Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M., Jr. (1978) *Biochemistry 17*, 1183.
Pownall, H. J., Pao, Q., Hickson, D., Sparrow, J. T., Kusserow, S. K., & Massey, J. B. (1981a) *Biochemistry 20*, 6630.
Pownall, H. J., Hickson, D., & Gotto, A. M., Jr. (1981b) *J. Biol. Chem. 256*, 9849.
Pownall, H. J., Knapp, R. D., Gotto, A. M., Jr., & Massey, J. B. (1983) *FEBS Lett. 159*, 17.
Reijngoud, D.-J., & Phillips, M. C. (1984) *Biochemistry 23*, 276.
Rosseneu, M., vanTornout, P., Lievens, M.-J., Schmitz, G., & Assmann, G. (1982) *Eur. J. Biochem. 128*, 455.
Scanu, A. M., Edelstein, C., & Shen, B. W. (1982) in *Lipid–Protein Interactions* (Jost, P., & Griffith, O. H., Eds.) p 259, Wiley-Interscience, New York.
Segrest, J. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M., Jr. (1974) *FEBS Lett. 38*, 247.
Segrest, J. P., Chung, B. H., Brouillette, C. G., Kanellis, P., & McGahan, R. (1983) *J. Biol. Chem. 258*, 2290.
Soutar, A. K., Garner, C. W., Baker, H. N., Sparrow, J. T., Jackson, R. L., Gotto, A. M., Jr., & Smith, L. C. (1975) *Biochemistry 14*, 3057.
Subczynski, W. K., & Hyde, J. S. (1983) *Biophys. J. 41*, 283.
Subczynski, W. K., & Hyde, J. S. (1984) *Biophys. J. 45*, 743.
Tall, A. R., Shipley, G. G., & Small, D. M. (1976) *J. Biol. Chem. 251*, 3749.
Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 2200.
Vaughn, W. M., & Weber, G. (1970) *Biochemistry 9*, 464.
Washburn, E. W., Ed. (1928) in *International Critical Tables*, 1st ed., Vol. 3, pp 257 and 272, McGraw-Hill, New York.
Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochemistry 17*, 1792.
Weber, G., & Farris, F. J. (1979) *Biochemistry 18*, 3075.
Weinberg, R. B., & Spector, M. S. (1985) *J. Biol. Chem. 260*, 4914.
Zacchariasse, K. A., Phuc, N. V., & Kozankiewicz, B. (1981) *J. Phys. Chem. 85*, 2676.

Role of Free Radical Processes in Stimulated Human Polymorphonuclear Leukocytes†

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ABSTRACT: Human polymorphonuclear leukocytes produce large quantities of superoxide when they attack and kill bacteria. However, superoxide is a weak oxidizing and reducing agent, and other more reactive oxygen species derived from reactions of superoxide are suggested to participate in the killing processes. To test the hypothesis that a reactive free radical or singlet oxygen is involved in bactericidal activity, human polymorphonuclear leukocytes were exposed to phagocytozable particles containing lipids that contain the easily autoxidized 1,4-diene moiety. After incubation the preparations were extracted and the extracts reduced with $NaBH_4$ to convert hydroperoxides to stable alcohols. Using gas chromatography/mass spectrometry to analyze the extracts, we were unable to detect products unless iron salts were added to the medium. The products obtained by extraction are those that would be expected if both free radical chain autoxidation and $^1O_2$ oxidation were taking place. In summary, we find that polymorphonuclear leukocytes do not cause peroxidation, implying that formation of strongly oxidizing free radicals is not an intrinsic property of the leukocyte. Added iron catalyzes peroxidation by activated leukocytes yielding an unusual distribution of hydroxylated products.

The human polymorphonuclear leukocyte (PMNL)[1] generates large quantities of superoxide and its disproportionation product H_2O_2 when it attacks bacteria. That the generation of these oxidants is essential for killing bacteria is implicit from studies on patients that suffer from chronic granulomatous disease (CGD). Characteristic traits of the CGD PMNL are the inability to produce superoxide (Curnutte et al., 1974) and the inability to kill phagocytozed bacteria (Quie et al., 1967; Kaplan et al., 1968). Because superoxide is both a weak oxidant and reductant (Sawyer & Valentine, 1981), other species have been sought as the agents responsible for killing. Several reports have suggested that singlet oxygen ($^1O_2$) (Rosen & Klebanoff, 1977) and hydroxyl radical (HO•) (Johnston et al., 1975; Tauber & Babior, 1977; Ambruso & Johnston, 1981; Sagone, 1981; Newburger & Tauber, 1982) are formed by reactions requiring superoxide or its disproportionation product H_2O_2. Recent studies on $^1O_2$ formation from superoxide disproportion have shown that this pathway is not operating to a significant extent (Foote et al., 1980a), and attempts to detect $^1O_2$ in the PMNL phagosome have been unsuccessful (Foote et al., 1980b).

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[1] Abbreviations: BHT, 2,6-di-*tert*-butyl-4-methylphenol; BSA, bovine serum albumin; CGD, chronic granulomatous disease; CML, carboxy-modified latex; GC/MS, gas chromatography/mass spectrometry; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OH, 2,2-dimethyl-5-hydroxypyrrolidinyl-1-oxy; DMPO-OOH, 2,2-dimethyl-5-hydroperoxy-pyrrolidinyl-1-oxy; H•, hydrogen atom; HO•, hydroxyl radical; HO_2•, perhydroxyl radical; HPLC, high-pressure liquid chromatography; HMS, hexose monophosphate shunt; NEM, *N*-ethylmaleimide; NHE, normal hydrogen electrode; $^1O_2$, singlet oxygen ($^1\Delta_g$); PBS, Dulbecco's phosphate-buffered saline; PMNL, polymorphonuclear leukocyte; SOD, superoxide dismutase.

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Evidence suggesting the formation of HO• is derived from several indirect experiments. However, these interpretations are not rigorous proof of HO• participation because qualifying assumptions must be made as to the chemical mechanisms involved in detecting HO•. For example, ethylene evolution from methional and 4-(thiomethyl)-2-oxobutyric acid, two probes routinely used to demonstrate HO• participation in PMNL-dependent processes, can be induced by radicals other than HO• (Pryor & Tang, 1978; Winston et al., 1983). Moreover, myeloperoxidase activity was recently shown to correlate with ethylene evolution in PMNLs (Rosen & Klebanoff, 1979), implying that ethylene evolution might not be a specific measure of free radical reaction. Another test for HO•, decarboxylation of [1-^{14}C]benzoic acid, is blocked by azide, a potent inhibitor of myeloperoxidase (Sagone et al., 1980), suggesting a nonradical mechanism for decarboxylation. More convincing evidence for HO• has been obtained through spin-trapping experiments with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which yields a DMPO-OH adduct when added to active PMNLs (Green et al., 1979; Rosen & Klebanoff, 1979). However, these results are ambiguous because the DMPO-OOH adduct reportedly can decompose to DMPO-OH (Finkelstein et al., 1982). Results of a study reporting the inhibition of bacterial killing by radical scavengers, superoxide dismutase, and/or catalase (Johnston et al., 1975) have been disputed (McCay et al., 1980).

Since there are no reports that unambiguously demonstrate formation of powerful, oxidizing oxy radicals by PMNLs, we attempted to provide such evidence by looking for peroxidation products from linoleic acid and from linoleyl alcohol administered to PMNLs on phagocytozable particles. Molecules like linoleic acid and linoleyl alcohol that contain doubly allylic methylene hydrogens undergo facile free radical autoxidation initiated by a variety of oxy radicals. Conjugated diene hydroperoxides are the predominant products. Linoleic acid (Thomas & Pryor, 1981; Thomas et al., 1982a) and linoleyl alcohol also give characteristic products when oxidized by $^1O_2$. Therefore, we could confirm other work regarding $^1O_2$ formation by PMNLs. The 1,4-dienes were either delivered in albumin–mineral oil emulsion or coated onto latex beads. Malondialdehyde formation from polyunsaturated fatty acid ester incorporated into mineral oil–bovine serum albumin emulsions had been reported (Stossel et al., 1974), and we expected to demonstrate the presence of conjugated diene hydroperoxides. Since hydroperoxides are initial products of free radical autoxidation, we felt that they would give a more quantitative measure of free radical oxidation than their degradation products like polyperoxides or malondialdehyde. However, oxidation was observed with latex beads only when iron salts were added to the medium. The distribution of products obtained in the presence of iron salts was unusual, implying that the oxidation catalyzed by iron may involve more than one chemical mechanism.

Materials and Methods

General. Linoleyl alcohol was purchased from NuChek Prep, Inc. Linoleic acid was from Calbiochem. [1-^{14}C]-Linoleic acid (54.9 mCi/mmol) was from New England Nuclear, Inc., and purified by high-pressure liquid chromatography (HPLC) before use. Carboxy-modified latex (CML) beads of 0.455- and 1.10-μm (DOW) and 0.732-μm (IDS SPHERES) diameter were from Duke Scientific Co. Dulbecco's phosphate-buffered saline (PBS) was from Grand Island Biological Co. Dextran T500 was from Pharmacia. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide was from Molecular Probes. All other chemicals were of the highest quality available from commercial sources.

PMNL Isolation and Other Assays. PMNLs were prepared by a procedure described in the literature (DeChatelet et al., 1982) using Dextran T500 in place of plasma gel. This method routinely yields a preparation that contains greater than 87% granulocytes with a viability of 95% or better as judged by the exclusion of trypan blue.

Stimulation of the respiratory burst was demonstrated by an increase in hexose monophosphate shunt activity (Long et al., 1981). Oxygen uptake was monitored on a YSI Model 53 oxymeter using a Clark electrode. Hydrogen peroxide was measured by the standard formate assay (DeChatelet & Shirley, 1981) and with a YSI Model 25 oxidase meter using a YSI Model 2510 oxidase probe. Superoxide production was demonstrated by using a cytochrome *c* assay described in the literature (Babior et al., 1973). Cell viability in experiments utilizing latex beads was assessed by measuring the amount of lactate dehydrogenase released into the medium. The above experiments were performed at the same bead/PMNL ratio (78:780) or the paraffin oil concentration used in the following experiments.

Stimulation with Linoleyl Alcohol–CML Beads. Linoleyl alcohol was introduced into the PMNL after adsorption on CML beads. A 1.0-mL suspension of beads was treated with 5 μL (4.3 mg) of linoleyl alcohol and then gently rotated overnight under Ar at 4 °C. The beads were counted on a light microscope. When the effect of iron salts was examined, bead solutions were prepared by treating 1 mL of coated beads with 0.05–0.1 mL of a 0.3–0.4 M solution of Fe(III) prepared by dissolving $FeCl_3 \cdot 7H_2O$ in deionized water and then filtering off solids. The beads (IDS SPHERES) showed good stability when mixed with this iron solution. The coated bead–Fe(III) solution was added in the same manner as the coated beads in the absence of Fe(III). Iron concentrations are measured by dissolving the salt in 0.8 N H_2SO_4 and monitoring the absorption at 305 nm (ϵ = 2206 L mol^{-1} cm^{-1}). Association of the latex beads with PMNLs was studied by phase contrast/fluorescence microscopy on a Leitz Laborlux 11 equipped with Leitz fluorescence and photomicrographic attachments. The degree of association is measured by the number of beads attached to PMNLs as compared to the total number of beads in the microscope field. Transmission electron micrographs were obtained on a Philips EM-400 microscope. PMNLs were washed 6 times with 10 mM ethylenediaminetetraacetic acid in PBS and then fixed with 2.5% glutaraldehyde. After postfixing with OsO_4, the cells were dehydrated and then embedded in epoxy resin. Sections of 600-Å thickness were stained with uranyl acetate and lead citrate and then observed at 80 kV.

A suspension of fresh cells (1.1 × 10^8 cells/mL) and glucose (1.1 mM) was prepared, and 3.2-mL aliquots were added to 50-mL Erlenmeyer flasks. One set of flasks was preincubated for 5 min at 37 °C, treated with 5 × 10^{10} latex beads, and then incubated with shaking for either 30 or 60 min before treatment with 7 mL of cold 1 mM *N*-ethylmaleimide (NEM). A second set of flasks containing control cells was kept on ice and treated with 7 mL of 1 mM NEM before adding beads. The PMNLs were pelleted at 900*g*, washed with PBS containing 1 mM NEM, and then repelleted. The pellet was treated with 1 mL of methanol and vortexed, and 1 mL of water was added and then extracted 3 times with 15-mL portions of 1:1 hexane–diethyl ether containing 0.001% 2,6-di-*tert*-butyl-4-methylphenol (BHT). The combined extracts were dried in vacuo and then reduced with methanolic $NaBH_4$ (0 °C). The solution was neutralized with 0.1 M H_2SO_4,

treated with 20 mL of water, and extracted 2 times with 5-mL portions of 1:1 hexane–diethyl ether. The extracts were washed with water until the washings were neutral. Samples were plated onto Whatman silicar K TLC plates and developed with 1:1 hexane–diethyl ether. Appropriate regions of the plates were removed, and the silica gel was extracted 18 h with 1:1 hexane–diethyl ether (BHT added). Samples were stored in 100 μL of hexane at −70 °C under Ar until analysis.

Preparation of Standards. Linoleyl alcohol was purified as follows. Linoleyl alcohol (1 g) was treated with methanolic $NaBH_4$, neutralized, and extracted into 1:1 hexane–diethyl ether. After evaporation, the oily residue was applied to a 2.5 cm × 30 cm column of silica gel G, then nonpolar contaminants were eluted with 500 mL of hexane–diethyl ether–2-propanol (475:25:0.05), and linoleyl alcohol was eluted with 250 mL of hexane–diethyl ether–2-propanol (125:125:0.05).

To prepare the free radical autoxidation products of linoleyl alcohol, a film of linoleyl alcohol (200 mg) was subjected to a stream of clean, dry air as described for the free radical autoxidation of arachidonic acid (Porter et al., 1979; Thomas et al., 1982b). The residue was reduced with cold methanolic $NaBH_4$. The reduced products, called dienediols, were purified by HPLC with the following conditions: flow rate, 2 mL/min; solvent, hexane–2-propanol–acetic acid (492:8:0.5); column, 3.9 mm × 300 mm μ-Porasil (Waters); monitor wavelength, 236 nm. Singlet oxygen derived products were prepared by dye-sensitized photooxidation with methods described by Higgins et al. (1968). A solution of 0.71 g of linoleyl alcohol and 2 mg of BHT in 200 mL of methanol containing 5×10^{-4} M methylene blue was cooled to 15 °C, sparged with O_2, and then irradiated for 7.5 h with a 650-W DWY tungsten–halogen lamp running at 70 V. The photolysate was treated with $NaBH_4$ overnight at 2 °C. After evaporation the residue was mixed with 50 mL of water and 50 mL of diethyl ether and acidified to pH 2. After the layers were separated, the aqueous layer was extracted twice. The combined organic layers were washed with water until the washings were neutral and then dried over Na_2SO_4. Separation of unreacted alcohol from oxidized alcohol was performed on a 2.5 cm × 30 cm silica gel G column by the procedure for purifying linoleyl alcohol. Dienediols (0.34 g, 45%) were stripped from the column with 250 mL of ethyl acetate. Purification was achieved with the HPLC system described above by simultaneously monitoring the eluant at 210 and 236 nm. To isolate the hydroperoxides after dye-sensitized photooxygenation, the solvent was removed in vacuo at room temperature and the residue dissolved in 0.5 mL of hexane and put on a 5 mm × 5 cm column of silica gel. The unreacted linoleyl alcohol and peroxide were separated from the dye by eluting with 10% hexane–diethyl ether. The individual peroxides can then be purified with the methods described for the corresponding alcohols.

Analysis of Linoleyl Alcohol and Products Extracted from PMNL. Mass spectrometric analysis was performed on a Ribermag Model R10-10C quadrupole mass analyzer interfaced to a Girdel gas chromatograph. Data acquisition and reduction were performed on a PDP-8/A minicomputer. Separation and quantitation of linoleyl alcohol and products were obtained on 30 m × 0.25 mm i.d. DB-1, 30 m × 0.25 mm i.d. DB-5, or 6 m × 0.25 mm i.d. DB-1 fused silica WCOT capillary columns (J & W, Inc.) that terminated at the source. Alcohols were converted to trimethylsiloxy ethers with bis(trimethylsilyl)trifluoroacetamide in pyridine. Methyl stearate was added to the samples before GC/MS analysis to quantitate linoleyl alcohol recovery, and 1,12-dihydroxy-9(*Z*)-octadecene was added to oxidized samples before derivatization to standardize recoveries. The following parameters were used in GC/MS analysis: injector temperature, 250 °C; interface temperature, 270 °C; oven program, 50–250 °C at 10 °C/min; helium flow, 35 cm/s; source temperature, 200 °C; filament current, 0.3 mA; electron voltage, 42 V.

Stimulation with [1-^{14}C]Linoleic Acid–Paraffin Oil. Linoleate was introduced to the PMNL after incorporation into paraffin oil. An emulsion was prepared from 0.3 mL of [1-^{14}C]linoleic acid (25 μCi/mL) in paraffin oil and 0.9 mL of bovine serum albumin (BSA, 20 mg/mL) and then opsonized with human serum. Fresh cells were divided into 4-mL aliquots (1×10^8 cells/mL) in PBS, preincubated for 5 min at 37 °C, and then treated with 0.1 mL of emulsion. Control PMNLs were immediately treated with 1 mM cold NEM in PBS. Test PMNLs were allowed to incubate for 60 min before treatment with NEM. The PMNLs were centrifuged at 225*g* and washed with cold buffer. Cell pellets were suspended in 1 mL of methanol. The solutions were diluted with water, acidified to pH 2 with 5 N HCl, and then extracted 3 times with 5-mL portions of 1:1 hexane–diethyl ether. The solvent was removed in vacuo, and the samples were reduced at 0 °C with methanolic $NaBH_4$. The samples were acidified to pH 2 and then extracted 2 times with 5-mL aliquots of 1:1 hexane–diethyl ether. The combined organic layers were washed with water until the washings were neutral. After the extraction solvent was removed in a stream of Ar, the samples were taken up in HPLC solvent and stored under Ar at −70 °C. When Oil Red O uptake was examined, the cells were pelleted at 900*g*, resuspended in cold PBS, and pelleted again, and the pellet was suspended in dioxane. After centrifugation to remove suspended matter, the optical density was measured at 524 nm.

High-performance liquid chromatographic analysis of linoleic acid oxidation products as their 4′-bromophenacyl esters was performed as previously described (Thomas et al., 1982a). Authentic hydroxylated linoleate standards for both the $^1O_2$ and free radical autoxidation products were prepared as described (Thomas & Pryor, 1980; Thomas et al., 1982a) and used as carriers. Fractions were collected every 0.5 min in 7-mL minivials, dried in a stream of air, and then counted on a Packard Tricarb 4000.

RESULTS

Characterization of Products from Linoleyl Alcohol Oxidation. Photooxidation of linoleyl alcohol followed by $NaBH_4$ reduction yields four hydroxylated products. These compounds have been isolated by HPLC on a μ-Porasil column. The bis(trimethylsiloxyl) derivatives of these diols elute as two peaks from the GC/MS with a total ion-integrated area ratio of 1.14:1. The first peak (13.45 min, 6 M DB-1) has intense fragment ions at *m/z* 185 and 315 while the second (13.68 min, 6 M DB-1) has strong ions at *m/z* 225 and 355. Ion fragments *m/z* 185 and 315, formed by carbon–carbon bond homolysis adjacent to the trimethylsiloxyl moiety, are characteristic of the nonconjugated diols 1,12-bis(trimethylsiloxy)-9,13-octadecadiene and 1,10-bis(trimethylsiloxy)-8,12-octadecadiene. The second pair of fragments are characteristic of the conjugated dienes 1,9-bis(trimethylsiloxy)-10,12-octadecadiene and 1,13-bis(trimethylsiloxy)-9,11-octadecadiene. The latter two conjugated dienes are a substantial fraction of the products from reduced, autoxidized linoleyl alcohol. The corresponding trans,trans isomers, prepared by autoxidation, show strong ion fragments of *m/z* 225 and 355 and elute from 6 M DB-1 at 13.95 and 14.05 min, respectively. A detection limit of less than 1 ng was determined by analyzing samples

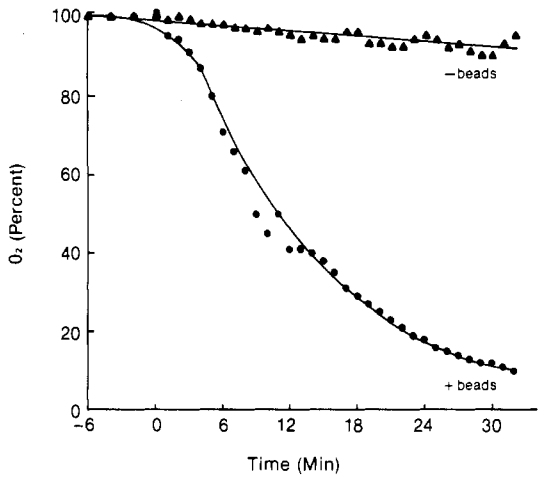

FIGURE 1: Oxygen uptake plotted as percentage of an air-saturated solution for PMNLs treated with beads (+ beads) or without beads (−beads). The solution contained 1.7×10^6 PMNLs/mL and 1.1 mM glucose in PBS. Linoleyl alcohol coated beads (1.2×10^9) were added to initiate oxygen uptake. The total volume was 2 mL.

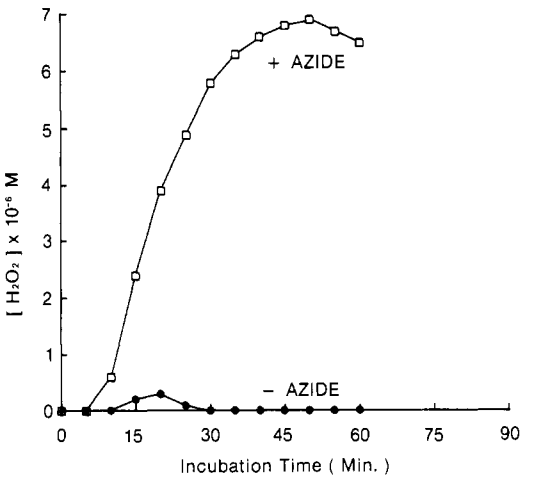

FIGURE 2: Hydrogen peroxide secretion (μM) as a function of time. Azide (1 mM) was used to inhibit myeloperoxidase (+azide). An uninhibited run is shown for comparison (−azide). Conditions are described in Figure 1. The concentration of beads was 1.7×10^9/mL. The volume is 3 mL.

of $^1O_2$ diols of known concentration.

Response of PMNLs to Latex Particles Coated with Linoleyl Alcohol. Linoleyl alcohol coated beads stimulated responses normally associated with phagocytosis and bacterial killing. At a concentration of 1.6×10^9 beads/mL, these beads stimulate a 1.9-fold increase in HMS activity (data not shown) and a 21-fold increase in SOD inhibitable cytochrome *c* reduction (data not shown). Both responses were measured 60 min after the beads were added. Oxygen uptake for both control and bead-treated PMNLs is shown in Figure 1. Hydrogen peroxide release was demonstrated by the formate assay (DeChatelet & Shirley, 1981) (data not shown) and by the H_2O_2-sensing electrode (Figure 2) with 1 mM sodium azide to inhibit myeloperoxidase. Control cells incubated with 1 mM azide in the absence of beads displayed approximately 50-fold less H_2O_2 production than bead-treated cells (data not shown). After a 60-min incubation, the release of lactate dehydrogenase was approximately the same for both control (11.2 ± 2.8%) and bead-treated (13.3 ± 0.1%) cells, demonstrating comparable viability.

Linoleyl alcohol was extracted after the beads were pelleted at 9000*g* according to the procedure described for extracting the PMNL pellet (Materials and Methods). GC/MS analysis of linoleyl alcohol extracted from the beads that were incubated in the presence or absence of PMNLs displayed linoleyl alcohol recoveries that were the same within experimental error (data not shown). On the basis of the amount of linoleyl alcohol extracted from a 30-min incubation, we estimate that there are approximately one to three beads associated with PMNL after centrifugation. The analysis is based on the assumption that the beads are uniformly coated with linoleyl alcohol. Both bead concentration and linoleyl alcohol were determined experimentally, and the linoleyl alcohol/bead ratio was found to be approximately 8.6 fg/bead with Dow beads. Because iron salts are generally insoluble at physiologic pH, it was necessary to ascertain the distribution of iron in our bead–Fe(III) solutions when they were presented to the PMNLs. Determination of the iron concentrations showed that the iron was associated with the beads (data not shown).

Phase-contrast microscopy showed that PMNLs rapidly associate with 1.1 μm diameter beads coated with linoleyl alcohol and the fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide (data not shown). After 5 min 45% of the beads are associated with the PMNLs while at 45 min 96% of the beads are associated with PMNLs. Transmission electron microscopy of washed PMNLs showed that approximately 75% of the beads were inside the cell (data not shown).

Stability of Linoleyl Alcohol Hydroperoxides. Hydroperoxide stability was established by examining the recovery of diols from PMNLs exposed to latex beads coated with linoleyl alcohol containing 2.5 wt % $^1O_2$-derived hydroperoxides. The mixture was prepared by combining 1 μL of photooxidized linoleyl alcohol (Materials and Methods) with 20 μL of pure linoleyl alcohol and then coating the cells with 5 μL of the mixture. The amount of conjugated diene was determined by absorption at 236 nm and then corrected for the amount of nonconjugated peroxide that does not absorb at 236 nm. Reduced extracts from both control and stimulated PMNL preparations contained conjugated and nonconjugated diols. The coated beads were shown to support autoxidation by allowing them to stand in buffer. After several days, reduced extracts were found to contain conjugated dienols.

Analysis for Peroxidized Products of Linoleyl Alcohol. Although apparently stimulated by beads, $NaBH_4$-reduced extracts of PMNLs incubated with linoleyl alcohol coated beads did not contain diols. These results are based on six experiments for which a total of 11 stimulated PMNL preparations and 11 unstimulated control preparations were analyzed. However, when iron salts were added to the medium, oxidation products were found. The use of iron-treated beads resulted in the identification of dienols after $NaBH_4$ reduction. Controls treated with NEM show fewer products, less than 10% that seen with stimulated cells. A typical GC/MS trace is shown in Figure 3, where the selected ion chromatograms are displayed. Trace e displays the internal standard 1,12-dihydroxy-9(*Z*)-octadecene. By comparison to the internal standard, we estimate that approximately 10% of the linoleyl alcohol associated with PMNLs was oxidized to hydroperoxides. These results are based on five experiments for which a total of 10 stimulated and 10 unstimulated preparations were analyzed.

Response of PMNLs to Particles Containing Linoleic Acid. An emulsion of mineral oil, BSA, and tracer amounts of [^{14}C]linoleic acid was used to probe for the presence of $^1O_2$ or free radical involvement during stimulation of the respiratory burst in human PMNL. We examined uptake of the mineral oil–BSA emulsion by substituting Oil Red O for

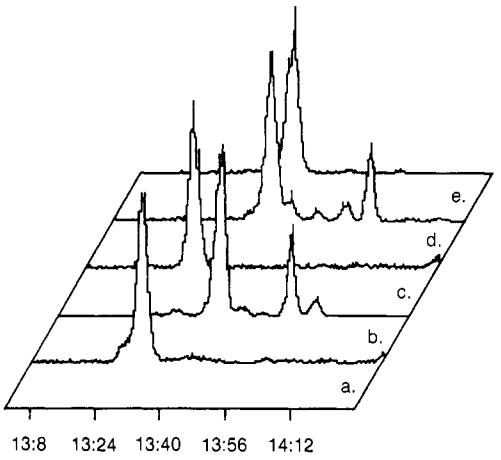

FIGURE 3: GC/MS selective ion traces of ion intensity vs. time (min:s) for extracts from linoleyl alcohol coated latex beads in the presence of PMNLs and added iron: (trace a) m/z 185, 1,12-bis(trimethylsiloxy)-9,13-octadecadiene; (trace b) m/z 225, 1,9-bis(trimethylsiloxy)-10,12-octadecadiene; (trace c) m/z 315, 1,10-bis(trimethylsiloxy)-8,12-octadecadiene; (trace d) m/z 355, 1,13-bis(trimethylsiloxy)-9,11-octadecadiene; (trace e) m/z 187, 1,12-bis(trimethylsiloxy)-9-octadecene, 2 ng of internal standard. Analysis conditions are given under Materials and Methods.

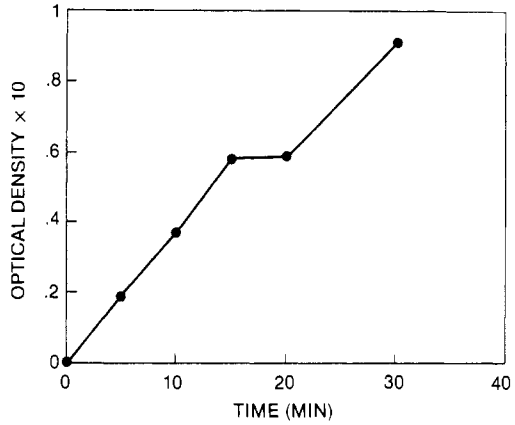

FIGURE 4: Oil Red O uptake by phagocytozing PMNLs as a function of time. The method for preparing the oil–BSA emulsion and the administration to the PMNLs are described under Materials and Methods. The assay consisted of 1 mL of 1×10^7 PMNLs/mL and 0.1 mL of linoleate–Oil Red O–BSA emulsion that were incubated at 37 °C for varying lengths of time.

mineral oil (Stossel et al., 1972). Figure 4 shows the result of treating PMNLs with the Oil Red O–BSA emulsion for varying lengths of time. A linear relationship between optical density and time was observed. Figure 5 shows that the mineral oil emulsion stimulates the HMS as monitored by the release of $^{14}CO_2$ from [1-^{14}C]glucose. Release of $^{14}CO_2$ continues for approximately 40 min at which time there was no further increase in $^{14}CO_2$ evolution. In addition, as seen in Figure 6, the activity depended upon the number of PMNLs employed at a constant dose of oil emulsion.

Analysis for Peroxidized Products of Linoleic Acid. Analysis of the ^{14}C distribution by HPLC indicated that no monohydroxylated products were formed (data not shown). These observations are based on analysis of 12 stimulated PMNL preparations and their paired, unstimulated controls. Tracing radioactivity recovery shows that approximately 33% (4.14 nmol) of the radiolabel associates with the stimulated PMNLs while only 10% of the label associates with the control cells. Approximately 30% of the radioactivity in the stimulated cells was extracted while approximately 40% of the radioactivity associated with control cells could be recovered. Recovery from HPLC was approximately 90%. When [^{14}C]-linoleic acid was allowed to autoxidize and then analyzed by HPLC, radioactivity coeluted with authentic monohydroxylated conjugated dienes used as carriers (Thomas et al., 1982a).

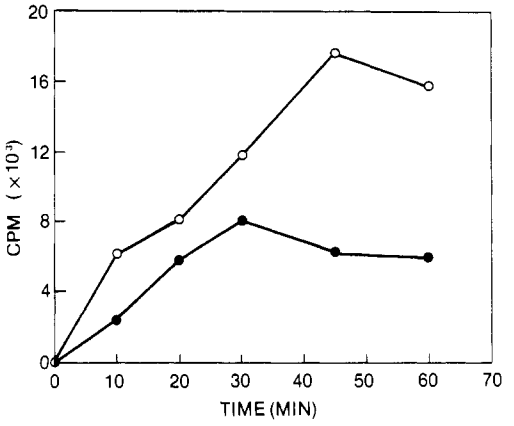

FIGURE 5: Hexose monophosphate shunt activity of phagocytozing (○) and resting (●) PMNLs. The phagocytic stimulus was 0.1 mL of the linoleate–mineral oil–BSA emulsion. Cell concentration is 1.7 $\times 10^6$ PMNLs/mL with a total volume of 3 mL. Activity is measured by $^{14}CO_2$ evolution (expressed as counts per minute). This analysis was performed as described in the literature (Stossel et al., 1972).

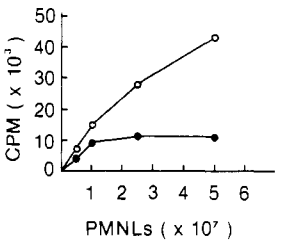

FIGURE 6: Effect of PMNL number on hexose monophosphate shunt activity. The phagocytic stimulus was 0.1 mL of linoleate–mineral oil–BSA emulsion. The conditions are as described in Figure 5.

DISCUSSION

In the absence of added iron salts we find that the $NaBH_4$-reduced extracts from PMNLs activated by the addition of linoleyl alcohol coated latex beads do not contain dienediols. The easily attainable GC/MS sensitivity of 1 ng would have allowed the detection of less than 1% oxidation of linoleyl alcohol. The degree of oxidation that could have been detected with [^{14}C]-linoleic acid was 0.1% (500 dpm, 4.13 pmol). Because linoleic acid recoveries were low, the absence of linoleic acid oxidation products would not by itself convincingly prove that peroxidation does not take place when PMNLs are stimulated with a mineral oil–BSA emulsion. However, the linoleic acid study supports the result obtained with linoleyl alcohol.

The oxidized products that are detected in $NaBH_4$-reduced extracts after simultaneous addition of iron and linoleyl alcohol coated beads include both conjugated and nonconjugated dienediols. Only small amounts of conjugated dienediols were found in the control preparations. The appearance of both cis,trans- and trans,trans-conjugated dienediols is consistent with a free radical autoxidation mechanism as described by Porter et al. (1980) for linoleic acid. The nonconjugated dienediols 1,10-dihydroxy-8,12-octadecadiene and 1,12-dihydroxy-9,13-octadecadiene could arise by H$^\bullet$ abstraction from the monoallylic positions C8 and C14, respectively, followed by addition of O_2 at C10 and C12 of the allylic radical intermediate. However, H$^\bullet$ abstraction at the monoallylic sites would be expected to give two other dienediols by addition of

O_2 to the allylic radical at C8 and C14. Addition of oxygen to both ends of an allylic radical has been observed for oleate (Neff & Frankel, 1980) and for linoleate (Haslbeck & Grosch, 1983). Since no other dienediols were found in this study, H• abstraction from the monoallylic position is not a likely mechanism for the formation of the nonconjugated dienediols.

Both the nonconjugated dienediols and the cis,trans-conjugated dienediols detected in PMNL preparations are readily prepared by dye-sensitized $^1O_2$ oxidation of linoleyl alcohol. However, the mechanism by which $^1O_2$ could be generated by the PMNL is not clear. Formation of $^1O_2$ from superoxide in an electron-transfer reaction is impossible unless the energy difference between product state and reactant state is greater than the energy gap between O_2 ($^3\Sigma_g^-$) and $^1O_2$ ($^1\Delta_g$). Assuming a reduction potential of −0.33 V (vs. NHE) for superoxide at pH 7 (Bielski et al., 1985), then the electron acceptor must have a reduction potential of >0.65 V (vs. NHE). The reaction O_2^- + Fe(III) → $^1O_2$ + Fe(II) could take place only if the Fe(III) salt in our system has a reduction potential similar to that of Fe(III) in water, 0.77 V (Weast, 1981). An alternative mechanism for the formation of $^1O_2$ would be a reaction between H_2O_2 and ^-OCl (Khan & Kasha, 1963); the ^-OCl presumably derived from PMNL myeloperoxidase (Harrison & Schultz, 1976). However, since oxidized products are not detected in the absence of iron salts, the direct reaction of H_2O_2 with ^-OCl is not a likely source of $^1O_2$. Iron salt–H_2O_2 mixtures in acetonitrile are known to exhibit a degree of selectivity in their oxidation of organic substrates (Sugimoto & Sawyer, 1984; Groves & Van Der Puy, 1976), and it was proposed that $^1O_2$ or a ferryl complex might give rise to the oxidation products (Sugimoto & Sawyer, 1984). In spite of the solvent differences, it is tempting to draw a parallel between the work in acetonitrile and our results, where the product distributions are reminiscent of those obtained with $^1O_2$.

One result of our study implies that in the absence of added iron salts PMNLs do not produce sufficient quantities of those species that are capable of oxidizing linoleyl alcohol. This conclusion is different from earlier work in which thiobarbituric acid reactive material was detected when linolenates dissolved in mineral oil emulsions were introduced to PMNLs (Stossel et al., 1974). However, iron is ubiquitous in many biological preparations, and iron contamination may have influenced their results. An additional complication could be the lack of specificity of the thiobarbituric acid test for malondialdehyde. Our result also differs from what would be expected if 1,4-dienes were exposed to a high flux of HO_2 that might be found in an acidic phagosome. HO_2 will initiate free radical chain autoxidation (Gebicki & Bielski, 1981; Bielski et al., 1983) with a rate constant that is much lower than disproportionation. Therefore, H• abstraction is likely to be at a competitive disadvantage with respect to disproportionation, thereby preventing initiation of free radical autoxidation.

A comparison of the two methods used to test for oxy radicals shows that the linoleyl alcohol coated bead is superior to linoleic acid in a BSA–mineral oil emulsion. Both methods were demonstrated to activate the respiratory burst by use of several characteristic tests. With the BSA–mineral oil emulsion we find 3 times as much label is taken up by PMNLs incubated at 37 °C as compared to controls kept on ice. This is consistent with a more active phagocytosis by incubated cells, an observation that was also demonstrated by using Oil Red O instead of mineral oil. However, recovery of [^{14}C]linoleate was uniformly poor from both stimulated and control PMNLs. Since fatty acids are readily incorporated into PMNLs at concentrations similar to those used in this study (Chilton et al., 1982), we presume that most of the missing linoleic acid was incorporated into cellular phospholipids. Most phospholipids are not soluble in hexane–diethyl ether and would remain behind with other cellular residue. In contrast, we could recover all of the linoleyl alcohol used in our experiments. In addition, when hydroperoxides of linoleyl alcohol were added to beads, these products were recovered after phagocytosis by activated PMNLs. Although we can rationalize the loss of linoleic acid through incorporation into lipids, the good recovery of both linoleyl alcohol and hydroperoxides obtained with the linoleyl alcohol–latex bead method makes for a more definitive interpretation of our observations.

In summary, we have obtained two significant findings from our study. The first is that the product distribution from the iron-catalyzed oxidation implies a process that is more complex than "simple" free radical chain autoxidation. The products might be best described as originating from a process involving both $^1O_2$ oxidation and free radical chain autoxidation. However, a mechanism for the formation of $^1O_2$ is not readily apparent. Our observation of an iron-augmented oxidative process is consistent with a previous report by Ambruso and Johnston (1981). Our second finding is that there is no peroxidation of susceptible molecules in the absence of iron salts. The absence of both free radical and $^1O_2$ oxidation products could imply that linoleyl alcohol is insensitive to the flux of oxidants normally found in phagocytozing PMNLs and that added iron salts are required to increase the oxidant flux to a level that yields detectable amounts of products. However, since, 1,4-dienes like linoleyl alcohol are extremely susceptible to free radical attack, this interpretation implies that only the most labile cell components are susceptible to free radical oxidation. We feel that our observations justify postulating that the PMNL produces negligible flux radicals capable of initiating polyunsaturated fatty acid oxidation. We suggest that the iron salts carried with the phagocytozed particle are responsible for the oxidation of susceptible molecules and that iron-catalyzed reactions may be the source of HO• found in some studies. Work is in progress to determine the mechanism responsible for oxidizing linoleyl alcohol in the phagocytic vacuole and to ascertain the role of iron in the oxidation processes.

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Registry No. Fe, 7439-89-6; H_2O_2, 7722-84-1; $HO(CH_2)_7CH{=}CHCH(OH)CH_2CH{=}CH(CH_2)_4Me$, 104995-35-9; $HO(CH_2)_8CH{=}CHCH_2CH(OH)CH{=}CHBu$, 104995-36-0; $HO(CH_2)_8CH(OH)(CH{=}CH)_2Bu$, 104995-37-1; $HO(CH_2)_8(CH{=}CH)_2CH(OH)(CH_2)_4Me$, 104995-38-2; linoleyl alcohol, 506-43-4; linoleate, 60-33-3.

REFERENCES

Ambruso, D. R., & Johnston, R. B. (1981) *J. Clin. Invest. 67*, 352–360.

Babior, B. M., Kipnes, R. S., & Curnutte, J. T. (1973) *J. Clin. Invest. 52*, 741–744.

Bielski, B. H. J., Arudi, R., & Sutherland, M. W. (1983) *J. Biol. Chem. 258*, 4759–4761.

Bielski, B. H. J., Cabelli, D. E., Arudi, R., & Ross, A. B. (1985) *J. Phys. Chem. Ref. Data 14*, 1041–1100.

Chilton, F. H., O'Flaherty, J. T., Walsh, C. E., Thomas, M. J., Wykle, R. L., DeChatelet, L. R., & Waite, B. M. (1982) *J. Biol. Chem. 257*, 5402–5407.

Curnutte, J. T., Whitten, D. M., & Babior, B. M. (1974) *N. Engl. J. Med. 290*, 593–597.
DeChatelet, L. R., & Shirley, P. S. (1981) *J. Immunol. 126*, 1165–1169.
DeChatelet, L. R., Long, G. D., Shirley, P. S., Bass, D. A., Thomas, M. J., Henderson, P. W., & Cohen, M. S. (1982) *J. Immunol. 129*, 1589–1593.
Finkelstein, E., Rosen, G. M., & Rauckman, E. J. (1982) *Mol. Pharmacol. 21*, 262–265.
Foote, C. S., Shook, F. C., & Abakerli, R. A. (1980a) *J. Am. Chem. Soc. 102*, 2503–2504.
Foote, C. S., Abakerli, R. A., Clough, R. L., & Shook, F. C. (1980b) *Dev. Biochem. 11B*, 222–230.
Gebicki, J. M., & Bielski, B. H. J. (1981) *J. Am. Chem. Soc. 103*, 7020–7022.
Green, M. R., Hill, H. A. O., Okolow-Zubkowska, M. J., & Segal, A. W. (1979) *FEBS Lett. 100*, 23–26.
Groves, J. T., & Van Der Puy, M. (1976) *J. Am. Chem. Soc. 98*, 5290–5297.
Harrison, J. E., & Schultz, J. (1976) *J. Biol. Chem. 251*, 1371–1374.
Haslbeck, F., & Grosch, W. (1983) *Lipids 18*, 706–713.
Higgins, R., Foote, C. S., & Cheng, H. (1968) *Adv. Chem. Ser. No. 77*, 102–117.
Johnston, R. B., Kule, B. B., Misra, H. P., Lehmeyer, J. E., Webb, L. S., Baehner, R. L., & Rajagopalan, K. V. (1975) *J. Clin. Invest. 55*, 1357–1372.
Kaplan, E. L., Laxdol, T., & Quie, P. G. (1968) *Pediatrics 41*, 591–599.
Khan, A. U., & Kasha, M. (1963) *J. Chem. Phys. 39*, 2105–2106.
Long, G. D., DeChatelet, L. R., O'Flaherty, J. T., McCall, C. E., Bass, D. A., Shirley, P. S., & Parce, J. W. (1981) *Blood 57*, 561–566.
McCay, P. B., Noguchi, T., Fong, K. L., Lai, E. K., & Poyer, J. L. (1980) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. 4, pp 155–186, Academic, New York.
Neff, W. E., & Frankel, E. N. (1980) *Lipids 15*, 587–590.
Newburger, P. E., & Tauber, A. I. (1982) *Pediatr. Res. 16*, 856–860.
Porter, N. A., Logan, J., & Kontoyiannidou, V. (1979) *Org. Chem. (N.Y.) 44*, 3177–3181.
Porter, N. A., Weber, B. A., Weenen, H., & Khan, J. A. (1980) *J. Am. Chem. Soc. 102*, 5597–5601.
Pryor, W. A., & Tang, R. H. (1978) *Biochem. Biophys. Res. Commun. 81*, 498–503.
Quie, P. G., White, J. G., Holmes, B., & Good, R. A. (1967) *J. Clin. Invest. 46*, 668–679.
Rosen, H., & Klebanoff, S. J. (1977) *J. Biol. Chem. 252*, 4803–4810.
Rosen, H., & Klebanoff, S. J. (1979) *J. Clin. Invest. 64*, 1725–1729.
Sagone, A. L. (1981) in *Oxygen and Oxy-Radicals in Chemistry and Biology* (Rogers, M. A. J., & Powers, E. L., Eds.) pp 719–724, Academic, New York.
Sagone, A. L., Jr., Decker, M. A., Wells, R. M., & Democko, C. (1980) *Biochim. Biophys. Acta 628*, 90–97.
Sawyer, D. T., & Valentine, J. S. (1981) *Acc. Chem. Res. 14*, 393–400.
Stossel, T. P., Mason, R. T., Hartwig, J., & Vaughan, M. (1972) *J. Clin. Invest. 51*, 615–624.
Stossel, T. P., Mason, R. J., & Smith, A. L. (1974) *J. Clin. Invest. 54*, 638–645.
Sugimoto, H., & Sawyer, D. T. (1984) *J. Am. Chem. Soc. 106*, 4283–4285.
Tauber, A. I., & Babior, B. M. (1977) *J. Clin. Invest. 60*, 374–379.
Thomas, M. J., & Pryor, W. A. (1980) *Lipids 15*, 544–548.
Thomas, M. J., & Pryor, W. A. (1981) in *Oxygen and Oxy-Radicals in Chemistry and Biology* (Rogers, M. A. J., & Powers, E. L., Eds.) pp 761–763, Academic, New York.
Thomas, M. J., Mehl, K. S., & Pryor, W. A. (1982a) *J. Biol. Chem. 257*, 8343–8347.
Thomas, M. J., O'Flaherty, J. T., Cousart, S., & McCall, C. E. (1982b) *Prostaglandins 23*, 265–272.
Weast, R. C. (1981) *CRC Handb. Chem. Phys. 62*, D-133.
Winston, G. W., Best, L., & Cederbaum, A. I. (1983) in *Oxy Radicals and Their Scavenger Systems* (Cohen, G., & Greenwald, R. A., Eds.) Vol. 1, pp 145–150, Elsevier, New York.