IODINATION OF BACTERIA: A BACTERICIDAL MECHANISM

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Neutrophilic polymorphonuclear (PMN) leukocytes are exceptionally rich in peroxidase. Agner (1) has estimated the peroxidase content of human myeloid leukemic leukocytes to be about 1–2% of the dry weight of the cells, whereas Schultz and Kaminker (2) have suggested that the normal human neutrophil has a peroxidase content of greater than 5% of the dry weight of the cell. This enzyme was originally called verdoperoxidase by Agner (1) due to its green color; however, the name myeloperoxidase was subsequently suggested with the finding that the milk peroxidase, lactoperoxidase, was also green in color, and this name is now generally employed. Recently, the name neutrophil peroxidase has been suggested (3) since a second cell of myeloid origin, the eosinophil, is also extremely rich in peroxidase, and the eosinophil peroxidase appears to differ in several respects from the neutrophil enzyme.

Peroxidases alone have not been shown to exert an antibacterial effect. However, it is possible, at least theoretically, for a peroxidase to exert an antibacterial effect indirectly by catalyzing the conversion of a substance with low antibacterial properties into one with high antibacterial properties. Thus Kojima (4) has reported that peroxidase and H_2O_2 considerably increase the germicidal power of a number of phenols, presumably by conversion of the phenol to the corresponding quinone. In addition, lactoperoxidase has been found to inhibit the growth of a number of organisms in complete growth medium when combined with thiocyanate ions and H_2O_2; and this antimicrobial system appears to be present naturally in milk and in saliva (5–17). Thiocyanate ions can be replaced by iodide ions (10, 11, 13) and lactoperoxidase by a purified preparation of myeloperoxidase (13, 14). This demonstration of a myeloperoxidase-mediated antibacterial system prompted a study of the role of myeloperoxidase in the killing of bacteria by leukocytes. This publication will consider the bactericidal effect of myeloperoxidase, iodide, and H_2O_2. A preliminary report of this work has appeared elsewhere (18).

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Materials and Methods

Determination of Bactericidal Effect.—

The components indicated in the tables were placed in 15 × 100 mm test tubes and were incubated in an atmosphere of air for 30 min at 37°C with shaking (120 oscillations per minute in an Eberbach water bath shaker). Aliquots (0.1 ml) were removed at intervals, and the viable cell count was determined by the pour plate method using Trypticase soy agar after suitable dilutions in water. In the initial experiments, 0.9% NaCl was employed as the diluent. However, the finding that chloride ions influenced the bactericidal effect (18) prompted the change to water. The cells were plated immediately after dilution and, under these conditions, suspension in water had no effect on the viable cell count.

Iodide Incorporation into TCA-Precipitable Material.—

Isolated bacteria: The components indicated in the tables were incubated in 10 × 75 mm test tubes at 37°C with shaking (120 oscillations per minute) for 30 min unless otherwise indicated. The reaction was terminated by the addition of 0.1 ml of 0.1 M sodium thiosulfate. 1 ml of cold 10% trichloroacetic acid (TCA) was added, the precipitate collected by centrifugation at 2000 *g* for 5 min in an International PR-2 centrifuge and washed four times with 1.0 ml of cold 10% TCA. The test tube containing the washed precipitate was placed directly into a plastic counting tube (Packard Instrument Co., Downers Grove, Ill.) and counted in a Packard Auto-Gamma well scintillation counter. The results are expressed as mμmoles of iodide converted to a TCA-precipitable form.

Intact guinea pig leukocytes: All glassware was siliconized prior to use. Male guinea pigs weighing between 250 and 350 g were injected intraperitoneally with 20 cc of 12% casein. The animals were sacrificed 16 hr later by decapitation, and the blood was collected by allowing it to drip into a 50 ml centrifuge tube. The peritoneal cavity was opened and the peritoneal fluid removed with a Pasteur pipette and passed through four layers of cheesecloth into a 50 ml centrifuge tube. The cells were collected by centrifugation at 500 *g* for 5 min and suspended in HBG, which is a modified Hanks' solution containing glucose and crystalline bovine albumin (19). The white blood cell density was determined with a hemocytometer and the preparation was immediately employed.

The components, indicated in the legends, were incubated in 10 × 75 mm test tubes at 37°C for 60 min with shaking. The reaction was terminated and the TCA precipitate was prepared and counted as described above.

Radioautographic Studies.—

Isolated bacteria: Heat-killed *Lactobacillus acidophilus* (6.5×10^6 organisms) were incubated in a reaction mixture which contained 100 μmoles of acetate buffer pH 5.0, 0.1 μmole of sodium iodide containing 5–10 μc of ^{125}I, 5 μmoles of glucose, 0.5 μg of glucose oxidase, 150 *o*-dianisidine units of myeloperoxidase, and water to a final volume of 0.5 ml, for 30 min at 37°C with shaking. Controls were employed in which either myeloperoxidase, or glucose and glucose oxidase, or all three were deleted from the reaction mixture. The reaction was stopped and the proteins precipitated and washed with TCA as described above. In some experiments, the bacteria were collected by centrifugation and washed with water without treatment with TCA. In each instance, the washed material was suspended in 0.2 ml of water and cover slip smears were prepared using clean unsubbed cover slips. The smears were fixed in absolute methanol for 4 min and washed in water for 1 hr. The cover slips were mounted with the smear exposed on subbed slides with methacrylate. The smears were coated with Kodak NTB2 emulsion (1 part emulsion to 2 parts of 1% Kodak Photo-Flo 200 in water) using the dipping machine described by Kopriwa (20). After appropriate exposure at 6°C, the radioautograms were developed, and the smears stained with a mixture of methylene blue and azure II (21).

White blood cell–enriched human blood preparation: All glassware was siliconized prior to use. Human blood (10 ml) was defibrinated with glass beads in a 25 ml Erlenmeyer flask. After centrifugation at room temperature at 1800 *g* for 5 min, approximately 4/5 of the plasma was removed, and the remainder of the plasma was gently mixed with the buffy coat and the upper 1/5 of the RBC layer. This WBC-enriched blood preparation was removed with a Pasteur pipette and was immediately employed.

An aliquot (0.25 ml) of the blood preparation was incubated at 37°C with shaking in 10 × 75 mm test tubes with 2–4 μc of carrier-free Na-^{125}I and, where indicated, with *L. acidophilus* (0.4–1.5 × 10^6 organisms) and Tapazole (0.5 μmole). The final volume was 0.26 ml. Cover slip smears were prepared at intervals up to 30 min. The smears were fixed in methanol, washed in water, and radioautograms prepared as described above.

Determination of Peroxidase Activity.—

Peroxidase activity was determined by the *o*-dianisidine method (22). One unit of activity is that causing an increase in absorbancy of 0.001/min at 460 mμ in a Cary M-15 spectrophotometer.

Materials.—

Escherichia coli (ATCC No. 11775) was grown on Trypticase soy agar and broth (Baltimore Biological Laboratories, Baltimore, Md.) and *L. acidophilus* (ATCC No. 4357) on Lactobacillus Selective (LBS) agar and broth (Baltimore Biological Laboratories). A 16-hr culture in broth was washed two times with water and suspended in water to the required absorbancy at 540 mμ (Coleman Jr. spectrophotometer) just prior to use. Myeloperoxidase was prepared in highly purified form (i.e., to the end of step 6) from canine pyometral pus by the method of Agner (23). One preparation of myeloperoxidase used in this study was kindly supplied by Dr. Cecil Yip. Lactoperoxidase was prepared from bovine milk by the method of Morrison and Hultquist (24). Glucose oxidase (fungal type IV, 88.7 μM units/mg), catalase (bovine liver, 2 times crystallized, 40,000 Sigma units/mg), NAD, NADH, NADP, NADPH,[1] and reduced glutathione were obtained from Sigma Chemical Co., St. Louis, Mo. Ergothioneine was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. 1-Methyl-2-mercaptoimidazole (Tapazole) was kindly supplied by Eli Lilly and Co., Indianapolis, Ind. Na-^{131}I, carrier free in 0.1 N NaOH, and Na-^{125}I, carrier free in 0.1 N NaOH, were obtained from New England Nuclear Corp., Boston, Mass.

The WBC particulate preparation was prepared from guinea pig peritoneal lavage leukocytes as follows: The cells were washed once with water and suspended in water to make a 5% suspension (w/v). The 5% suspension was homogenized in a Potter-Elvehjem homogenizer, the homogenate centrifuged at 20,000 *g* for 15 min, and the particulate fraction resuspended to the original volume of the 5% homogenate with water.

RESULTS

Bactericidal Effect of Myeloperoxidase, Iodide, and H_2O_2.—

Myeloperoxidase alone did not exert a bactericidal effect on *E. coli* under the conditions employed in Table I. However, if myeloperoxidase was combined with iodide ions and H_2O_2, a bactericidal effect was readily apparent. This was the case when the iodide and H_2O_2 concentrations were decreased to levels at

[1] NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

which either alone, or both in combination, had little or no effect on the viable cell count. The bactericidal effect was abolished by preheating the myeloperoxidase at 90° C for 10 min. Myeloperoxidase could be replaced in this bactericidal system by a guinea pig WBC particulate preparation or by lactoperoxidase;

TABLE I

Bactericidal Effect of Peroxidase, Iodide, and H_2O_2

Supplements	Viable cell count
	organisms/ml
None	6.2×10^6
MPO	6.4×10^6
Iodide	6.1×10^6
H_2O_2	5.1×10^6
MPO + iodide	5.8×10^6
MPO + H_2O_2	5.0×10^6
Iodide + H_2O_2	4.9×10^6
MPO + iodide + H_2O_2	0
MPO (heated) + iodide + H_2O_2	4.7×10^6
WBC particulate	5.2×10^6
WBC particulate + iodide + H_2O_2	0
WBC particulate (heated) + iodide + H_2O_2	4.7×10^6
LPO	6.3×10^6
LPO + iodide + H_2O_2	0
LPO (heated) + iodide + H_2O_2	3.9×10^6
MPO + thyroxine	5.6×10^6
Thyroxine + H_2O_2	4.0×10^6
MPO + thyroxine + H_2O_2	0
MPO + triiodothyronine	5.1×10^6
Triiodothyronine + H_2O_2	4.2×10^6
MPO + triiodothyronine + H_2O_2	0
MPO + thyronine + H_2O_2	5.0×10^6
Glucose + glucose oxidase	3.0×10^6
MPO + glucose + glucose oxidase	3.3×10^6
Iodide + glucose + glucose oxidase	3.0×10^6
MPO + iodide + glucose + glucose oxidase	0

The reaction mixture contained sodium acetate buffer pH 5.0, 200 μmoles; *E. coli*, 6.0×10^6 organisms per ml; water to a final volume of 2.0 ml; and the supplements indicated below as follows: myeloperoxidase (MPO), 150 *o*-dianisidine units; WBC particulate, 150 *o*-dianisidine units; lactoperoxidase (LPO), 150 *o*-dianisidine units; iodide, 0.1 μmole; H_2O_2, 0.01 μmole; thyroxine, 0.1 μmole; triiodothyronine, 0.1 μmole; thyronine, 0.1 μmole; glucose, 0.5 μmole; glucose oxidase, 0.5 μg. The peroxidases were heated to 90°C for 10 min where indicated.

H_2O_2 could be replaced by an H_2O_2-generating system such as glucose and glucose oxidase, and iodide ions could be replaced by the iodinated compounds, thyroxine or triiodothyronine, but not by the deiodinated derivative, thyronine. Iodide ions also could be replaced by bromide or chloride ions (18). The effect

of halides other than iodide will be considered in more detail in a later publication.

All the components of the bactericidal system, myeloperoxidase, iodide, and H_2O_2, were required for extensive microbial killing under the conditions employed in Table I. H_2O_2 is a known antibacterial agent and it had a considerable bactericidal effect on *E. coli* when added alone at concentrations higher than those employed in Table I (Table II). Of interest was the finding that the bactericidal effect of H_2O_2 at relatively high concentrations was increased by the addition of iodide ions at pH 5.0. Myeloperoxidase was not required under these conditions. This bactericidal effect of H_2O_2 and iodide at relatively high concentrations was not observed at pH 7.0.

Effect of Preincubation.—

Iodide ions are readily oxidized by peroxidase and H_2O_2 with the formation of iodine, a known antibacterial agent; and the first assumption was that the

TABLE II

Bactericidal Effect of Iodide and H_2O_2

Supplements	Viable cell count
	organisms/ml
None	5.8×10^6
H_2O_2 (0.5 μmole)	3.6×10^5
Iodide (0.5 μmole)	4.8×10^6
H_2O_2 (0.5 μmole) + iodide (0.5 μmole)	0
H_2O_2 (0.05 μmole)	1.1×10^6
H_2O_2 (0.05 μmole) + iodide (0.5 μmole)	3.4×10^3

The conditions were as described in Table I except that the supplements were added in the amounts indicated.

antibacterial effect of myeloperoxidase, iodide, and H_2O_2 was due to the accumulation of iodine in the reaction mixture. However, the following experiment suggested that this was not the case.

Myeloperoxidase, iodide, and H_2O_2 were preincubated in acetate buffer pH 5.0 for periods of up to 30 min at 37°C. The oxidation of iodide during the preincubation period was indicated by the production of a light brown color when the iodide and H_2O_2 concentrations were sufficiently high. The organisms were added at the completion of the preincubation period and the viable cell count was determined at the end of a further 30 min incubation period.

Table III demonstrates that the preincubation of myeloperoxidase, iodide, and H_2O_2 for 5 min in the absence of the organisms resulted in a decrease in the bactericidal effect, and this effect was largely abolished by a 30 min preincubation period. A similar effect of preincubation was observed when lactoperoxidase was substituted for myeloperoxidase. Thus, the bactericidal effect of myeloperoxidase, iodide, and H_2O_2 does not appear to be due to the

relatively stable iodide oxidation products such as iodine, which accumulate in the reaction mixture during the preincubation period. The organisms must be present in the reaction mixture *during* iodide oxidation for optimum killing which suggests the involvement of unstable or volatile intermediates of iodide oxidation.

Iodination of Bacteria.—

Myeloperoxidase catalyzes the formation of iodine-carbon covalent bonds when incubated with iodide ions, H_2O_2 (or preferably an H_2O_2-generating system), and a suitable iodine acceptor such as tyrosine or tyrosine residues of protein (25, 26). A characteristic of the iodination reaction catalyzed by peroxidase is that the iodine acceptor must be present in the reaction mixture *during*

TABLE III

Effect of Preincubation

Preincubation period	Viable cell count
min	*organisms/ml*
0	0
1	0
5	2.2×10^3
15	2.1×10^5
30	2.5×10^6

Myeloperoxidase, iodide, and H_2O_2 in the amounts described in Table I were preincubated at 37°C in acetate buffer pH 5.0 for the periods indicated. *E. coli* (4×10^6 organisms per ml) were then added and the viable cell count was determined at the end of a further 30 min incubation period.

iodide oxidation for optimum iodination. The similarity to the bactericidal effect of myeloperoxidase, iodide, and H_2O_2 in this regard prompted a study of the iodination of bacteria by the peroxidase system.

Table IV demonstrates the conversion of iodide into a TCA-precipitable form in a reaction mixture which contained myeloperoxidase, iodide labeled with ^{131}I, H_2O_2, and heat-killed *E. coli* in acetate buffer pH 5.0. Little or no iodination was observed in the absence of H_2O_2 or myeloperoxidase or in the presence of myeloperoxidase preheated to 90° C for 10 min. The iodination observed when the *E. coli* organisms were deleted from the reaction mixture was less than 5% of that observed in the presence of *E. coli*. Myeloperoxidase could be replaced by lactoperoxidase, heat-killed *E. coli* by live organisms, and H_2O_2 by glucose and glucose oxidase. Indeed glucose and glucose oxidase were more effective in this regard than reagent H_2O_2.

The conversion of iodide to a TCA-precipitable form requires the presence of the bacteria under the conditions employed in Table IV. Support for the iodina-

tion of the bacteria by myeloperoxidase, iodide, and H_2O_2 under these conditions was supplied by radioautographic studies. Fig. 1 demonstrates the localization of silver grains over organisms (heat-killed *L. acidophilus*) which had been incubated with myeloperoxidase, iodide labeled with ^{125}I, glucose, and glucose oxidase at pH 5.0. The reaction was terminated by the addition of sodium thiosulfate, and the bacteria were precipitated and washed with TCA prior to the preparation of the radioautograms. A similar localization was observed when live bacteria were employed, and when the bacteria were collected by centrifugation and washed with water instead of TCA. No localization of silver

TABLE IV

Iodination of E. coli

Supplements	Iodide incorporation
	mμmoles
MPO + H_2O_2 + *E. coli*	36.1
MPO + H_2O_2	0.6
MPO + *E. coli*	0.1
H_2O_2 + *E. coli*	0.2
MPO (heated) + H_2O_2 + *E. coli*	0.3
MPO + glucose + glucose oxidase + *E. coli*	68.2
LPO + H_2O_2 + *E. coli*	33.2
LPO + H_2O_2	1.5
LPO + *E. coli*	0.1
LPO (heated) + H_2O_2 + *E. coli*	0.2
LPO + glucose + glucose oxidase + *E. coli*	73.1

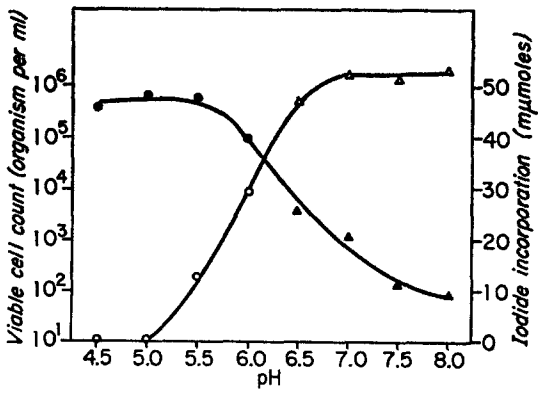
The reaction mixture contained sodium acetate buffer pH 5.0, 100 μmoles; Na-^{131}I, 0.1 μmole (0.2 μc); water to a final volume of 0.5 ml; and the supplements indicated below as follows: MPO, 100 *o*-dianisidine units; LPO, 75 *o*-dianisidine units; H_2O_2, 0.1 μmole; glucose, 5 μmoles; glucose oxidase, 0.5 μg; and *E. coli*, 1×10^8 organisms heated at 100°C for 10 min. MPO and LPO were heated to 90°C for 10 min where indicated.

grains over bacteria was observed if either myeloperoxidase or glucose and glucose oxidase or all three were deleted from the reaction mixture.

Comparison of Iodination and the Bactericidal Effect.—

The properties of the iodination reaction catalyzed by myeloperoxidase were comparable to those of the peroxidase-mediated antibacterial system in all the parameters measured. Preincubation of myeloperoxidase, iodide, and H_2O_2 in the absence of the bacteria decreased iodination as it did the bactericidal effect. Both the bactericidal effect and iodination were high at pH 5.0 and fell as the pH was increased (Text-fig. 1). No loss in viability was observed over the pH range 7.0–8.0 under the conditions employed in Text-fig. 1. However, a peroxidase-dependent bactericidal effect could be readily demonstrated at

pH 7.0 if the H_2O_2 and iodide concentrations were increased. Table V demonstrates the effect of a number of inhibitors on both the bactericidal effect and iodination produced by myeloperoxidase, iodide, and H_2O_2. Cyanide and azide are inhibitors of peroxidase-catalyzed reactions, and they inhibit both the bactericidal effect and iodination. Thiocyanate ions were also inhibitory. This is an apparent paradox, since thiocyanate ions when combined with myeloperoxidase and H_2O_2 exert an antibacterial effect as measured by the decrease in growth of organisms in complete growth medium (13, 14). Myeloperoxidase, thiocyanate, and H_2O_2, however, did not have a bactericidal effect on *E. coli* in the concentrations employed in Table V. The bactericidal effect and iodination were also inhibited by a number of sulfhydryl compounds and by the reduced pyridine



TEXT-FIG. 1. Effect of pH on iodination and on the bactericidal effect. Iodination (closed symbols) was performed as described in Table IV, and the bactericidal effect (open symbols) was determined as described in Table I except that the amount of iodide employed was 0.005 μmole. The pH was varied as indicated. Acetate (○, ●) and phosphate (△, ▲) buffers were employed.

nucleotides, NADH and NADPH. NAD and NADP were without effect at the concentrations employed. Catalase (200 μg/ml) was also inhibitory to both iodination and the bactericidal effect.

Iodination by Intact Leukocytes.—

Table VI demonstrates the conversion of iodide to a TCA-precipitable form in a reaction mixture which contained intact guinea pig peritoneal lavage leukocytes and heat-killed *E. coli* in a modified Hanks' solution containing glucose, bovine albumin, and 10% serum. Smears prepared at the end of the incubation period indicated extensive phagocytosis of the bacteria by PMN leukocytes. Iodination was only slightly decreased on the deletion of serum from the reaction mixture; however, when either the leukocytes or heat-killed *E. coli* were deleted, iodination was largely abolished. Heat-killed *E. coli* could be replaced

TABLE V

Effect of Inhibitors

Inhibitors	Viable cell count	Iodide incorporation
μmoles/ml	*organisms/ml*	*mμmoles*
None	0	34.6
Cyanide (5.0)	5.0×10^6	—
Cyanide (0.5)	1.1×10^4	0.2
Cyanide (0.05)	0	28.4
Cyanide (0.005)	—	31.9
Azide (0.5)	5.4×10^6	0.1
Azide (0.05)	5.3×10^6	25.0
Azide (0.005)	0	32.7
Thiocyanate (0.5)	—	0.1
Thiocyanate (0.05)	4.6×10^6	2.3
Thiocyanate (0.005)	6.4×10^6	22.7
Thiocyanate (0.0005)	4.2×10^3	—
Perchlorate (0.5)	0	34.3
Tapazole (0.5)	—	0.1
Tapazole (0.05)	7.2×10^6	7.9
Tapazole (0.005)	7.2×10^6	26.7
Tapazole (0.0005)	1.7×10^2	—
Thiourea (0.5)	—	0.1
Thiourea (0.05)	8.4×10^6	2.7
Thiourea (0.005)	2.0×10^6	25.7
Thiourea (0.0005)	1.9×10^2	—
Glutathione (reduced) (0.5)	—	0.1
Glutathione (reduced) (0.05)	3.3×10^6	23.2
Glutathione (reduced) (0.005)	0	29.3
Cysteine (0.5)	—	0.1
Cysteine (0.05)	6.4×10^6	32.4
Cysteine (0.005)	0	37.2
Ergothioneine (0.5)	—	0
Ergothioneine (0.05)	4.3×10^6	6.5
Ergothioneine (0.005)	1.2×10^2	24.0
Ergothioneine (0.0005)	0	—
Thiosulfate (0.5)	—	0.1
Thiosulfate (0.05)	3.4×10^6	36.1
Thiosulfate (0.005)	0	35.4
NADH (0.5)	—	0.1
NADH (0.05)	7.4×10^6	15.6
NADH (0.005)	1.0×10^5	27.2
NADH (0.0005)	0	—
NAD (0.05)	0	28.3
NADPH (0.5)	—	0.1
NADPH (0.05)	8.7×10^6	14.8
NADPH (0.005)	7.4×10^6	22.8
NADPH (0.0005)	0	—
NADP (0.05)	0	35.4

The bactericidal effect of myeloperoxidase, iodide, and H_2O_2 was determined as described in Table I except that the amount of iodide employed was 0.01 μmole. Iodination of heat killed *E. coli* by myeloperoxidase, iodide, and H_2O_2 was determined as described in Table IV. The inhibitors were added in the amounts indicated.

by live organisms. The iodination of the TCA-precipitable material by intact leukocytes in the presence of bacteria was inhibited by Tapazole.

The fixation of iodide by neutrophils which contained intracellular bacteria could be demonstrated radioautographically. White blood cell–enriched human blood was incubated with Na-^{125}I with or without bacteria. Smears were fixed in methanol, washed in water, and radioautograms prepared. No localization of iodide was observed when the blood was incubated with iodide alone (Fig. 2 *a*). However, when bacteria (either heat-killed or live *L. acidophilus*) were added, they were readily phagocytosed by neutrophilic PMN leukocytes, and iodide was fixed by the neutrophils which contained intracellular bacteria (Fig. 2 *b* and *c*). Neutrophils of the same smear which did not contain intracellular bacteria did not fix iodide. The silver grains were concentrated over the cyto-

TABLE VI

Iodination by Intact Leukocytes

Supplements	Iodide incorporation
	mμmole × 10^{-3}
WBC + *E. coli* + serum	28.3
WBC + *E. coli*	23.7
WBC + serum	0.3
E. coli + serum	0.1
WBC + *E. coli* + serum + Tapazole	0.04

The reaction mixture contained 0.5 mμmole of Na-^{131}I (0.2 μc), HBG to a final volume of 0.5 ml, and the following supplements were indicated: 6 × 10^6 guinea pig white blood cells, 10% guinea pig serum, 1 × 10^8 *E. coli* (heated at 100°C for 30 min), and 0.5 μmole of Tapazole.

plasm of the cell in association with the phagocytosed bacteria; indeed, it was possible in many instances to localize a line of silver grains over an intracellular bacterium. Iodide fixation, under these conditions, was prevented by Tapazole (Fig. 2 *d*).

Silver grains were not concentrated over erythrocytes, lymphocytes, or eosinophils. The phagocytosis of bacteria by eosinophils was not a striking phenomenon under the experimental conditions employed. Of eight eosinophils in a typical smear, seven contained no intracellular bacteria, and one contained a single bacterium under conditions in which neutrophils contained an average of five intracellular bacteria per cell. Monocytes were actively phagocytic under the experimental conditions employed, although the number of bacteria per cell was less than in neutrophils. Monocytes also contain peroxidase positive granules (27), and a concentration of silver grains over monocytes containing intracellular bacteria was observed, although it was not as striking as that over neutrophils.

DISCUSSION

Myeloperoxidase, iodide, and H_2O_2 have a bactericidal effect on *E. coli* which is considerably greater than that exerted by the individual components of the system. Iodide is oxidized by myeloperoxidase and H_2O_2 with the formation of iodine. However, the bactericidal effect does not appear to be primarily due to the iodine formed. The preincubation of myeloperoxidase, iodide, and H_2O_2, which results in the formation of iodine prior to the addition of the bacteria, greatly decreased the bactericidal effect. The bacteria must be present in the reaction mixture during the process of iodide oxidation for optimum killing. In this and in all the other parameters measured, there was a close correspondence between the bactericidal effect and the iodination of the bacteria by the myeloperoxidase-iodide-H_2O_2 system. This suggests that the death of the organisms under these conditions is associated with, and may therefore be a consequence of, their iodination.

The iodination reaction occurs in the intact neutrophilic PMN leukocyte after the phagocytosis of bacteria. It cannot be said with certainty that the bacteria are iodinated under these conditions. However, the light microscopic radioautographic studies reported here at least suggest that this is the case. Extensive iodination is always associated with the phagocytosis of bacteria, and it is possible, in many instances, to localize lines of silver grains directly over the ingested organisms. In addition, when polystyrene latex particles (0.81 μ diameter) are substituted for bacteria, iodine fixation by the cells is very much diminished although not abolished (Klebanoff, unpublished data).

The conversion of iodide to organic form in the mammalian organism occurs predominately in the thyroid gland as a step in the synthesis of the thyroid hormones, and the current view is that the iodination reaction which occurs in the thyroid gland is catalyzed by a thyroid peroxidase and requires iodide ions and H_2O_2. It is probable that the iodination which occurs in the intact leukocyte results from a similar mechanism.

Myeloperoxidase is present in the neutrophil in high concentration. Histochemical studies (27) suggest its localization in the cytoplasmic (lysosomal) granules of the cell, and this is supported by the demonstration of peroxidase activity in a granular fraction of human leukocytes which also contains β-glucuronidase and acid phosphatase but which does not contain cytochrome oxidase or other oxidative enzymes (28). The cytoplasmic granules of the neutrophil rupture after phagocytosis, with the discharge of their contents into the phagocytic vacuole containing the ingested particle (29–31).

Iodide is present in very low concentrations in plasma (less than 1 μg/100 ml). However, the uptake of iodide by normal human leukocytes has been reported to be 1200 times the uptake by erythrocytes (32). Thyroxine and triiodothyronine also are preferentially taken up by leukocytes (32), and the deiodination of these hormones by leukocyte preparations has been reported (33).

The process of phagocytosis and granule rupture is associated with a burst of leukocyte metabolic activity (for review, see reference 34). Among the many metabolic alterations which occur, two may be particularly pertinent to this discussion. First, it has been reported (35) that H_2O_2 is formed in the leukocyte during the period of accelerated metabolism which follows phagocytosis; and second, the intracellular pH in the vicinity of the ingested particle has been reported to be distinctly acid (36), i.e. in the vicinity of the pH optimum of the iodination reaction, due presumably to the formation of lactic acid

The process of phagocytosis and granule rupture initiates a sequence of events which leads in most instances to the killing and degradation of the ingested bacteria. A number of antibacterial systems have been reported to be present in PMN leukocytes (for review, see reference 37). It is possible that among these is the leukocyte peroxidase, myeloperoxidase, acting with an oxidizable substance such as iodide (or chloride) ion and H_2O_2 formed either by leukocyte or microbial metabolism.

SUMMARY

Myeloperoxidase, iodide, and H_2O_2 have a bactericidal effect on *Escherichia coli*. Myeloperoxidase can be replaced in this system by lactoperoxidase or by a guinea pig leukocyte particulate preparation, H_2O_2 by an H_2O_2-generating system such as glucose and glucose oxidase, and iodide by thyroxine or triiodothyronine. The bactericidal effect was high at pH 5.0 and fell as the pH was increased.

Preincubation of myeloperoxidase, iodide, and H_2O_2 for 30 min before the addition of the bacteria largely prevented the bactericidal effect. Thus, the organisms must be present in the reaction mixture during iodide oxidation for maximum killing, which suggests the involvement of labile intermediates of iodide oxidation rather than the more stable end products of oxidation such as iodine.

Iodination of the bacteria by the myeloperoxidase-iodide-H_2O_2 system was demonstrated chemically and radioautographically. Iodination and the bactericidal effect were similarly affected by changes in experimental conditions in all the parameters tested (effect of preincubation, pH, and inhibitors).

Phagocytosis of bacteria by guinea pig leukocytes was associated with the conversion of iodide to a trichloroacetic acid–precipitable form. Iodide was localized radioautographically in the cytoplasm of human leukocytes which contained ingested bacteria. Iodide fixation was not observed in the absence of phagocytosis or in the presence of Tapazole.

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BIBLIOGRAPHY

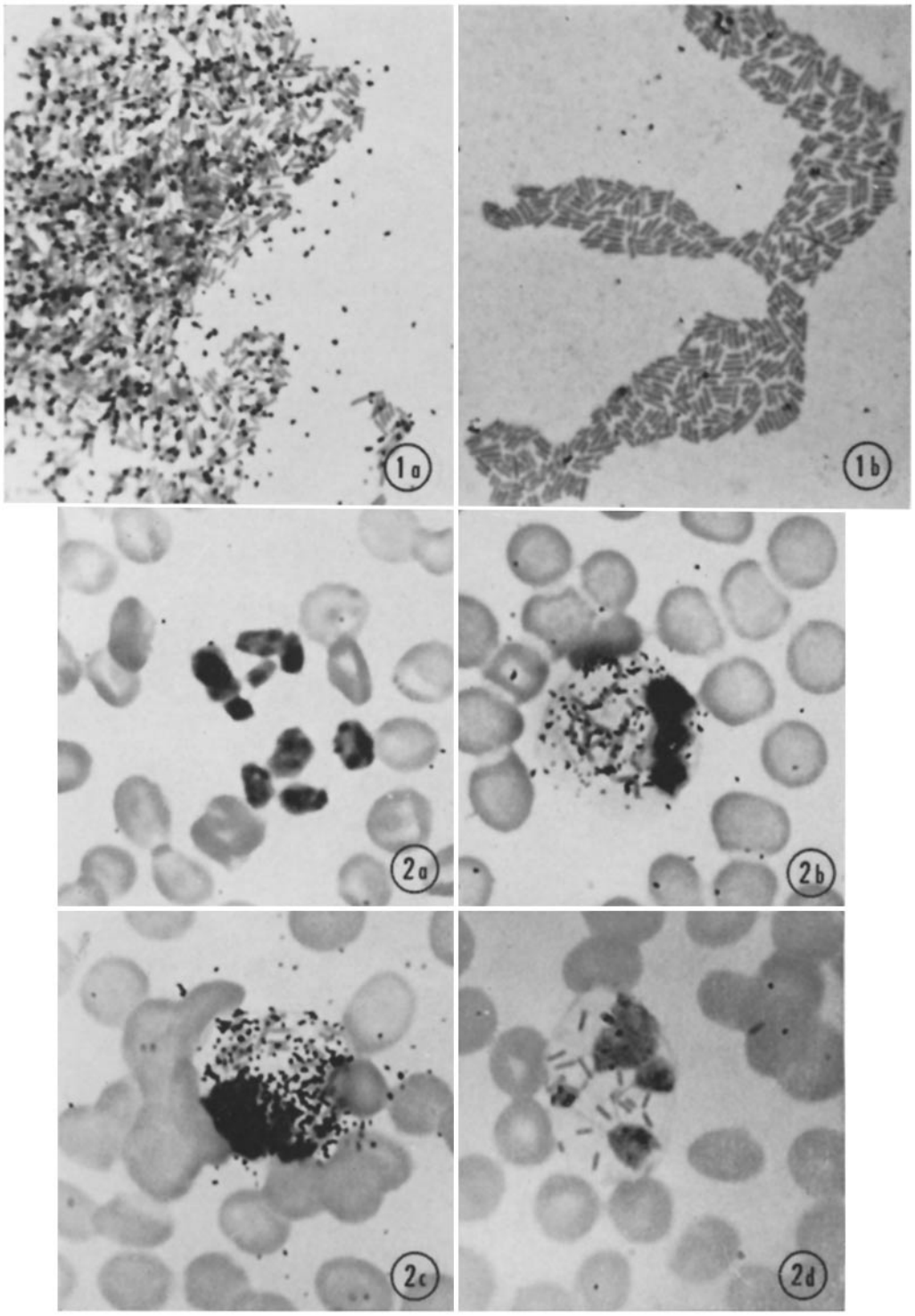
1. Agner, K. 1941. Verdoperoxidase. A ferment isolated from leucocytes. *Acta. Physiol. Scand. Suppl.* **11**:7.
2. Schultz, J. and K. Kaminker. 1962. Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. *Arch. Biochem. Biophys.* **96**:465.
3. Archer, G. T., G. Air, M. Jackas, and D. B. Morell. 1965. Studies on rat eosinophil peroxidase. *Biochim. Biophys. Acta.* **99**:96.
4. Kojima, S. 1931. Studies on peroxidase. II. The effect of peroxidase on the bactericidal action of phenols. J. Biochem. **14**:95.
5. Hanssen, F. S. 1924. The bactericidal property of milk. *Brit J. Exptl. Pathol.* **5**:271.
6. Wright, R. C., and J. Tramer. 1958. Factors influencing the activity of cheese starters. The role of milk peroxidase. *J. Dairy Res.* **25**:104.
7. Portmann, A., and J. E. Auclair. 1959. Relation entre la lacténine L_2 et la lactoperoxidase. *Lait.* **39**:147.
8. Stadhouders, J., and H. A. Veringa. 1962. Some experiments related to the inhibitory action of milk peroxidase on lactic acid streptococci. *Neth. Milk Dairy J.* **16**:96.
9. Jago, G. R., and M. Morrison. 1962. Anti-streptococcal activity of lactoperoxidase. III. *Proc. Soc. Exptl. Biol. Med.* **111**:585.
10. Dogon, I. L., A. C. Kerr, and B. H. Amdur. 1962. Characterization of an antibacterial factor in human parotid secretions active against Lactobacillus casei. *Arch. Oral Biol.* **7**:81.
11. Zeldow, B. J. 1963. Studies on the antibacterial action of human saliva. III. Cofactor requirements of a Lactobacillus bactericidin. *J. Immunol.* **90**:12.
12. Reiter, B., A. Pickering, and J. D. Oram. 1964. An inhibitory system - lactoperoxidase/thiocyanate/peroxide - in raw milk. *In* Proceedings of the Fourth International Symposium on Food Microbiology, Goteborg, Sweden 297.
13. Klebanoff, S. J., and R. G. Luebke. 1965. The antilactobacillus system of saliva. Role of salivary peroxidase. *Proc. Soc. Exptl. Biol. Med.* **118**:483.
14. Klebanoff, S. J., W. H. Clem, and R. G. Luebke. 1966. The peroxidase - thiocyanate - hydrogen peroxide antimicrobial system. *Biochim. Biophys. Acta.* **117**:63.
15. Mickelson, M. N. 1966. Effect of lactoperoxidase and thiocyanate on the growth of Streptococcus pyogenes and Streptococcus agalactiae in a chemically defined culture medium. *J. Gen. Microbiol.* **43**:31.
16. Oram, J. D., and B. Reiter. 1966. The inhibition of streptococci by lactoperoxidase, thiocyanate and hydrogen peroxide. The effect of the inhibitory system on susceptible and resistant strains of group N streptococci. *Biochem. J.* **100**:373.
17. Oram, J. D., and B. Reiter. 1966. The inhibition of streptococci by lactoperoxidase, thiocyanate and hydrogen peroxide. The oxidation of thiocyanate and the nature of the inhibitory compound. *Biochem. J.* **100**:382.
18. Klebanoff, S. J. 1967. A peroxidase-mediated antimicrobial system in leukocytes. *J. Clin. Invest.* **46**:1078.
19. Cohn, Z. A., and S. I. Morse. 1959. Interactions between rabbit polymorphonuclear leucocytes and staphylococci. *J. Exptl. Med.* **110**:419.
20. Kopriwa, B. M. 1967. A semiautomatic instrument for the radioautographic coating technique. *J. Histochem. Cytochem.* **14**:923.

21. Richardson, K. C., L. Jarett, and E. H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35**:313.
22. Klebanoff, S. J. 1965. Inactivation of estrogen by rat uterine preparations. *Endocrinology.* **76**:301.
23. Agner, K. 1958. Crystalline myeloperoxidase. *Acta Chem. Scand.* **12**:89.
24. Morrison, M., and D. E. Hultquist. 1963. Lactoperoxidase. II. Isolation. *J. Biol. Chem.* **238**:2847.
25. Klebanoff, S. J., C. Yip, and D. Kessler. 1962. The iodination of tyrosine by beef thyroid preparations. *Biochim. Biophys. Acta.* **58**:563.
26. Taurog, A., and D. E. Gamble. 1966. Enzymatic iodination of thyroglobulin and other proteins with various peroxidases. *Federation Proc.* **25**:347.
27. Kaplow, L. S. 1965. Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood.* **26**:215.
28. Schultz, J., R. Corlin, F. Oddi, K. Kaminker, and W. Jones. 1965. Myeloperoxidase of the leucocyte of normal human blood. III. Isolation of the peroxidase granule. *Arch. Biochem. Biophys.* **111**:73.
29. Hirsch, J. G., and Z. A. Cohn. 1960. Degranulation of polymorphonuclear leukocytes following phagocytosis of microorganisms. *J. Exptl. Med.* **112**:1005.
30. Hirsch, J. G. 1962. Cinemicrophotographic observations on granule lysis in polymorphonuclear leukocytes during phagocytosis. *J. Exptl. Med.* **116**:827.
31. Zucker-Franklin, D., and J. G. Hirsch. 1964. Electron microscope studies on the degranulation of rabbit peritoneal leukocytes during phagocytosis. *J. Exptl. Med.* **120**:569.
32. Siegel, E., and B. A. Sachs. 1964. *In vitro* leukocyte uptake of ^{131}I-labeled iodide, thyroxine and triiodothyronine, and its relation to thyroid function. *J. Clin. Endocrinol. Metab.* **24**:313.
33. Kurland, G. S., M. V. Krotkov, and A. S. Freedberg. 1960. Oxygen consumption and thyroxine deiodination by human leukocytes. *J. Clin. Endocrinol. Metab.* **20**:35.
34. Karnovsky, M. L. 1962. Metabolic basis of phagocytic activity. *Physiol. Rev.* **42**: 143.
35. Iyer, G. Y. N., D. M. F. Islam, and J. H. Quastel. 1961. Biochemical aspects of phagocytosis. *Nature.* **192**:535.
36. Rous, P. 1925. The relative reaction within living mammalian tissues. *J. Exptl. Med.* **41**:379.
37. Hirsch, J. G. 1965. Neutrophil and eosinophil leucocytes. *In* The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press Inc., New York. 245.

PLATE 80

FIG. 1 *a* demonstrates the localization of silver grains over heat-killed *L. acidophilus* incubated with myeloperoxidase, iodide labeled with ^{125}I, glucose and glucose oxidase at pH 5.0. Fig. 1 *b* demonstrates the absence of localization when bacteria were incubated with iodide alone. × 3500.

FIG. 2 *a* demonstrates the absence of the fixation of iodide by neutrophils incubated with iodide alone. Location of iodide in neutrophils is apparent in Fig. 2 *b* after a 15 min incubation with Na-^{125}I and heat-killed *L. acidophilus*, and this is more pronounced after a 30 min incubation (Fig. 2 *c*). In Fig. 2 *d*, no localization of iodide was observed after a 30 min incubation when Tapazole was added in addition to heat-killed *L. acidophilus*. Intracellular bacteria, however, are readily apparent. × 3500.



(Klebanoff: Iodination of bacteria)