethane (○), 0.1 mM [¹⁴C]lysine (■), 0.2 mM [¹⁴C]leucine (□), 0.2 mM [¹⁴C]glucosamine (▲), and 0.2 mM [¹⁴C]methylamine (△).

[³H]taurine concentrations, with HOCl to initiate the reaction. HOCl was generated by adding a solution of OCl⁻ in dilute base to the buffered solution of [³H]taurine. After HOCl and [³H]taurine had reacted, the acceptor protein was added and the incubation continued. For each [³H]taurine concentration, the maximum amount of incorporation was obtained at a HOCl/[³H]taurine ratio of 2. The amount of incorporation was proportional to the initial [³H]taurine concentration, but the amount incorporated was only 3–4% of the label in [³H]taurine.

Figure 3 compares incorporation of label from other radioactively labeled amines into RNase. With all amines tested, incorporation was less than 10% of the amount of amine. Greater incorporation was obtained with histamine than with any other amine, so that the imidazole nitrogens of histamine did not interfere. With 1,2-diaminoethane, the maximum incorporation was obtained at a HOCl/amino group ratio of 2, or a HOCl/amine ratio of 4. Also, incorporation was about 2-fold greater than with taurine at the same concentration (Figure 2). Therefore, the two amino groups of diaminoethane reacted independently, and incorporation of the diamine was the same as for a 2-fold higher concentration of the monoamine, taurine. In other experiments, similar results were obtained with 1,4-diaminobutane (putrescine).

Figure 3 shows that similar results were obtained with lysine, though the maximum incorporation was lower than with other diamines. This decrease was probably due to loss of oxidizing equivalents and ^{14}C label in oxidative decarboxylation of the α-amino acid moiety (Paul et al., 1970; Zgliczynski et al., 1971). Consistent with this interpretation, less incorporation was obtained with leucine than with lysine. Incorporation of glucosamine was low, probably due to loss of oxidizing equivalents in reactions involving the reducing sugar moiety. Very little incorporation was obtained with methylamine (CH_3NH_2), possibly due to demethylation reactions (Sidgwick et al., 1966).

In experiments with MPO, replacing Cl^- with bromide (Br^-), iodide (I^-), or the pseudohalide thiocyanate (SCN^-) did not result in incorporation. Instead, these substances inhibited when added in the presence of Cl^-. The amount of Br^-, I^-, and SCN^- required to block incorporation was 0.5 mM, 10 μM, and 0.1 mM, respectively, with 0.2 M Cl^- at pH 5. Similarly, incorporation of [^3H]taurine could not be initiated with bromine (Br_2), iodine (I_2), or hypothiocyanite ($OSCN^-$) (Thomas, 1981). Adding taurine to solutions of these agents at pH 7 did not alter their absorption spectra, indicating that they did not react with taurine under these conditions.

Results in Figures 1–3 suggested that dichloramine derivatives were involved in incorporation. Yields of the monochloramine (RNHCl) and dichloramine (RNCl$_2$) derivatives of taurine were calculated from absorption at 252 and 300 nm (Gray et al., 1978). Molar extinction coefficients for RNHCl of 429 at 252 nm and 36 at 300 nm were determined by adding HOCl to 10 mM taurine in 0.1 M NaCl–0.1 M NaOH up to a HOCl/taurine ratio of 0.05 and assuming that all the absorption was due to RNHCl. Molar extinction coefficients for RNCl$_2$ of 190 at 252 nm and 370 at 300 nm were determined by adding 0.25 mM increments of HOCl at 1-min intervals to 1 mM taurine in 0.1 M NaCl–0.1 M phosphate, pH 7, and assuming that all the absorption at the HOCl/taurine ratio of 2 was due to RNCl$_2$. When RNHCl or RNCl$_2$ solutions prepared in this way were adjusted to pH 5–8, the absorption did not change, indicating that the extinction coefficients were independent of pH in this range.

Figure 4 shows results of adding HOCl into a solution of taurine. At low HOCl/taurine ratios, the yields of RNHCl and RNCl$_2$ were proportional to HOCl, and the relative yields were influenced by pH. At pH 8, RNCl$_2$ was almost undetectable, whereas at pH 5, the RNCl$_2$/RNHCl ratio was about 0.3. In other experiments, the RNCl$_2$/RNHCl ratio was also influenced by taurine concentration. With 10, 20, and 50 mM taurine at pH 5, the RNCl$_2$/RNHCl ratio was about 0.5, 0.25, and 0.1. In contrast, the RNCl$_2$/RNHCl ratio was not altered by adding more taurine 15 s after the addition of HOCl or by altering the pH in the range of 5–8. Therefore, the reaction
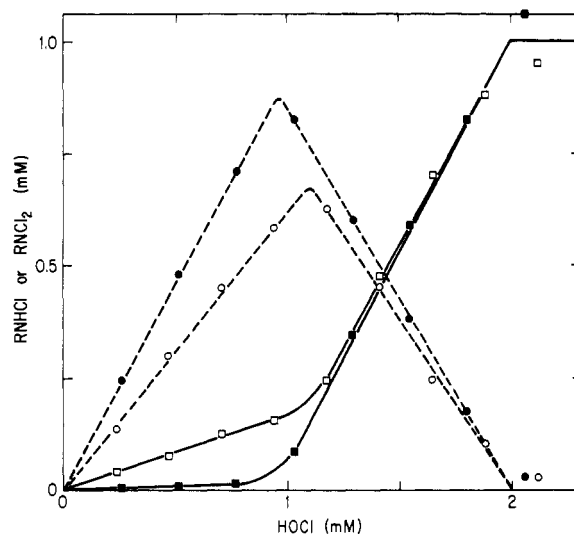

FIGURE 4: Formation of RNHCl and RNCl$_2$ derivatives of taurine. Additions of HOCl were made at 1-min intervals to 1 mM taurine in 0.2 M NaCl–0.1 M phosphate at pH 5 (O, □) or pH 8 (●, ■), and the yield of RNHCl [dashed lines (O, ●)] and RNCl$_2$ [solid lines (□, ■)] was calculated as described from absorbance at 252 and 300 nm. the reference cuvette contained the same components without OCl^-.

of HOCl with taurine to form RNHCl and RNCl$_2$ appeared essentially irreversible.

Figure 4 also shows that at HOCl/taurine ratios >1, the yield of RNCl$_2$ increased sharply and reached a maximum at 2. Under these conditions, similar yields of RNCl$_2$ were obtained at pH 5 or 8. At the HOCl/taurine ratio of 2, the reaction of HOCl with taurine appeared quantitative according to the equation

$$2HOCl + RNH_2 \rightarrow RNCl_2 + 2H_2O$$
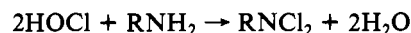
The ability of the solution to oxidize Nbs was consistent with the calculated yield of RNCl$_2$, indicating that no significant portion of the oxidizing equivalents had been lost. Adding excess taurine to the RNCl$_2$ solution did not increase the absorbance at 252 or 300 nm, indicating that no detectable amount of free HOCl was present. Adding HOCl in excess did not increase the absorbance at 252 or 300 nm, when corrected for absorbance of the excess HOCl or OCl^-. Also, none of the oxidizing equivalents could be extracted into organic solvents until the HOCl/taurine ratio exceeded 2, whereas when HOCl was added into pH 7 buffer without taurine, 80% of the oxidizing equivalents were extracted at a 1/1 ratio of ethyl acetate to aqueous volume.

If the order of addition shown in Figure 4 was reversed, so that increasing amounts of taurine were added into a solution of HOCl at pH 7, then RNCl$_2$ was the major product at all HOCl/taurine ratios, and little or no RNHCl was observed. The yield of RNCl$_2$ reached a maximum when the amount of taurine added was half the initial concentration of HOCl. Adding more taurine at pH 7 did not decrease the amount of RNCl$_2$ or increase the amount of RNHCl, within 1 h at 37 °C. Slow redistribution of the Cl(+1) moiety was observed only at extremes of pH, as described in a later section. These results also indicated that RNHCl, RNCl$_2$, and HOCl were not in equilibrium, in that the yield of products depended on the order of addition of HOCl and taurine rather than on their final concentrations.

In other experiments, incorporation at pH 8 was measured after adding HOCl up to 2 mM into 10 mM solutions of amines at pH 5, after adding HOCl up to a HOCl/amino group ratio of 2 at pH 5 or 8, or after adding amines into a
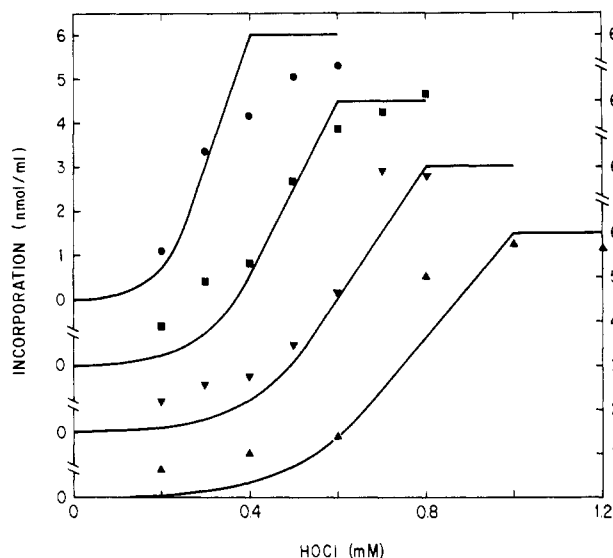
FIGURE 5: Inhibition by amines. Reaction mixtures (75 μL) containing ^{3}[H]taurine in 0.12 M NaCl–50 mM phosphate, pH 7, without other amines (●) or with $(CH_3)_2NH$ (■), CH_3NH_2 (▼), or NH_4^+ (▲) were supplemented with HOCl, incubated 5 min at 25 °C, diluted with 25 μL of 2 mM RNase, and incubated 1 h at 37 °C. Final concentrations were 0.2 mM [^{3}H]taurine, 0.2 mM $(CH_3)_2NH$, 0.2 mM CH_3NH_2, and 0.1 mM NH_4^+.

HOCl solution at pH 7. Under all conditions, incorporation was proportional to $RNCl_2$, indicating that RNHCl did not participate or interfere.

Inhibitors. Figure 5 shows that incorporation of label from [^{3}H]taurine into RNase was inhibited if an unlabeled amine was present during the formation of the [^{3}H]$RNCl_2$ derivative. Because the labeled and unlabeled amines competed for reaction with HOCl, larger amounts of HOCl were required to produce [^{3}H]$RNCl_2$. With an equal concentration of [^{3}H]-taurine and unlabeled dimethylamine, 1.5 times as much HOCl was required, consistent with reaction of $(CH_3)_2NH$ with only 1 mol of HOCl. In the presence of an equal concentration of methylamine, 2 times as much HOCl was required, consistent with reaction of CH_3NH_2 with 2 mol of HOCl. With NH_4^+ at 0.5 times the [^{3}H]taurine concentration, 2.5 times as much HOCl was required, consistent with reaction of NH_3 with 3 mol of HOCl. Therefore, these compounds competed by reacting with the predicted amounts of HOCl, although it is unlikely that CH_3NCl_2 or NCl_3 was present, in that these substances are unstable. Trimethylamine, $(CH_3)_3N$, would not form a N–Cl derivative, and this compound did not inhibit at HOCl/[^{3}H]taurine ratios up to about 2. Some inhibition was observed at the highest ratios, possibly due to demethylation of trimethylamine.

Unlabeled amines did not inhibit when they were added after formation of labeled $RNCl_2$ at pH 7. For example, unlabeled lysine or taurine at 10 mM did not inhibit incorporation of label from 0.2 mM [^{3}H]$RNCl_2$, when the amines were added along with the acceptor protein at pH 7. Mild reducing agents such as glucose also did not inhibit at 10 mM concentrations. However, readily oxidized substances such as sulfhydryl compounds (e.g., Nbs or reduced glutathione, GSH), thioethers (e.g., methionine), or diketo compounds (e.g., 2,4-pentanedione) inhibited incorporation by reducing $RNCl_2$.

Figure 6 shows that different patterns of inhibition were obtained with methionine and glutathione disulfide (GSSG). When methionine was added along with the acceptor protein to the preformed $RNCl_2$ derivative, the amount of methionine required to inhibit incorporation depended on the $RNCl_2$ concentration and did not increase upon raising the acceptor
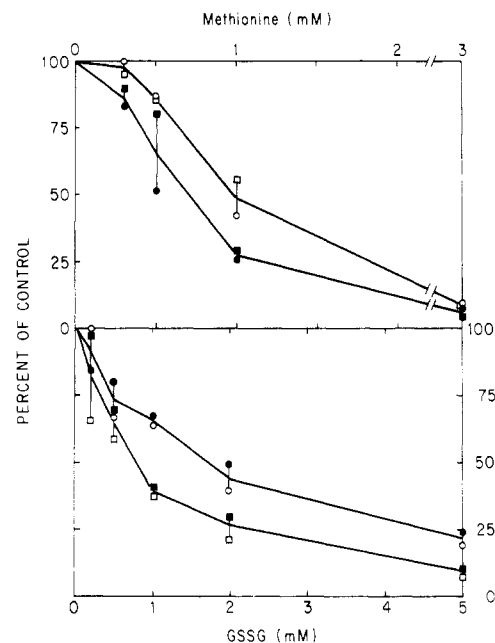

FIGURE 6: Inhibition by methionine and GSSG. Reaction mixtures (75 μL) containing [^{3}H]taurine in 0.12 M NaCl–50 mM phosphate, pH 7, were supplemented with HOCl at a 2/1 ratio to [^{3}H]taurine, incubated 5 min at 25 °C, diluted with 25 μL of RNase solution containing methionine (top) or GSSG (bottom), and incubated 1 h at 37 °C. Final concentrations of methionine and GSSG are shown. Other final concentrations were as follows: $RNCl_2$ = 0.4 mM with protein = 0.5 (○) or 0.15 mM (□); $RNCl_2$ = 0.2 mM with protein = 0.5 (●) or 0.15 mM (■) (top); $RNCl_2$ = 0.4 (○) or 0.2 mM (●) with protein = 0.5 mM; $RNCl_2$ = 0.4 (□) or 0.2 mM (■) with protein = 0.15 mM (bottom).

protein concentration. The concentration of methionine required for 50% inhibition increased from 0.75 to 1.5 mM upon raising the $RNCl_2$ concentration from 0.2 to 0.4 mM. In contrast, the amount of GSSG required to inhibit incorporation did not increase upon raising the $RNCl_2$ concentration. Instead, the amount required for 50% inhibition increased from 0.85 to 2 mM (2.4-fold) when the protein concentration was increased from 0.15 to 0.5 mM (3.3-fold). These results suggested that methionine reduced $RNCl_2$ whereas GSSG competed with RNase as the acceptor for the incorporation reaction.

Phenols competed for incorporation in the same way as GSSG. With 0.5 mM RNase at pH 7, concentrations of tyrosine, phenol, and cresol required for 50% inhibition were about 1, 1, and 0.1 mM, respectively. Similar results were obtained with imidazole compounds. Concentrations of histidine, histamine, and imidazole required for 50% inhibition were about 0.02, 3, and 30 mM, respectively. No inhibition was observed with tryptophan or other α-amino acids at concentrations up to 10 mM. These results suggested that histidine, tyrosine, and cystine residues were the principal protein moieties involved in incorporation into RNase.

Acceptor Proteins and Poly(amino acids). RNase was selected as an acceptor protein of known amino acid composition and molecular weight, with no sulfhydryls, and with high solubility from pH 5 to pH 8. Similar amounts of incorporation were measured by precipitating labeled RNase with acid or with a 9/1 ratio of acetone to aqueous volume. Also, labeled RNase was separated from low molecular weight labeled compounds by chromatography on Sephadex G-25. The amount of label in fractions that contained RNase was equal to the amount measured by acid precipitation. Similar results were obtained with other proteins. With bovine serum albumin, no incorporation was obtained unless the amount of ox-
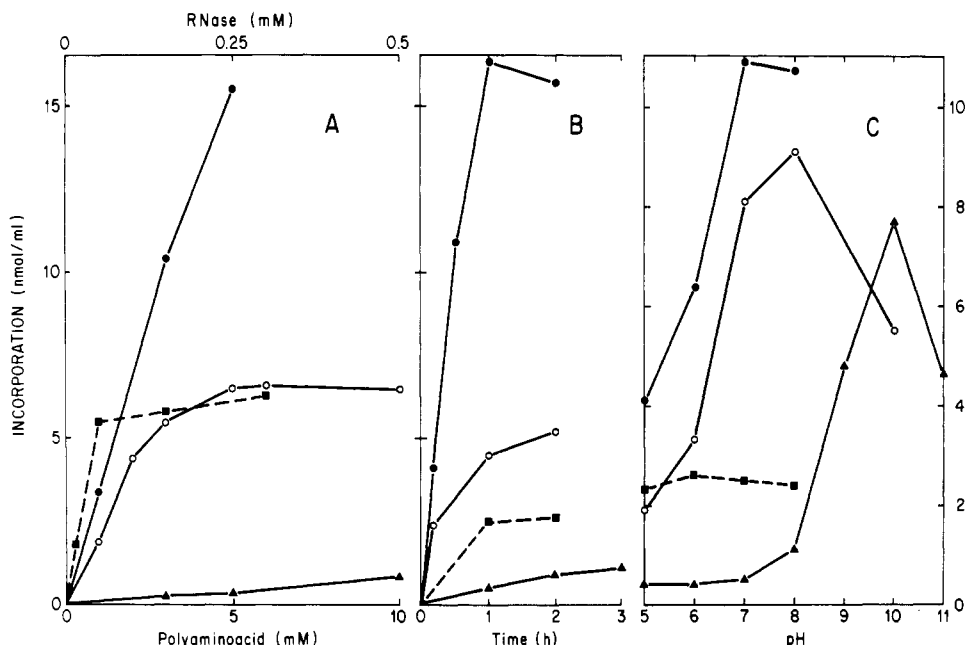
FIGURE 7: Comparison of protein and poly(amino acid) acceptors. Reaction mixtures (75 μL) containing [³H]taurine in 0.12 M NaCl–5 mM phosphate, pH 7, were supplemented with HOCl at a 2/1 ratio to [³H]taurine, incubated 5 min at 25 °C, and diluted to a final [³H]RNCl₂ concentration of 0.2 mM with 25 μL of buffered solutions of polyhistidine (●), RNase (○), polytyrosine (■), or polylysine (▲). Mixtures at pH 7 were incubated 1 h at 37 °C, with the indicated final concentrations of acceptors (A). Mixtures at pH 7 with polyhistidine (5 mM), RNase (0.5 mM), polytyrosine (1 mM), or polylysine (10 mM) were incubated for the indicated periods at 37 °C (B). Reaction mixtures at the indicated pH containing polyhistidine (3 mM), RNase (0.5 mM), polytyrosine (1 mM), or polylysine (10 mM) were incubated 1 h at 37 °C (C). Mixtures containing polytyrosine were 50% (v/v) in dimethyl sulfoxide.

idizing agent was greater than the amount required to oxidize all the protein sulfhydryls.

In Figures 1–3, RNase was 0.5 mM so that the concentration of any amino acid residue in RNase was greater than the initial concentration of the labeled amine and much greater than the amount of incorporation. In other experiments, RNase was incubated with a 2/1 mixture of HOCl/unlabeled taurine and then washed by acetone precipitation. This pretreatment did not alter the ability of RNase to act as an acceptor with HOCl and [³H]taurine. These observations indicated that the maximum amount of incorporation was not limited by the amount of reactive functional groups of the acceptor protein.

Incorporation was also obtained with polyhistidine, -tyrosine, or -lysine as the acceptor but not with polytryptophan, -arginine, -asparagine, -(glutamic acid), -serine, or -(O-acetyl-tyrosine). Although incorporation was not limited by the amount of amino acid residues, an acceptor concentration dependence was observed (Figure 7A). Concentrations of poly(amino acids) are given as the concentration of amino acid residues, whereas RNase concentration is the concentration of polypeptide molecules. Polyhistidine was the best acceptor of the poly(amino acids), and polyhistidine at 5 mM (0.7 mg/mL) was a better acceptor than RNase at 0.25 mM (3.3 mg/mL). At this concentration, RNase would contain residues equivalent to 1 mM histidine, 1.5 mM tyrosine, 2.5 mM lysine, and 1 mM cystine.

Figure 7B shows that the reaction required at least 1 h to go to completion. In other experiments, the time required was longer at low pH or at low acceptor concentrations. Incorporation had a high pH optimum, particularly with polylysine as the acceptor (Figure 7C). There was little incorporation into polylysine at pH 7, which accounts for the observation that free lysine did not inhibit incorporation of [³H]taurine into RNase at pH 7. No pH dependence was observed with polytyrosine, probably due to the high concentration of dimethyl sulfoxide used to solubilize this acceptor.

In other experiments, the rate of disappearance of RNCl₂ in the presence of protein was measured from the loss of ability to oxidize Nbs. All the RNCl₂ was consumed within 3 h, although only 2–4% of the label in RNCl₂ was incorporated. Therefore, the major limitation to the amount of incorporation was the consumption of RNCl₂ in reactions that did not result in incorporation, such as oxidation of methionine or other amino acid residues. Also, the rate of loss of RNCl₂ was about the same at pH 5 or 8. Therefore, the incorporation reaction did not make major contribution to the disappearance of RNCl₂.

In similar experiments with the RNCl₂ derivative of [³H]taurine at 0.2 mM and with 3 mM polyhistidine as the acceptor at pH 8, incorporation was complete within 6 h at 37 °C, although the total amount of oxidizing equivalents had decreased by only 30%. As described below, the reaction of RNCl₂ with amino groups at high pH converted RNCl₂ to RNHCl with little or no loss of oxidizing equivalents. Therefore, the reaction of RNCl₂ with histidine residues or N-terminal amino groups of polyhistidine could convert [³H]RNCl₂ to [³H]RNHCl, bringing the incorporation reaction to an end.

Decomposition of RNHCl and RNCl₂. The RNHCl derivative of taurine was stable for at least 24 h at neutral to alkaline pH. At pH 5, solutions containing primarily RNHCl underwent a series of reactions. In the initial phase (1–4 h at 37 °C) about 1 mol of RNCl₂ was obtained per 2 mol of RNHCl lost, and there was only a small loss of oxidizing equivalents. These results were consistent with the dismutation of RNHCl at low pH (Gray et al., 1978).

$$2RNHCl \rightarrow RNCl_2 + RNH_2$$

After prolonged incubations (24–48 h), the yield of RNCl₂ had fallen to about 0.5 mol/2 mol of RNHCl lost, and about half the total oxidizing equivalents were lost, apparently due to decomposition of RNCl₂.
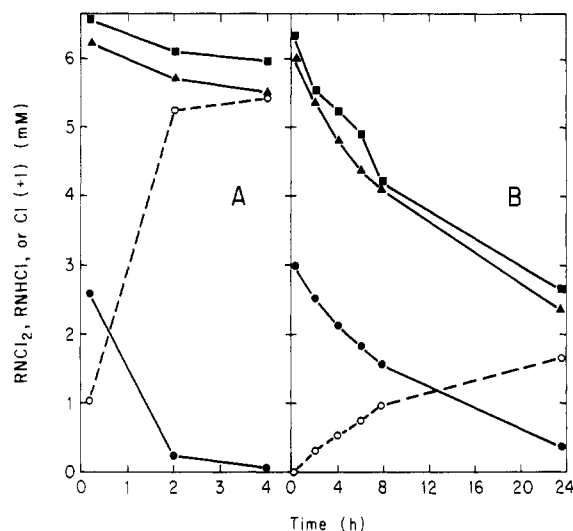
In contrast to observations with RNHCl, the RNCl₂ de-

FIGURE 8: Decomposition of $RNCl_2$ in the presence and absence of RNH_2. The $RNCl_2$ derivative of taurine was diluted to 3 mM with an equal volume of 0.2 M phosphate, pH 10, containing 60 mM taurine (A) or without taurine (B) and incubated at 37 °C. Concentrations of $RNCl_2$ (●) and RNHCl (○) were calculated from absorbance at 252 and 300 nm. Concentration of Cl(+1) was determined by oxidation of Nbs (■) or calculated as the sum of RNHCl plus 2 times $RNCl_2$ (▲).

rivative of taurine was more stable at low pH. Decomposition was studied by forming $RNCl_2$ at pH 7, adjusting to the desired pH, and then incubating at 37 °C. At intervals, portions of the reaction mixture were taken, and the rate of loss of oxidizing equivalent determined by the loss of ability of the solution to oxidize Nbs. Also, the rate of loss of $RNCl_2$ was measured from the loss of absorbance at 300 nm, corrected for changes in the concentration of RNHCl as measured from absorbance at 252 nm. About 1%, 3%, and 10% of the $RNCl_2$ was lost in the first hour at pH 5, 8, and 10, respectively.

At intervals, [³H]$RNCl_2$ solutions at pH 5, 8, or 10 were adjusted to pH 7 and incubated with 0.5 mM RNase for 1 h at 37 °C. The ability to incorporate into protein was lost as the $RNCl_2$ disappeared; there was no accumulation of an incorporating species. Therefore, if a decomposition product was involved in incorporation, this product was short-lived.
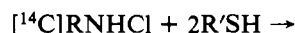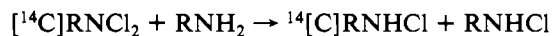
The rate of loss of $RNCl_2$ at high pH and the yield and identity of decomposition products were influenced by adding taurine (RNH_2) or other amines. Figure 8A shows results obtained with a RNH_2/$RNCl_2$ ratio of 10. The $RNCl_2$ derivative disappeared within 1–2 h at 37 °C, and about 2 mol of RNHCl was obtained per mol of $RNCl_2$ lost. Therefore, the total amount of Cl(+1), calculated as the sum of RNHCl plus 2 times $RNCl_2$, remained nearly constant. Also, there was only a small loss of oxidizing equivalents, as measured by the ability of the solution to oxidize Nbs. These results were consistent with the reaction of $RNCl_2$ with RNH_2 to yield two RNHCl, which was reported to occur at high pH (Gray et al., 1978).

$$RNCl_2 + RNH_2 \rightarrow 2RNHCl$$

Figure 8B shows that the loss of $RNCl_2$ was much slower in the absence of added RNH_2. Over half the total Cl(+1) and oxidizing equivalents were lost. About 0.5 mol of RNHCl was obtained per mol of $RNCl_2$ lost. As described below, about 0.5 mol of $RNCl_2$ was converted to other products. The increase in absorbance at 252 nm appeared to be due to formation of RNHCl rather than to formation of the other products, in that this absorbance could be eliminated by adding an amount of sulfhydryl compound sufficient to eliminate the

remaining oxidizing equivalents.

Following complete decomposition of the $RNCl_2$ derivative of [¹⁴C]taurine, the remaining oxidizing equivalents were reduced with dithiothreitol (R′SH), and the reaction mixture was subjected to thin-layer chromatography. With an RNH_2/[¹⁴C]$RNCl_2$ ratio of 10, as in Figure 8A, most of the label was recovered as [¹⁴C]taurine, with an R_f value of 0.3. The [¹⁴C]taurine obtained under these conditions was due to formation of the [¹⁴C]RNHCl derivative, which was reduced to [¹⁴C]taurine by R′SH.

$$[^{14}C]RNCl_2 + RNH_2 \rightarrow {}^{14}[C]RNHCl + RNHCl$$

$$[^{14}C]RNHCl + 2R'SH \rightarrow$$
$$[^{14}C]RNH_2 + R'SSR' + H^+ + Cl^-$$

When decomposition of [¹⁴C]$RNCl_2$ occurred in the absence of RNH_2 as in Figure 8B, about half the label was recovered as [¹⁴C]taurine after reduction, due to formation of about 0.5 mol of [¹⁴C]RNHCl/mol of [¹⁴C]$RNCl_2$. The other half of the label was recovered in one or more decomposition products with R_f values of 0.5–0.6 and in a product with an R_f of 0.7. None of these high R_f products were converted to [¹⁴C]taurine by treatment with excess dithiothreitol, sodium borohydride, or acid.

When histidine, tyrosine, or GSSG was present during decomposition of the [¹⁴C]$RNCl_2$ derivative of taurine, there was an increase in labeled products with low mobility (R_f 0–0.2). These products were presumably the result of the incorporation reaction. The amino acids or GSSG had no effect when added after decomposition of [¹⁴C]$RNCl_2$. The time course of incorporation into histidine at pH 10 paralleled the decomposition of [¹⁴C]$RNCl_2$, as measured by appearance of the high R_f decomposition products. Decomposition and incorporation were about 85% complete after 6 h at 37 °C with 0.2 mM histidine and 0.2 mM [¹⁴C]$RNCl_2$. After decomposition was complete, the amount of label in low R_f incorporation products with 0.2 mM histidine, tyrosine, and GSSG was about 5%, 3%, and 2% of the label originally in 0.2 mM [¹⁴C]$RNCl_2$.

The percentage of incorporation decreased when the ratio of the acceptor to [¹⁴C]$RNCl_2$ was greater than 10. Because histidine, tyrosine, and GSSG contain amino groups, these compounds had the same effect as excess unlabeled taurine on the recovery of label from [¹⁴C]$RNCl_2$ in [¹⁴C]RNHCl.

Incorporation with Granule Extracts. Extracts from leukocyte granules contain MPO and other proteins that would be present in the phagolysosome when the antimicrobial activity of MPO is expressed. Such extracts were found to have a high content of substances with primary amino groups, which competed with added labeled amines for reaction with HOCl. With a protein concentration of 0.55 mg/mL, the concentration of primary amines was estimated as 0.25 mM by reaction with fluorescamine, relative to taurine as the standard. The fluorescent intensity of fluorescamine derivatives varies widely (deSilva & Strojny, 1975), so that the absolute concentration could not be determined in this way.

Extracts were supplemented with a low concentration of [³H]taurine, 0.2 M Cl⁻, and increasing amounts of H_2O_2. Incorporation was higher at pH 5 than at pH 7, and the maximum incorporation was obtained with 0.4–0.5 mM H_2O_2. This amount of H_2O_2 was about twice the estimated amount of amines. Chromatography of labeled extract on Sephadex G-100 was used to detect incorporation products, as shown in Figure 9. The distribution of label was not identical with the protein distribution. The major portion of acid-precipitable label was obtained in components of relatively low molecular
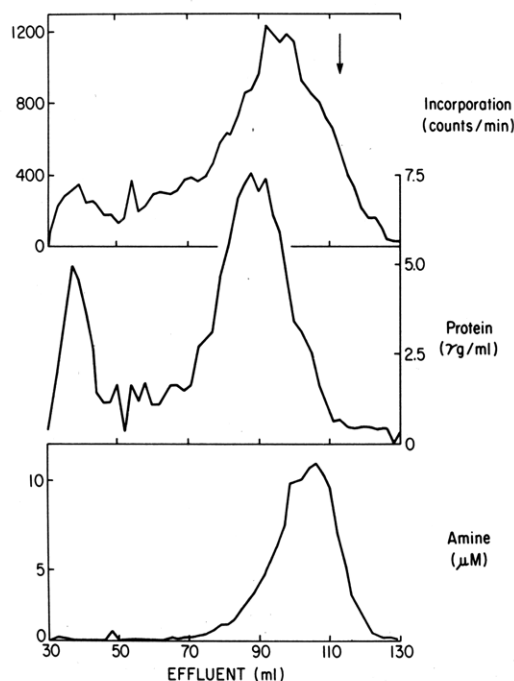
FIGURE 9: Chromatography of labeled extract. Granule extract (0.55 mg of protein) in 1 mL of 0.2 M NaCl–20 mM phosphate, pH 5, with 10 μM [³H]taurine was supplemented with 0.4 mM H₂O₂, incubated 1 h at 37 °C, and then chromatographed on Sephadex G-100. Portions of effluent fractions were taken for determination of total label, acid-precipitable label, protein, and primary amines. The arrow indicates the peak fraction of nonprecipitable label.
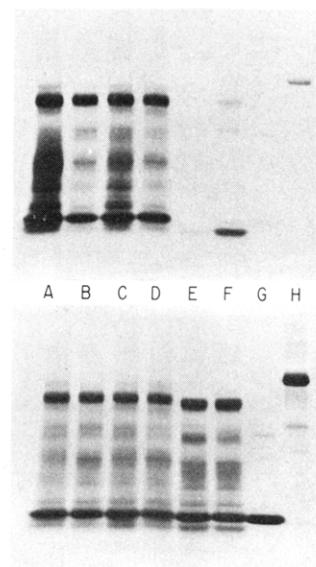

FIGURE 10: Gel electrophoresis of labeled granule extract followed by fluorography (top) and protein staining (bottom). Reaction mixtures (0.2 mL) containing granule extract (0.11 mg of protein) in 0.12 M NaCl–20 mM phosphate at pH 5 (A) or pH 7 (B) with 10 μM [³H]taurine were supplemented with 0.4 mM H₂O₂. Alternatively, reaction mixtures at pH 5 (C) and pH 7 (D) were supplemented with 0.4 mM HOCl. In (E–H), the RNCl₂ derivative of [³H]taurine (20 μM) was diluted with an equal volume of granule extract (0.11 mg of protein) in 0.12 M NaCl–25 mM phosphate at pH 5 (E) or pH 7 (F) or with the molecular weight markers lysozyme (14 000) (G) or lactoferrin (80 000) (H) at pH 7. Incubations were continued for 1 h at 37 °C.

weight (25 000–10 000). Extracts from human leukocyte granules contain a class of "arginine-rich" polypeptides in this molecular weight range, and these polypeptides also have a high histidine content (Olsson & Venge, 1972). These granule components may be the major acceptors for the incorporation reaction. In other experiments, a similar distribution of label was obtained at pH 7 by adding HOCl rather than H₂O₂ or by reacting [³H]taurine with HOCl and then adding the preformed ³[H]RNCl₂ to the extract.

Figure 9 also shows that a large amount of fluorescamine-reactive substances was observed with apparent molecular weights of 15 000–3000. The molecular weight distribution of these amines overlapped with that of the acid-precipitable labeled components and with that of proteins. However, the highest amounts of amines were obtained in fractions that contained little protein as determined by the dye-binding assay or from absorbance at 230 or 280 nm.

Presumably, the major form of incorporation that was obtained under these conditions was the incorporation of the low molecular weight, unlabeled amines into proteins. However, this method detected only incorporation of label from [³H]-taurine, as a representative of the amines that were present. Only 0.6% of the label added was incorporated. If it is assumed that [³H]taurine and the unlabeled amines reacted homogeneously with HOCl, and were incorporated to the same extent, the amount of incorporation was 2.7 nmol of amines/mg of protein.

In experiments at pH 5 with labeled amines at 10 μM, H₂O₂ at 0.4 mM, and a different extract preparation, the relative amounts of incorporation were similar to results in Figure 3. Incorporation as a percentage of label added was as follows: 1,2-diaminoethane, 4.0%; histamine, 2.9%; putrescine, 2.4%; taurine, 1.3%; lysine, 0.5%; leucine, 0.2%; glucosamine, methylamine, 0.1%.

Figure 10 (top) shows incorporation of label from [³H]-taurine into granule extract polypeptides as determined by gel electrophoresis and fluorography. A similar distribution of label was obtained by adding H₂O₂ (lanes A and B) or HOCl (lanes C and D), and incorporation was higher at pH 8 (lanes A and C). The distribution of label appeared much different from that determined by chromatography (Figure 9). Part of the low molecular weight, acid-precipitable, labeled components shown in Figure 9 were lost upon acidifying and centrifuging the reaction mixture and in fixing, staining, and preparing the gel for fluorography. For this reason, a larger proportion of the label observed on gels was in the higher molecular weight polypeptides.

When the extract was labeled by adding the preformed RNCl₂ derivatives of [³H]taurine (lanes E and F), incorporation was higher at pH 7 than at pH 5, and the distribution of label was different from that in lanes A–D. A larger proportion of the incorporation was into the low molecular weight components that were not recovered on the gel. Also, several of the labeled polypeptides migrated with a lower apparent molecular weight than in lanes A–D.

Figure 10 (bottom) shows the apparent molecular weight distribution of polypeptides as determined by staining for protein. When incorporation was obtained by adding H₂O₂ or HOCl into the extract (lanes A–D), there was an increase in the apparent molecular weight of a number of polypeptides, compared to samples in which the preformed [³H]RNCl₂ derivative was added (lanes E and F). This increase in apparent molecular weight may be due to incorporation of the low molecular weight fluorescamine-reactive substances into the polypeptides. Samples incubated with the low concentration of preformed [³H]RNCl₂ (lanes E and F) appeared identical with untreated samples (results not shown).

Discussion

The results indicate that MPO catalyzes incorporation of primary amines into derivatives of proteins and that HOCl

and the $RNCl_2$ derivative of the amine are intermediates in the reaction. This reaction accounts for the MPO-mediated incorporation of label from $[^{14}C]$glucosamine in leukocytes and provides evidence that HOCl and N–Cl derivatives are formed in leukocytes.

In these studies on MPO-catalyzed incorporation of amines in vitro, the highest incorporation was obtained when the reaction was performed sequentially. First, MPO-catalyzed oxidation of Cl^- to HOCl was carried out at the low pH optimum of MPO (Zgliczynski et al., 1977).

$$H_2O_2 + Cl^- + H^+ \rightarrow H_2O_2 + HOCl$$
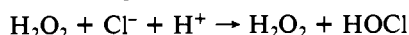
Also, oxidation of Cl^- was carried out in the presence of the amine, so as to prevent inactivation of MPO by HOCl (Zgliczynski et al., 1968; Harrison & Schultz, 1976) and to prevent reduction of HOCl by H_2O_2 (Held & Hurst, 1978; Thomas, 1979a). The HOCl reacted with RNH_2 to yield RNHCl and with RNHCl to yield $RNCl_2$.

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+$$

$$RNH_2 + HOCl \rightarrow RNHCl + H_2O$$

$$RNHCl + HOCl \rightarrow RNCl_2 + H_2O$$
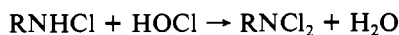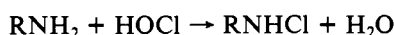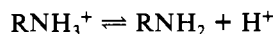
At low HOCl/amine ratios, low pH favored formation of $RNCl_2$ possibly by lowering the concentration of RNH_2 relative to that of RNHCl or by acid-catalysis of the reaction of HOCl with RNHCl (Morris, 1967). Also, $RNCl_2$ could be obtained at low pH from the slow dismutation of RNHCl (Gray et al., 1978). At pH 5

$$2RNHCl \rightarrow RNCl_2 + RNH_2$$

Next, the acceptor protein was added and the pH was raised. Whereas formation of $RNCl_2$ was favored at low pH, the subsequent incorporation reaction was favored at high pH. However, $RNCl_2$ could also react with amino groups of the acceptor molecule at high pH, resulting in conversion of $RNCl_2$ to RNHCl. At pH 8–10

$$RNCl_2 + R'NH_2 \rightarrow RNHCl + R'NHCl$$
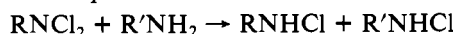
This reaction interfered with incorporation by depleting $RNCl_2$.

When the reaction was not performed sequentially, less incorporation was obtained. The acceptor protein would compete with RNH_2 and RNHCl for reaction with HOCl, interfering with formation of $RNCl_2$. Under these conditions, the highest incorporation was obtained at low pH. Therefore, the pH dependence of formation of $RNCl_2$ appeared more significant than the pH dependence of the subsequent incorporation reaction.

In most reactions involving $RNCl_2$ or other N–Cl derivatives, the Cl(+1) moieties form the reactive portion of the molecule, as in the sulfhydryl oxidation reaction that was used to quantitate RNHCl and $RNCl_2$.

$$RNHCl + 2R'SH \rightarrow RNH_2 + R'SSR' + H^+ + Cl^-$$

$$RNCl_2 + 4R'SH \rightarrow RNH_2 + 2R'SSR' + 2H^+ + 2Cl^-$$
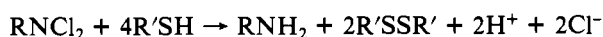
However, it appeared that $RNCl_2$ can undergo reactions in which the RN moiety becomes the activated portion of the molecule and attacks functional groups of proteins.

Incorporation of label from $RNCl_2$ could be due to direct attack of $RNCl_2$ on amino acid residues or to attack by a reactive intermediate formed in the decomposition of $RNCl_2$. Two observations suggested that an intermediate was involved: (1) both incorporation and decomposition were favored at high pH and (2) the rate of decomposition was comparable to the rate of incorporation. However, the amount of incorporation was small, so that it is possible that high pH favored both

decomposition and incorporation, which proceeded by separate, competing mechanisms.

An analogy to the incorporation reaction is offered by the N,N-dichlorosulfonamides (Campbell & Johnson, 1978). These compounds undergo reactions with a wide variety of functional groups, primarily involving attack by Cl(+1). However, they also undergo reactions with certain functional groups including disulfides and thioethers that result in insertion (incorporation) of the RSO_2N moiety. The mechanism involves oxidative attack on the acceptor functional group to give an intermediate that rearranges to the product containing RSO_2N. Decomposition of RSO_2NCl_2 is not involved, though the reaction may be initiated by a species derived from RSO_2NCl_2, such as RSO_2NCl^- or RSO_2NH^-. These reactions were thought to be unique to RSO_2NCl_2 and the related N,N-dichlorocarbamates. However, results presented here indicate that similar reactions are obtained with $RNCl_2$ derivatives of a wide variety of primary amines, though the yield of incorporation products is low.

Although the results are similar, incorporation reactions of RSO_2NCl_2 and $RNCl_2$ may proceed by different mechanisms. There are a number of reactive intermediates that might be formed during decomposition of $RNCl_2$ or in other reactions involving N–Cl derivatives. Intermediates such as $RNCl^-$ (Yagil & Anbar, 1962) or $R'\!—CH\!=\!NCl$ (Stelmaszynska & Zgliczynski, 1978) have been proposed.

$$RNCl_2 + OH^- \rightarrow RNCl^- + OCl^- + H^+$$

$$R'\!—CH_2\!—NCl_2 \rightarrow R'\!—CH\!=\!NCl + Cl^- + H^+$$
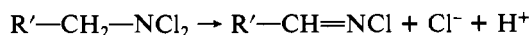
Although incorporation of amines probably accompanies antimicrobial action by HOCl or N–Cl derivatives in leukocytes, the characteristics of the incorporation reaction suggest that it would not contribute significantly to antimicrobial action. That is, the reaction is quantitatively minor, is slow, has a high pH optimum, and results in modification of tyrosine and histidine residues. In the latter respect, the incorporation reaction resembles iodination of proteins (Morrison & Schonbaum, 1976). Iodination of aromatic amino acid residues can accompany antimicrobial action but is not directly related to loss of microbial viability (Pincus & Klebanoff, 1971; Klebanoff & Clark, 1977; Thomas & Aune, 1978). Measurements of chemical modification of microbial components in vitro by HOCl (Knox et al., 1948), N–Cl derivatives (Thomas, 1979a,b; Kohl et al., 1980), or the $MPO–H_2O_2–Cl^-$ system (Thomas, 1979a,b) indicate that antimicrobial action is correlated with oxidation of microbial sulfhydryl components. It has also been proposed that oxidation of cofactors or enzyme prosthetic groups (Albrich et al., 1981) may contribute to killing.

Nevertheless, incorporation of labeled amines into microbial proteins, granule components, or phagocytized particles may provide a method for studying MPO action in vivo. The utility of the method is limited in that there is no direct quantitative relation between incorporation of label and the amount of HOCl or N–Cl derivatives. However, the sensitivity of the method is limited only by the specific activity of the labeled amine and the extent to which the specific activity is diluted by other amines that may be present. In this study, extracts from leukocyte granules contained high concentrations of primary amines. These amines competed with labeled amine for reaction with HOCl, so that high concentrations of HOCl were required for incorporation. However, incorporation of label was observed even in the presence of the competing amines. The origin and identity of these amines remain to be determined, and it is not known whether these substances

would be present in the phagolysosome.

MPO-catalyzed incorporation of labeled amines may also provide a method to evaluate the effect of exogenous amines on MPO-mediated antimicrobial action in leukocytes. There are a number of routes by which amines could enter the phagolysosome. Primary amines, like other weak bases, tend to become concentrated in acidic cellular compartments, in that they diffuse through membranes in the unchanged form and then are trapped by conversion to the charged, protonated form. The pH within the phagolysosome may fall as low as pH 5 (Mandel, 1970; Jensen & Bainton, 1973). Amines from the extracellular medium may also be taken up in the entrapped volume that accompanies the phagocytized particle. Glucosamine may be transported into leukocytes (Swendsen & DeChatelet, 1981) and may enter the phagolysosome by transport or diffusion. Leukocytes also contain high levels of endogenous amines, including taurine (Soupart, 1962) and polyamines (Rennert & Shukla, 1978). The entry of amines into the phagocytic vesicle could influence antimicrobial activity by yielding N–Cl derivatives with differing antimicrobial activity.

Acknowledgments

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