Iodination by stimulated human neutrophils

Studies on its stoichiometry, subcellular localization and relevance to microbial killing

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(Received 14 June 1982/Accepted 18 October 1982)

Myeloperoxidase of phagocytic leucocytes is thought to utilize H_2O_2 to oxidize halides, which then react with and kill ingested microbes. This hypothesis was based largely on the incorporation of radiolabelled iodide into cells that had phagocytosed bacteria. The present studies investigated the stoichiometry of these reactions and the subcellular localization and electrophoretic pattern of the cellular components that became iodinated. 1. The stoichiometry of the reactions are such that only a small proportion (less than 0.3%) of the total oxygen consumed is utilized for iodination. Iodination after stimulation with the soluble stimulus phorbol myristate acetate (PMA), which is not known to involve the azurophil granules and their contained myeloperoxidase, was comparable with that occurring after bacterial ingestion. 2. Analytical subcellular fractionation of cells that had phagocytosed bacteria localized about 25% of the radioactivity to the membranes, and most of the residual radioactivity distributed with the bacteria and dense granules. In cells stimulated with PMA, more of the radioactivity was associated with the membranes, but about half was still associated with the dense granules. 3. Autoradiographs after dodecyl sulphate/polyacrylamide-gel electrophoresis of cells stimulated with opsonized bacteria gave a similar distribution of iodinated components to that obtained with cells that had been stimulated with PMA or iodinated with Iodogen. These patterns of iodination were very different from those obtained when bacteria alone were iodinated with Iodogen or myeloperoxidase and H_2O_2 . Preparations in which bacteria had been phagocytosed did not show evidence of iodination of bacterial proteins or coating opsonins. Thus positive evidence for the iodination of bacteria has not been produced, and the role of iodination in the microbicidal process of neutrophils remains to be established.

The main phagocytic leucocytes in the circulation are neutrophils and monocytes. Upon phagocytosis, myeloperoxidase (EC 1.11.1.7) is released from the cytoplasmic granules into the phagocytic vacuole, where it comes into contact with the superoxide (Babior *et al.*, 1973) and H_2O_2 (Iyer *et al.*, 1961) generated by a burst of non-mitochondrial respiration.

If radiolabelled iodide is incubated with stimulated cells, some radioactivity becomes attached to cellular components, with which it is co-precipitated by trichloroacetic acid (Klebanoff, 1967). It was pro-

Abbreviations used: PMA, phorbol myristate acetate; SDS, sodium dodecyl sulphate.

posed that the functional role of myeloperoxidase is the peroxidation of iodide, with H_2O_2 as substrate, to generate toxic, reactive oxidation products, which then react with and destroy the ingested micro-organism (Klebanoff, 1967, 1968).

A wide body of evidence has been collected which appears to support this iodination theory. Autoradiographic evidence indicates that iodination occurs in close proximity to phagocytosed bacteria (Klebanoff, 1970), and the specific radioactivity of phagocytic vacuoles isolated from neutrophils that had phagocytosed droplets of paraffin oil was greater than that of the cells as a whole (Root & Stossel, 1974). Iodination is decreased in cells from

patients with a congenital absence of myeloperoxidase (Pincus & Klebanoff, 1971; Lehrer, 1972), and in normal cells treated with the haem ligands azide and cyanide (Pincus & Klebanoff, 1971). In a system *in vitro*, myeloperoxidase will kill micro-organisms in the presence of H_2O_2 and iodide (Klebanoff, 1968). However, as more recent review articles indicate (Sbarra *et al.*, 1976; Root & Cohen, 1981), the biological role of myeloperoxidase and iodination is not clearly understood.

The stoichiometry of the iodination reaction has not been related to that of oxygen consumption, and iodination of the ingested organism has not been clearly demonstrated. This is relevant, as PMA, a potent soluble stimulus of the respiratory burst, which is thought not to result in degranulation of the myeloperoxidase-containing azurophil granules (White & Estensen, 1974), and does not provide a bacterial substrate, also induces neutrophil iodination (De Chatelet *et al.*, 1976).

More recently prominence has been given to the possibility that chloride rather than iodide might be the natural substrate for myeloperoxidase-dependent peroxidation (see Sbarra *et al.*, 1976; Root & Cohen, 1981), because chloride is more abundant in the cell and because myeloperoxidase functions as a chloroperoxidase (Morrison & Schonbaum, 1976). However, the theoretical basis for this chlorination theory remains largely an extrapolation from that of iodination.

We have therefore undertaken studies to investigate the stoichiometry of the iodination reaction in relation to that of oxygen consumption, and to examine the subcellular localization of this reaction after the stimulation of respiration by the addition of bacteria or PMA to the cells. We have also attempted to identify the degree to which the ingested bacterium becomes iodinated. Our results indicate that the hypothesis that myeloperoxidase promotes bacterial killing by causing iodination of the ingested organism (Pincus & Klebanoff, 1971; Klebanoff, 1968) requires more substantial proof than is currently available.

Materials and methods

Purification of neutrophils

Neutrophils were isolated from normal human blood taken into heparin (5 units/ml; preservative-free; Paines and Byrne, Greenford, Middx., U.K.) by dextran sedimentation of erythrocytes, centrifugation through Ficoll/sodium metrizoate and haemolysis of residual erythrocytes (Segal *et al.*, 1980) and suspended in RPMI 1640 medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) containing heparin (5 units/ml).

Preparation of bacteria

Staphylococcus aureus, live, heat-killed and [3H]-

glucose-labelled, were isolated and opsonized with human immunoglobulin G as described previously (Segal *et al.*, 1981) and resuspended (5×10^{10} organisms/ml) in RPMI 1640 medium containing heparin (5 units/ml).

Relationship of iodination to oxygen consumption

Incubations were conducted in a rapidly stirred thermostatically controlled incubation chamber attached to an oxygen electrode (Rank, Bottisham, Cambridge, U.K.). In general a sample of the cell suspension (1 ml containing 5×10^7 – 2×10^8 cells) was incubated in the chamber for 1 min with $20 \mu Ci$ of ^{131}I (as NaI; Amersham International; IBS.3) in NaCl (0.15 M; $10 \mu l$). The chamber was then sealed and incubated for a further 1 min before the addition of the stimulus, bacteria (in a ratio of 10/cell) or PMA [Sigma Chemical Co., St. Louis, MO, U.S.A.; $10 \mu l$ of 1 mg/ml solution in dimethyl sulphoxide (BDH, Poole, Dorset, U.K.)].

After a predetermined amount of oxygen consumption, usually about 150 nmol, which was reached about 3 min after the addition of the stimulus, the chamber was opened and samples (usually $400 \mu l$) were taken into ice-cold trichloroacetic acid (2 ml of 5%, w/v; Fisons, Loughborough, Leics., U.K.) in KI (100 mM). After 10 min at $4^\circ C$ the samples were filtered through glass-fibre paper (Whatman GF/C) and washed extensively with the cold trichloroacetic acid/KI solution. In some experiments, to establish the extent of iodide incorporation into extracellular protein, the incubation mixture was taken directly on to the filters, washed with cold NaCl (0.15 M) and then with cold trichloroacetic acid and KI. Radioactivity was determined in a Wallac Autogamma counter.

Covalent linkage of proteins to latex particles

Catalase and superoxide dismutase were covalently bound to the surface of carboxylated latex as described by Muller *et al.* (1980). Samples of the activated latex (0.5 ml) were suspended in 1.5 ml of 0.2 M-sodium carbonate buffer, pH 9.67, and mixed with 50 mg of catalase (Sigma, from bovine liver; 31 000 units/mg; EC 1.11.1.6) or 6.0 mg of superoxide dismutase (Sigma, from bovine blood: 3000 units/mg; EC 1.15.1.1) in 1 ml of carbonate buffer. After further coupling had been blocked with glycine, the latex particles were washed extensively in phosphate-buffered saline (100 mM-sodium phosphate/0.15 M-NaCl, pH 7.4). The concentration of latex-coupled catalase, as measured by the method of Sun & Poole (1975), was $20 \mu g/ml$, indicating that 0.4% of the catalase had been bound. The concentration of coupled superoxide dismutase was $15 \mu g/ml$ (0.3%) as assayed by the inhibition of cytochrome *c* reduction in a xanthine/xanthine oxidase O_2^- -generating system (Beauchamp &

Fridovich, 1971). Enzyme activity in a sample of the particles coated with catalase was destroyed by heating in a boiling-water bath for 10 min.

Inhibition of iodination and the peroxidase activity of myeloperoxidase by azide

The concentration-dependence of the inhibition by azide of iodination by stimulated neutrophils and of myeloperoxidase-dependent peroxidation was examined. To 1.4×10^7 neutrophils in 0.5 ml of RPMI medium were simultaneously added ^{131}I ($6 \mu\text{Ci}$), NaN_3 ($10 \mu\text{l}$ of various concentrations) and PMA ($5 \mu\text{g}$ in $5 \mu\text{l}$ of dimethyl sulfoxide). After incubation for 4 min at 37°C the reactions were terminated by taking portions into cold trichloroacetic acid (5%).

Peroxidase activity was assayed by the peroxidation of *o*-dianisidine (Bretz & Baggiolini, 1974) at pH 7.5, in 0.1 M-sodium phosphate buffer containing 0.1% Triton X-100, by the addition of $10 \mu\text{l}$ of the cell suspensions (2.8×10^5 neutrophils) to the incubation medium containing various concentrations of NaN_3 and H_2O_2 . This pH was used because of evidence that the pH within the vacuole soon after phagocytosis is slightly alkaline (Segal *et al.*, 1981).

Analytical subcellular fractionation of iodinated cells

Neutrophils in RPMI 1640 medium (5 ml; 1.8×10^8 cells/ml) were incubated at 37°C in the chamber attached to the oxygen electrode for 30 s with $25 \mu\text{Ci}$ of ^{131}I before the addition of heat-killed [^3H]-glucose-labelled opsonized bacteria (1 ml containing 4×10^4 organisms, 4.7×10^6 c.p.m.) or PMA ($50 \mu\text{g}$ in $50 \mu\text{l}$ of dimethyl sulfoxide). After incubation for 4 min the cell mixtures were mixed with 50 ml of ice-cold Hanks balanced salt solution (Flow Laboratories), centrifuged at $250 g$ for 3 min at 4°C , resuspended in a further 50 ml of ice-cold Hanks balanced salt solution and then again centrifuged at $250 g$. The cell pellets were resuspended in 10 ml of 9.0% (w/v) sucrose. Samples were taken, for lysostaphin lysis of extracellular bacteria (Segal *et al.*, 1981) to assess the degree of phagocytosis. The cells were disrupted in a Dounce homogenizer (Kontes Glass, Vineland, NJ, U.S.A.) with 50 strokes of a tight-fitting B pestle. The homogenates were centrifuged at $300 g$ for 3 min at 4°C . The pellets were rehomogenized and centrifuged as described above. The supernatants were pooled and 5 ml of this post-nuclear supernatant (containing 80 and 84% of the radioactivity in two experiments) was loaded on a sucrose density gradient, consisting of 2.0 ml of 20% sucrose above a 25 ml linear gradient extending from 35 to 55% (dense gradient), or from 20 to 45% (light gradient), sucrose on a 3 ml cushion of 60% sucrose, and centrifuged at 26000 rev./min in an AH 627 swinging-bucket rotor

in a OTD 50B Sorvall centrifuge (integrated angular velocity = $2.71 \times 10^{11} \text{ rad}^2 \cdot \text{s}^{-1}$, g_{max} . 125 000, g_{min} . 54 500, g_{av} . 89 900). The gradients were unloaded from the top of the tubes in 20 1.7 ml fractions by pumping dense (60%, w/v) sucrose into the bottom of the tube with a peristaltic pump. All sucrose solutions contained EDTA (1 mM, pH 7.4) and heparin (5 units/ml).

Extracts from cells enriched in various cell components were prepared from cells stimulated with PMA or opsonized bacteria. The stimulated cells were washed as described above and then homogenized in 9.0% sucrose containing pepstatin A ($10 \mu\text{l}/\text{ml}$ of a 1 mg/ml solution in ethanol), Trasylol [20 000 Kallikrein Inactivator units/ml; Bayer (U.K.) Ltd., Haywards Heath, Sussex, U.K.] and phenylmethanesulphonyl fluoride ($4 \mu\text{l}/\text{ml}$ of a 100 mM solution in ethanol; Sigma) in addition to the EDTA (1 mM) and heparin (5 units/ml). The post-nuclear supernatants were separated on continuous sucrose density gradients as described above, and fractions containing sucrose at concentrations between 27 and 36% (membranes), 40 and 43% (specific granules), 47 and 52% (azurophil granules) and 54 and 57% (corresponding to phagocytic vacuoles from cells exposed to bacteria) were pooled, precipitated with cold trichloroacetic acid and centrifuged at 200 000 g for 45 min in a Sorvall T 865.1 angle rotor. SDS/polyacrylamide-gel electrophoresis and autoradiography were conducted on the solubilized pellets as described below.

Lactate dehydrogenase (EC 1.1.1.27) released from the cells was measured by centrifuging samples ($500 \mu\text{l}$) at 7000 g for 30 s in a Microfuge (Beckman) and measuring the enzyme activity in the supernatant and in the pellet after disruption with Triton X-100 (0.1%) by determining the decrease in fluorescence of NADH in the presence of pyruvate (Bergmeyer *et al.*, 1974). Malate dehydrogenase (EC 1.1.1.37) was measured as described by Kane & Peters (1975).

Myeloperoxidase (EC 1.1.1.7) was measured by the peroxidation of *o*-dianisidine (Bretz & Baggiolini, 1974), cytochrome b_{-245} by reduced-minus-oxidized difference spectroscopy (Segal & Jones, 1978) and unsaturated vitamin B_{12} -binding capacity by a radioassay using ^{57}Co -labelled vitamin B_{12} (Kane & Peters, 1975). Protein was measured as described by Lowry *et al.* (1951), α -galactosidase (EC 3.2.1.22) by the hydrolysis of fluorescent substrate (Rest *et al.*, 1978), and 5'-nucleotidase (EC 3.1.3.5) by the hydrolysis of [^3H]AMP by the method described by Douglas *et al.* (1972), modified by increasing the specific radioactivity of the AMP 100-fold, incubating at 37°C for 1 h and stopping the reaction with ZnSO_4 rather than trichloroacetic acid.

Samples of the fractions ($500 \mu\text{l}$) were precipitated with cold trichloroacetic acid (5%), filtered through

glass-fibre filters, counted for ^{131}I γ -ray emission and then, after the total decay of detectable ^{131}I radioactivity, for ^3H β -ray emission from the labelled bacteria.

Determination of the pattern of iodinated cell components by SDS/polyacrylamide-gel electrophoresis followed by autoradiography

Neutrophils ($1.5 \times 10^7/\text{ml}$) in RPMI 1640 medium containing heparin (5 units/ml) were incubated with $200\mu\text{Ci}$ of ^{131}I for 45 s before the addition of a stimulus of the respiratory burst, consisting of live or heat-killed opsonized bacteria (40/cell) or PMA ($5\mu\text{g}/\text{ml}$ in $5\mu\text{l}$ of dimethyl sulfoxide). The incubation mixture was taken into and then washed by repeated centrifugation at $7000g$ for 4 min at room temperature in a Beckman Microfuge and resuspension in ice-cold trichloroacetic acid (5%) containing KI (100 mM). Between 3×10^5 and 1.5×10^6 c.p.m. were incorporated into each sample.

The cells and bacteria, both before and after opsonization, were iodinated with Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril) (Markwell & Fox, 1978). The cells (1×10^7 in 1 ml of RPMI medium) or bacteria (1×10^9 in 1 ml of RPMI medium) were incubated for 30 min with $100\mu\text{Ci}$ of ^{131}I in glass scintillation vials coated with Iodogen. The cells and bacteria were then taken into trichloroacetic acid as described above. Approx. 1×10^6 c.p.m. were incorporated into each sample.

Myeloperoxidase was purified from human neutrophil granules as described by Matheson *et al.* (1981) and then by isoelectric focusing with Ampholines of pH 9–11 (LKB Instruments, Selsdon, Surrey, U.K.). Myeloperoxidase ($400\mu\text{g}$) was incubated with $330\mu\text{M-H}_2\text{O}_2$ and $5\mu\text{Ci}$ of ^{131}I in potassium phosphate (100 mM, pH 7.9) for 5 min at 37°C before precipitation with trichloroacetic acid.

Analytical polyacrylamide-gel electrophoresis was performed in polyacrylamide gradient slab gels (1 mm thick; 7.5–15%, w/v) which were prepared with a three-channel peristaltic pump and electrophoresed in the Laemmli (1970) buffer system as described by Thorpe *et al.* (1979). The washed trichloroacetic acid-precipitated samples were prepared for electrophoresis by boiling for 3 min in SDS 5%, w/v/2-mercaptoethanol (40 mM)/sucrose (5%, w/v)/Bromophenol Blue (0.0005%, w/v)/Tris/HCl, pH 6.8 (125 mM). This treatment solubilized 93% and 75% respectively of the radioactivity of cells and bacteria iodinated by the Iodogen method (Markwell & Fox, 1978). Gels were run at a constant voltage of 180 V, with air cooling from a fan.

After electrophoresis, the gels were stained in 0.2% Coomassie Brilliant Blue in methanol/water/acetic acid (2:35:3, by vol.). For autoradiography to reveal the radiolabelled bands, the Coomassie Blue-stained gel was dried on a filter paper in the

Bio-Rad slab-gel dryer (model 224) and exposed to Fuji-Rx X-ray film by using fast tungstate intensifying screens (Ilford, Basildon, Essex, U.K.). The films were developed with D-19 developer (Kodak, Hemel Hempstead, Herts., U.K.). Reagents and molecular-weight markers were obtained from Sigma unless otherwise indicated.

Results

Kinetics and stoichiometry of iodination

A linear relationship existed between iodination and oxygen consumption by neutrophils after stimulation with opsonized bacteria and latex, and with PMA (Fig. 1b). All the trichloroacetic acid-precipitable radioactivity was cell-associated, because similar amounts were incorporated whether the cells were taken directly into the acid or washed before protein denaturation (Fig. 1a). This lack of an effect of prior washing of the cells also shows that iodination is not caused by the joint action of soluble substrates and trichloroacetic acid-dependent acidification.

Table 1 shows the incorporation of ^{131}I into trichloroacetic acid-precipitable material, normalized with respect to oxygen consumption after activation of the cells with different stimuli. The effects of NaN_3 , catalase and superoxide dismutase on the extent of iodination are also shown. The stoichiometry of the relationship between the stimulation of iodination and oxygen consumption was very similar for the different stimuli. Maximally stimulated oxygen consumption after incubation times of about 5 min was generally 2.5–3 times that of unstimulated cells. Their high basal degree of iodination could not be decreased further, despite the addition of relatively large concentrations of KI to the solution of trichloroacetic acid so as to lessen non-specific adsorption. Catalase decreased iodination to approx. 50% of the maximal stimulated value. This effect was also observed when the enzyme was immobilized on the surface of the latex particles, whereas particles coated with heat-inactivated catalase increased iodination. Superoxide dismutase immobilized on the particles enhanced iodination by about 150%. NaN_3 was a potent inhibitor of iodination, which it decreased below the extent obtained in unstimulated cells.

The inhibitory effect of NaN_3 on the peroxidase activity of neutrophils was dependent on the concentrations of H_2O_2 (Fig. 2). At $0.5\text{ mM-H}_2\text{O}_2$ the approximate concentration required to inhibit iodination by 50% was about $200\text{--}300\mu\text{M-NaN}_3$, whereas at $5.0\text{ mM-H}_2\text{O}_2$ it was about $20\text{--}30\mu\text{M-NaN}_3$. The pattern of inhibition of peroxidase activity at this higher concentration of H_2O_2 was very similar to the pattern of inhibition by NaN_3 of iodination by cells stimulated with PMA (Fig. 2).

Fig. 1. Incorporation of radiolabelled iodide into trichloroacetic acid-precipitable material of stimulated neutrophils

(a) Distribution of radioactivity between the cells and extracellular fluid. Duplicate samples were taken at various times after the addition of opsonized bacteria to a suspension of neutrophils. One sample, including the extracellular fluid, was taken directly into cold trichloroacetic acid (Total radioactivity) and the other was washed free of extracellular fluid before denaturation (Cell-associated radioactivity). (b) Relationship between iodination and the increment in oxygen consumption by cells after stimulation with bacteria (○), or with latex particles (●) opsonized with immunoglobulin G, or with PMA (▲). (c) Effect of isotope dilution on the incorporation of radioactive iodide into trichloroacetic acid precipitates of PMA-stimulated neutrophils after standardized oxygen consumption (150nmol/ml). The results of two separate experiments are shown.

The concentration of iodide in the incubation mixture was calculated, by the inhibitory effect on iodination of the addition of known amounts of unlabelled iodide (Fig. 1c), to be between 25 and 50nmol/ml. Of the total iodide in the system, $0.7 \pm 0.2\%$, or less than 0.5nmol, was incorporated into cells stimulated with heat-killed bacteria, in which oxygen consumption was 180nmol within 3 min of stimulation, and iodination was $250 \pm 50\%$ (mean $\pm$ S.E.M., $n=3$) of that in the unstimulated state. This indicates an efficiency of iodide incor-

Table 1. Iodination by neutrophils exposed to various stimuli and inhibitors

In all cases the extent of iodination was compared with and expressed as a percentage of that after a reference stimulus, bacteria in (a), PMA in (b), and carboxylated latex particles in (c). Each series of comparisons is equal with respect to the stimulated increment in oxygen consumption (generally about 150nmol). Iodination by unstimulated cells is included for comparison.

	Mean	S.E.M.	n
(a) Cells + bacteria	100		
Cells + PMA	143	39	9
Cells + latex	96	32	4
(Cells)	37	10	3
Cells + bacteria + soluble catalase	56		1
+ bacteria + NaN ₃	33		1
(b) Cells + PMA	100		
Cells + PMA + catalase	53	1	3
Cells + PMA + azide	21	3	3
(Cells)	31	4	3
(c) Cells + latex	100		
+ latex + coupled superoxide dismutase	150		
+ latex + coupled catalase	57		
+ latex + denatured catalase	185		
(Cells)	17

Fig. 2. Inhibition by azide of iodination (■) by PMA-stimulated neutrophils, and of neutrophil peroxidase activity at H_2O_2 concentrations of 0.5 (○) and 5.0 (△) mM

For further details see the text.

poration of less than 1 atom for each 360 molecules of oxygen consumed.

The mechanism of the incorporation of iodide into resting cells was not investigated. It was markedly

decreased (by above 50%) in the presence of dimethyl sulphoxide, suggesting the possible role of hydroxyl or other radicals for which dimethyl sulphoxide acts as a scavenger (Repine *et al.*, 1979). Iodination remained steady over the time course used in these experiments. In cells incubated with the radiolabelled iodide for 2h before stimulation rather than the usual 1min, iodide incorporation into resting cells was increased from 3000 to 15000 c.p.m. However, the increment in iodination (7000 c.p.m.) upon stimulation with PMA was the same regardless of the duration of preincubation with the radiolabelled iodide. With occasional preparations of radiolabelled iodide the baseline extents of iodination of unstimulated cells were unusually high. We attributed these high background values to the accumulation of free iodine, and $\text{Na}_2\text{S}_2\text{O}_3$ was added to these preparations of KI in equimolar concentrations (1.7 nmol/5 mCi) to scavenge this iodine. This did not affect results other than to decrease baseline incorporation by unstimulated cells.

Analytical subcellular fractionation of iodinated neutrophils after stimulation with bacteria and PMA

Fig. 3 shows that the density profile of trichloroacetic acid-precipitable radioactivity in cells stimulated with bacteria has two peaks of activity in the gradient. About 25% of the radioactivity was in

Fig. 3. Subcellular localization of iodinated material from cells stimulated with bacteria and fractionated on continuous sucrose gradients

The relative activity in the different fractions as a percentage of that in the gradient as a whole is shown for ^{131}I (●; recovery 96%), myeloperoxidase (△; recovery 87.5%) and for ^3H glucose-labelled bacteria (□; recovery 61%). The density of sucrose in the gradient fractions is shown by ▲.

Fig. 4. Subcellular localization of trichloroacetic acid-precipitable iodinated material (a, ●, recovery 89%) from neutrophils stimulated with PMA and separated on a dense sucrose gradient (▲, % w/w).

Density distribution profiles of lactate dehydrogenase (b, ◆, recovery 125%, cytoplasm), vitamin B_{12} -binding protein (c, ○, recovery 125%, specific granules), myeloperoxidase (d, △, recovery 76%, azurophil granules), α -galactosidase (e, ◇, recovery 70%, dense azurophil granules) and protein (f, ■, recovery 95%) are shown. The relative activity in the different fractions as a percentage of that in the gradient as a whole is shown, except for protein, which is expressed in mg/ml.

the light region of the gradient usually occupied by membranes, and the rest was distributed with the granules and bacteria, of which 90% had been demonstrated to be intracellular by protection from lysostaphin lysis. The peak of activity associated with the bacteria was slightly denser and had a different profile from the main peak of myeloperoxidase activity. A considerable proportion of bacteria, radioactivity and myeloperoxidase sedimented through the gradient on to the cushion of dense sucrose.

Apart from the ^{131}I radioactivity in the region of the membranes, the similarity between the distribution of bacteria and iodinated cellular material strongly supported previous studies, in which it had been suggested that most of the iodide was incorporated into phagocytosed bacteria (Klebanoff, 1970) and phagocytic vacuoles (Root & Stossel, 1974). The pattern of iodination after stimulation with PMA (Fig. 4) was different in that more radioactivity was localized in the region of the plasma membrane. However, a large proportion of the radioactivity also distributed with the granules.

This radioactivity was not specifically located with myeloperoxidase, but was widely distributed with the granules, with a peak that coincided with that of α -galactosidase. The density of this latter peak of activity was very similar to that of the iodination and bacteria in the previous experiment (Fig. 3). This indicated that the co-distribution of radioactivity with the bacteria was not a reliable indication that the bacteria themselves had been iodinated.

In an attempt to resolve further the localization of the light peak of incorporated radioactivity, cells that had been iodinated after stimulation with PMA were fractionated on a shallow gradient (Fig. 5). Once again most of the radioactivity localized with the granules. The remainder was widely distributed throughout the gradient, without specific localization in any particular organelle.

Molecular-weight pattern of iodinated components: polyacrylamide-gel electrophoresis and autoradiography

The autoradiographs of solubilized iodinated trichloroacetic acid precipitates showed at least 20

Fig. 5. Subcellular localization of trichloroacetic acid-precipitable iodinated material (a, ●, recovery 72%), from neutrophils stimulated with PMA and separated on a light sucrose gradient (▲, % w/w)

Density distribution profiles of malate dehydrogenase (b, ▲, recovery 122%, cytoplasm and mitochondria), 5'-nucleotidase (c, △, recovery 130%, plasma membrane), cytochrome oxidase (d, □, recovery 89%, mitochondria) and myeloperoxidase (e, △, recovery 87%, azurophil granules) are shown. The relative activity in the different fractions as a percentage of that in the gradient as a whole is shown.

bands, varying from very high molecular weights to about 8000 (Figs. 6 and 7). A large variety of iodinated bands became apparent in the stimulated cells when compared with resting cells. Major bands of iodination were observed at molecular weights of about 85 000 and 75 000, four bands at between 60 000 and 40 000, four bands at between 27 000 and 20 000, and the most prominent bands were below 14 000. Stimulation with opsonized live bacteria gave a similar pattern to that with heat-killed bacteria, and there was little change with incubation times of between 2 and 10 min (Fig. 6). Stimulation of the cells with PMA gave a very similar pattern of iodination to that produced by bacterial phagocytosis (Fig. 6) and both were similar to that of cells iodinated with Iodogen.

In contrast, a very different but characteristic pattern of bands was produced from bacteria surface-iodinated either with Iodogen (Fig. 6) or with myeloperoxidase and H_2O_2 (results not shown).

Fig. 6. Autoradiographs of electrophoretic pattern of iodination of extracts of human neutrophils and bacteria: SDS/polyacrylamide-gel electrophoresis on polyacrylamide gradient gels (a–h, 7.5–15%; i, 5–15%)

(A) Unstimulated cells (9.1×10^4 c.p.m. of ^{131}I applied to gel, 12-day exposure to film); (B) cells stimulated with PMA (3.4×10^5 c.p.m., 12-day exposure); (c–f) cells exposed to *S. aureus* opsonized with immunoglobulin G, either heat-killed and incubated with cells for 2, 4 or 10 min (C, D and E respectively) or live (F, incubated for 4 min) (1.2×10^5 c.p.m., 4-day exposure). Cells (G, 1.4×10^5 c.p.m., 6-day exposure) and bacteria in the absence (H, 1.2×10^5 c.p.m., 6-day exposure) and presence of opsonizing immunoglobulin G (I, 5.0×10^5 c.p.m., 2-day exposure) were iodinated by the Iodogen method (Markwell & Fox, 1978). The bands corresponding to the heavy (above) and light (below) chains of immunoglobulin G are clearly visible in gel (I) (arrows). Molecular weights ($\times 10^{-3}$) are indicated by arrows at the left-hand side.

Iodogen labelling of unopsonized bacteria resulted in a unique series of bands (Fig. 6). Iodogen and myeloperoxidase + H_2O_2 treatment of opsonized bacteria resulted in radiolabelling of the heavy and light chains of the coating immunoglobulins (Fig. 6). However, the radiolabelled bands produced by the surface iodination of bacteria or their opsonizing immunoglobulins were not seen after the incubation of the organisms with cells.

These bands of iodination by neutrophils also did not correspond to those observed when purified human myeloperoxidase iodinated itself after incubation with H_2O_2 and ^{131}I (Figs. 7a and 7b).

Thus the object of enhanced iodination that occurred when bacteria were phagocytosed by neutrophils appeared to be components of the ingesting cell rather than those of the engulfed microbe. In an attempt to localize these bands to different structures within the cell, SDS/polyacrylamide-gel electrophoresis was conducted on fractions enriched in different organelles after sub-cellular fractionation of cells stimulated with PMA or opsonized bacteria. There was very little difference in the pattern of iodination of fractions enriched in plasma membranes, specific granules and azurophil granules (Fig. 7). In these studies the cells were homogenized in both the presence and the absence of a combination of proteinase inhibitors, which made no difference to the pattern of iodination. The densest fractions, corresponding to the distribution of phagocytosed bacteria, did not show any iodination of bands unique to the bacteria or opsonizing immunoglobulins (Figs. 7c and 7d).

Discussion

These studies seriously challenge the premises on which the theory of microbial killing by myeloperoxidase-mediated halogenation depend (Klebanoff, 1967; Pincus & Klebanoff, 1971). This theory is based on the observations that the respiratory burst is accompanied by iodination (Klebanoff, 1967; Pincus & Klebanoff, 1971) and that this is largely localized to the phagocytic vacuole (Root & Stossel, 1974) containing the ingested particle (Klebanoff, 1967, 1970). The stoichiometry of the reactions has not been previously closely examined, and the most essential point, that the engulfed organism is actually the object of the iodination reaction, has not been established.

The stoichiometry of the reaction has not been previously directly related to oxygen consumption. Our data indicates that less than one in 300 of the consumed oxygen molecules are utilized for iodination, inefficiency that is difficult to reconcile with the primary biological function of an enzyme as complex, abundant and with such rapid kinetics as myeloperoxidase (Agner, 1963; Odajima &

Fig. 7. Electrophoretic patterns of components from cells after stimulation with PMA (a and b) and opsonized *S. aureus* (c and d), after disruption and separation on sucrose gradients

Autoradiographs (a) (1×10^5 c.p.m./track, 3-day exposure) and (c) (1×10^4 c.p.m./track, 10-day exposure) of the corresponding Coomassie Blue-stained dried gels (b and d) are shown. The post-nuclear supernatants (A) and fractions enriched in membranes (B), specific (C) and azurophil (D) granules and phagocytic vacuoles (E in c and d) were precipitated with trichloroacetic acid and separated by SDS/polyacrylamide-gel electrophoresis on gradient gels of 7.5–15% polyacrylamide. Track (E) in (a) and (b) represents myeloperoxidase that has undergone self-iodination in the presence of ^{131}I and H_2O_2 . Molecular weights ($\times 10^{-3}$) are indicated by arrows.

Yamazaki, 1972). Not only is the efficiency low but the increment of iodination in stimulated cells is also surprisingly low. In our system oxygen consumption is increased about 20-fold after stimulation (Segal & Coade, 1978), whereas iodination only shows a relatively low 2–3-fold increase.

The concentration of iodide that we measured in our experimental system was 20–50 μM , which is in the region of that required to saturate the iodination reaction (Klebanoff & Clark, 1977; Olsson *et al.*, 1972) and which has been shown to kill bacteria effectively in the myeloperoxidase– H_2O_2 –halide system *in vitro* (Klebanoff, 1968).

PMA stimulates the respiratory burst of neutrophils and causes them to secrete the contents of their specific granules (White & Estensen, 1974). It has been thought not to affect the myeloperoxidase-containing azurophil granules. It was therefore surprising to find that for the same amount of stimulated oxygen consumption this agent enhanced

iodination even more than that induced by the phagocytosis of bacteria (Table 1, Fig. 1). It is possible in any cell suspension that a small proportion of cells are disrupted and release their granules into the suspending medium. It is also possible that, despite observations to the contrary, PMA does indeed cause the release of small amounts of myeloperoxidase, which comes into contact with the cell membrane. Our fractionation data revealed a normal specific activity of myeloperoxidase in homogenates of washed cells that had been stimulated with PMA, suggesting little, if any, release, and a small amount of peroxidatic activity in the region of the plasma membrane, possibly trapped on the initial step of the gradient. However, it does seem unlikely that a soluble stimulus causing little degranulation of myeloperoxidase would result in the same amount of iodination as a particulate physiological stimulus which produces H_2O_2 in the confines of a phagocytic vacuole containing relatively enor-

mous concentrations of H_2O_2 and enzyme, if the primary function of the oxidase system and myeloperoxidase was indeed that reaction.

We then localized the sites of iodination within the cell by analytical subcellular fractionation (Figs. 3 and 4). After the phagocytosis of bacteria, most of the iodination was located together with the bacteria in the denser regions of the gradients. There was, however, a slight discrepancy between the distribution of bacteria and iodination, in that the latter showed a small peak of activity in the light regions of the gradient normally occupied by the cell membranes, which were devoid of bacteria. Therefore, initially this experiment supported the concept that the ingested bacteria were the main target of iodination. However, when the distribution of iodination after the soluble stimulus PMA was determined, it was found that, although a greater proportion of the iodination occurred in the region of the membranes, more of the radioactivity co-distributed with the granules. In addition, the peak of radioactivity in the denser regions of the gradient was even denser than that of myeloperoxidase, corresponding most closely to that of α -galactosidase, which is recognized as having a greater density in sucrose-gradient studies (Rest *et al.*, 1978). This indicated that the co-distribution of iodinated material and bacteria in the phagocytosis experiment was not a reliable indication that the bacteria were themselves becoming radiolabelled. Although some evidence had been obtained previously as to the possible subcellular location of iodination, in retrospect it appears not to have been very specific. Electron-microscopic autoradiographs (Klebanoff, 1970) were not precise because of the relatively large size and sparseness of silver grains in relation to ingested bacteria, resulting in very inaccurate localization. The iodination of purified phagocytic vacuoles (Root & Stossel, 1974) was of interest because the particles used in those experiments were paraffin oil droplets and not bacteria. It was found that the phagocytic vacuoles contained a greater specific radioactivity than the remainder of the cell. However, 45% of radioactivity was associated not with the phagocytic vacuoles but with the residual cellular components.

Studies were then undertaken to identify further the target of the iodination reaction. We found that after SDS/polyacrylamide-gel electrophoresis of trichloroacetic acid-precipitated cells, the same autoradiographic pattern of iodinated cellular components was obtained whether the cells were stimulated with bacteria or with PMA, or when they were simply labelled by the Iodogen method (Markwell & Fox, 1978; Figs. 6 and 7). In particular, we were unable to detect any evidence of iodination of those protein bands that became iodinated when bacteria alone were labelled by

Iodogen or with myeloperoxidase and H_2O_2 . In addition, we did not observe bands of iodination which corresponded to the heavy and light chains of the opsonizing immunoglobulins, which became labelled when opsonized bacteria were iodinated by myeloperoxidase and H_2O_2 , or Iodogen (Fig. 6). These findings indicate that most of the iodination that accompanies bacterial phagocytosis involves the components of the phagocytic cell itself. We have not as yet attempted to identify these iodinated components, but they appear to be fairly equally distributed between the membranes and specific and azurophil granules (Fig. 7) and could include lipids (Hubbard & Cohn, 1976).

In recent years more prominence has been given to the possibility that chloride rather than iodide is the natural substrate for myeloperoxidase-dependent peroxidation (see Sbarra *et al.*, 1976; Root & Cohen, 1981). This is largely because it has been realized that, although I^- is much more efficient in the myeloperoxidase-dependent microbicidal test system *in vitro*, being active at concentrations of $1\mu M$, compared with $1mM$ for Cl^- (Klebanoff, 1968), Cl^- is much more abundant in the cell.

The other evidence for the participation of Cl^- in the killing system is the observation that ^{36}Cl is incorporated into organic material when neutrophils phagocytose bacteria (Zgliczywski & Stelmazynska, 1975). As in the iodination studies, no attempt was made to identify the object of chlorination as the ingested bacteria. In addition, only a very small amount of the oxygen consumed appears to have been involved in the chlorination process. Stimulated neutrophils consume oxygen at a rate of approx. $40nmol/min$ per 10^7 cells (Segal & Coade, 1978), whereas these chlorination studies determined an incorporation of ^{36}Cl at a rate of about $50pmol/min$ per 10^7 cells. The incorporation of ^{36}Cl into bacteria was also demonstrated with purified myeloperoxidase in the presence of H_2O_2 . The conditions used in this system *in vitro* may not be closely related to those that actually pertain in the phagocytic vacuole. For example, Zgliczywski & Stelmazynska (1975) used a pH of 4.6, whereas the actual pH within the phagocytic vacuole soon after phagocytosis appears to be between 7.8 and 8 (Segal *et al.*, 1981).

It is impossible by the techniques that we have used to exclude categorically that a few molecules critical to the integrity and viability of the bacterium are not being iodinated. It is not our intention to disprove the theory that myeloperoxidase plays an important antimicrobial role by means of the oxidation of halogens. However, because the primary basis of this theory is the apparent iodination of the ingested bacterium, we have presented our observations which throw considerable doubt on the conclusions drawn from these

earlier experiments. We have shown that only a small proportion of the oxygen that is consumed is utilized for iodination, and that the major location of iodination appears to be in cellular rather than bacterial components. The elucidation of the biological role and mechanism of action of myeloperoxidase requires further investigation.

We thank the Medical Research Council and Wellcome Trust for financial support, the South London Blood Transfusion Service for blood samples, and Mr. A. Yates for technical assistance.

References

- Agner, K. (1963) *Acta Chem. Scand.* **17**, 332–338
- Babior, B. M., Kipnes, R. S. & Curnutte, J. T. (1973) *J. Clin. Invest.* **52**, 741–744
- Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287
- Bergmeyer, H. U., Bernt, E. & Hess, B. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 737, Academic Press, New York and London
- Bretz, U. & Baggiolini, M. (1974) *J. Cell Biol.* **63**, 251–269
- De Chatelet, L. R., Shirley, P. S. & Johnston, R. B. (1976) *Blood* **47**, 545–554
- Douglas, A. P., Kerley, R. & Isselbacher, K. J. (1972) *Biochem. J.* **128**, 1329–1338
- Hubbard, A. L. & Cohn, Z. A. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., ed.), pp. 427–501, Chapman and Hall, London
- Iyer, G. Y. N., Islam, M. F. & Quastel, J. H. (1961) *Nature (London)* **192**, 535–541
- Kane, S. P. & Peters, T. J. (1975) *Clin. Sci. Mol. Med.* **49**, 171–182
- Klebanoff, S. J. (1967) *J. Exp. Med.* **126**, 1063–1078
- Klebanoff, S. J. (1968) *J. Bacteriol.* **95**, 2131–2138
- Klebanoff, S. J. (1970) in *Biochemistry of the Phagocytic Process: Localization and the Role of Myeloperoxidase and the Mechanism of the Halogenation Reaction* (Schultz, J., ed.), pp. 89–110, North-Holland, Amsterdam
- Klebanoff, S. J. & Clark, R. A. (1977) *J. Lab. Clin. Med.* **89**, 675–686
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lehrer, R. I. (1972) *J. Clin. Invest.* **51**, 2566–2572
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Markwell, M. A. K. & Fox, C. F. (1978) *Biochemistry* **17**, 4807–4817
- Matheson, N. R., Wong, P. S. & Travis, J. (1981) *Biochemistry* **20**, 325–330
- Morrison, M. & Schonbaum, G. R. (1976) *Annu. Rev. Biochem.* **45**, 861–888
- Muller, W. A., Steinman, R. M. & Cohn, Z. A. (1980) *J. Cell Biol.* **86**, 292–303
- Odajima, T. & Yamazaki, I. (1972) *Biochim. Biophys. Acta* **284**, 368–374
- Olsson, I., Olofsson, T. & Odeberg, H. (1972) *Scand. J. Haematol.* **9**, 483–491
- Pincus, S. H. & Klebanoff, S. J. (1971) *N. Engl. J. Med.* **284**, 744–750
- Repine, J. E., Eaton, J. W., Anders, M. W., Hoidal, J. R. & Fox, R. B. (1979) *J. Clin. Invest.* **64**, 1642–1651
- Rest, R. F., Cooney, M. H. & Spitznagel, J. K. (1978) *Biochem. J.* **174**, 53–59
- Root, R. K. & Cohen, M. S. (1981) *Rev. Infect. Dis.* **3**, 565–598
- Root, R. K. & Stossel, T. D. (1974) *J. Clin. Invest.* **53**, 1207–1215
- Sbarra, A. J., Selvaraj, J., Paul, B. B., Poskitt, P. K. F., Zgliczynski, J. M., Mitchell, F. W. & Louis, F. (1976) *Int. Rev. Exp. Pathol.* **16**, 249–271
- Segal, A. W. & Coade, S. B. (1978) *Biochem. Biophys. Res. Commun.* **84**, 611–617
- Segal, A. W. & Jones, O. T. G. (1978) *Nature (London)* **276**, 515–517
- Segal, A. W., Dorling, J. & Coade, S. (1980) *J. Cell Biol.* **85**, 42–59
- Segal, A. W., Geisow, M., Garcia, R., Harper, A. & Miller, R. (1981) *Nature (London)* **290**, 406–409
- Sun, A. S. & Poole, B. (1975) *Anal. Biochem.* **68**, 260–273
- Thorpe, R., Delacourte, A., Ayers, M., Bullock, C. & Anderton, B. H. (1979) *Biochem. J.* **181**, 275–284
- White, J. G. & Estensen, R. D. (1974) *Am. J. Pathol.* **75**, 45–60
- Zgliczywski, J. M. & Stelmazyszynska, T. (1975) *Eur. J. Biochem.* **56**, 157–162