Generation of Hydroxyl Radical by Enzymes, Chemicals, and Human Phagocytes In Vitro

DETECTION WITH THE ANTI-INFLAMMATORY AGENT, DIMETHYL SULFOXIDE

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ABSTRACT Methane (CH_4) production from the anti-inflammatory agent, dimethyl sulfoxide (DMSO), was used to measure ·OH from chemical reactions or human phagocytes. Reactions producing ·OH (xanthine/xanthine oxidase or Fe^{++}/EDTA/H_2O_2) generated CH_4 from DMSO, whereas reactions yielding primarily O_2^- or H_2O_2 failed to produce CH_4. Neutrophils (PMN), monocytes, and alveolar macrophages also produced CH_4 from DMSO. Mass spectroscopy using $\underline{d}_6$-DMSO showed formation of $\underline{d}_3$-CH_4 indicating that CH_4 was derived from DMSO. Methane generation by normal but not chronic granulomatous disease or heat-killed phagocytes increased after stimulation with opsonized zymosan particles or the chemical, phorbol myristate acetate. Methane production from DMSO increased as the number of stimulated PMN was increased and the kinetics of CH_4 production approximated other metabolic activities of stimulated PMN. Methane production from stimulated phagocytes and DMSO was markedly decreased by purportedly potent ·OH scavengers (thiourea or tryptophane) and diminished to lesser degrees by weaker ·OH scavengers (mannitol, ethanol, or sodium benzoate). Superoxide dismutase or catalase also decreased CH_4 production but urea, albumin, inactivated superoxide dismutase, or boiled catalase had no appreciable effect. The results suggest that the production of CH_4 from DMSO may reflect release of ·OH from both chemical systems and phagocytic cells. Interaction of the nontoxic, highly permeable DMSO with ·OH may explain the anti-inflammatory actions of DMSO and provide a useful measurement of ·OH in vitro and in vivo.

INTRODUCTION

Stimulated phagocytes use oxygen (O_2) and produce highly reactive O_2 metabolites (1, 2). Several O_2 metabolites, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and perhaps, singlet oxygen ($^1O_2$), are made by phagocytes and appear to be involved in their bactericidal and/or cytotoxic capabilities (1, 3). Because O_2^- and H_2O_2 may form ·OH in the presence of trace metals (4, 5), recent investigations have focused on determining the importance of ·OH by measuring the production of ethylene (C_2H_4) by stimulated phagocytes in the presence of thioethers, such as β-methyl-propionaldehyde (methional) or 2-keto-4-thiomethyl-butyric acid (KMB)[1] (6–10):

$$\cdot OH + \underset{\text{(methional)}}{HCOCH_2CH_2SCH_3} \rightarrow C_2H_4 + \tfrac{1}{2}(CH_3S)_2 + HCOOH$$
(ethylene)

$$\cdot OH + \underset{\text{(KMB)}}{CH_3SCH_2CH_2COCOOH} \rightarrow C_2H_4 + \tfrac{1}{2}(CH_3S)_2 + HCOOH + CO_2$$
(ethylene)

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[1] *Abbreviations used in this paper*: AM, alveolar monocytes; CGD, chronic granulomatosus disease; DMSO, dimethyl

Indeed, stimulated neutrophils (PMN), monocytes (MN) or alveolar marcophages (AM) do produce C_2H_4 from methional or KMB (6–10). However, the strict dependence of C_2H_4 production upon ·OH is in doubt (8, 10–14). Methional spontaneously forms C_2H_4, especially during prolonged incubations with tissues (8), and can also react with H_2O_2 to form C_2H_4 (10). The specificity of KMB as a detector of ·OH is also in question because C_2H_4 production from KMB may reflect, at least in part, reactions with $^1O_2$ or hypochlorous acid (10).

In the course of comparing the generation of C_2H_4 from KMB in the presence of opsonized zymosan particles or phorbol myristate acetate (PMA) stimulated PMN, we observed that the latter samples also synthesized methane (CH_4). Additional experiments indicated that the CH_4 arose from the dimethyl sulfoxide (DMSO) used as a solvent for PMA (15). Because others have used DMSO as a scavenger of ·OH (16–25), and because CH_4 might be produced as a result of this reaction (20–22), the present investigations were designed to examine the validity of using DMSO as a detector of ·OH.

METHODS

Reagents. Xanthine oxidase (grade I, buttermilk), KMB, catalase (bovine liver, crystallized twice), superoxide dismutase (SOD, type 1, 3,000 U/mg protein), thiourea (grade I), urea, scopoletin (7-OH-6-methoxycoumarin), zymosan A, DMSO (Grade I), sodium azide, L-tryptophane, mannitol, xanthine, horse-heart ferricytochrome *c*, and DL-histidine were obtained from Sigma Chemical Co., St. Louis, Mo. Ferrous sulfate (Baker Adamson, Morriston, N. J.), EDTA (Sigma Chemical Co., St. Louis, Mo.), H_2O_2 (Mallinckrodt, Inc., St. Louis, Mo.), sodium benzoate (J. T. Baker Chemical Co., Phillipsburg, N. J.), PMA (12-*O*-tetradeconyl-phorbol-13-acetate, Consolidated Midland Corp., Brewster, N. Y.), and human serum albumin (25%, Cutter Laboratories, Inc., Berkeley, Calif.) were used as purchased.

Recovery and preparation of human PMN, MN, and AM. This investigation was approved by the Human Volunteers Committee of the University of Minnesota. Blood was collected from healthy volunteers not taking medications and from two patients with proven X-linked chronic granulomatosus disease (CGD) in good health at the time of venipuncture. Blood (30 ml) was drawn into a plastic syringe that contained 1,000 U of sodium heparin and allowed to sediment with 5 ml of 6% dextran in saline (Dextran 75, Travenol Laboratories, Inc., Morton Grove, Ill.) for 90 min (26). PMN or MN were separated by differential centrifugation on Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.)-Hypaque (Winthrop Laboratories, New York), washed once and resuspended in Hanks' balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (26). Contaminating erythrocytes were lysed by adding 6 ml of ice-cold sterile distilled water and mixing gently for 35 s. Tonicity was rapidly restored with 2 ml of hypertonic (4×) Ca^{++} and Mg^{++} free HBSS. The mixture was then centrifuged at 170 *g* for 10 min. Cells in the pellet were then washed once more, resuspended in HBSS with Ca^{++} and Mg^{++}, counted, and used immediately. PMN preparations contained >98% PMN with only a few lymphocytes and rare erythrocytes, platelets or MN. Suspensions of MN were similarly prepared. The percentages of MN were determined by differential counting of 600 cells on Wright's stained smears and cytochemically confirmed by esterase stain. MN preparations contained ~30% MN, <1% PMN, and the remainder lymphocytes. AM were obtained by bronchoscopic sterile saline lavage of the unaffected subsegments of the lungs of patients undergoing evaluations for localized pulmonary disease or from healthy volunteers (9). AM were recovered from lavage fluids, washed once, and counted. The final preparations contained >90% AM and <2% PMN. More than 85% of the AM excluded trypan blue. Concentrations of lymphocytes or platelets that were comparable to those remaining in phagocyte preparations did not produce significant amounts of CH_4 from DMSO.

Preparation of pooled human serum, opsonized zymosan, or PMA. Pooled human serum was prepared from clotted blood from five or more control subjects, pooled, and frozen in aliquots at −70°C for less than 2 wk before use (27). Zymosan A, 50 mg, was washed with HBSS, opsonized with 1 ml pooled human serum at 37°C for 30 min, centrifuged at 700 *g* for 5 min, and resuspended in HBSS. PMA was dissolved in DMSO and stored in the dark at 4°C (15).

Measurement of CH_4 or C_2H_4 production. For studies of phagocytic cell function, siliconized (3 ml) glass tubes were prepared by sequentially adding DMSO or KMB, PMN, MN, or AM in HBSS, and then HBSS, opsonized zymosan or PMA in HBSS (final volume 1 ml). These concentrations of zymosan or PMA produced maximal rates of O_2 uptake, O_2^- formation, and chemiluminescence by PMN. The tubes were then rapidly sealed with rubber stoppers and incubated at 37°C in a shaking water bath.

For studies with chemical reactions, tubes were prepared by successive additions of DMSO, KMB, phosphate-buffered HBSS, pH 7.8, EDTA, and/or xanthine. The tubes were then capped and either H_2O_2 or xanthine oxidase was injected through the rubber stopper. Subsequently, the reactions were mixed, incubated at 30°C in a water bath for 20 min, and placed on ice.

Samples of the headspace gas in each tube were introduced into a gas chromatograph (series 1400, flame ionization detector, Varian Associates, Palo Alto, Calif.) via a 0.25-ml gas sampling loop (10, 28). A ⅛ in. × 6 ft stainless steel column packed with Carbosieve B 60/80 mesh (Supelco, Inc., Bellefonte, Pa.) was used. The injector, detector, and column temperatures were 200°, 200°, and 120°C, respectively. The retention time for CH_4 was 0.5 min and for C_2H_4 was 2.5 min. Analyses of CH_4 or C_2H_4 standards (100 ppm, Scott Specialty Gases, division of Scott Environmental Technology, Inc., Plumsteadville, Pa.) were included with each experiment. The CH_4 or C_2H_4 contents of each test sample were determined by comparing the peak heights of experimental samples with those of standards. The CH_4 present in lab air was subtracted from each reading. The concentration of the gas in the headspace (2 ml) was initially measured in parts per million and was expressed as picomoles of CH_4 or C_2H_4.

Measurement of d_3-CH_4 by gas chromatography/mass spectroscopy. d_3-CH_4 produced from d_6-DMSO (99.5% atom % Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.) was identified using a Finnigan model 1015 gas chromatography/mass spectroscope (Finnigan Corp., Sunnyvale, Calif.) equipped with a 5 ft × 2 mm i.d. glass column packed with Carbosieve-B 60/80 mesh (Supelco, Inc.) maintained at 160°C

sulfoxide; HBSS, Hanks' balanced salt solution; KMB, 2-keto-4-thiomethylbutyric acid; m/e, mass/charge; MN, monocytes; PMA, phorbol myristate acetate; PMN, neutrophils; SOD, superoxide dismutase.

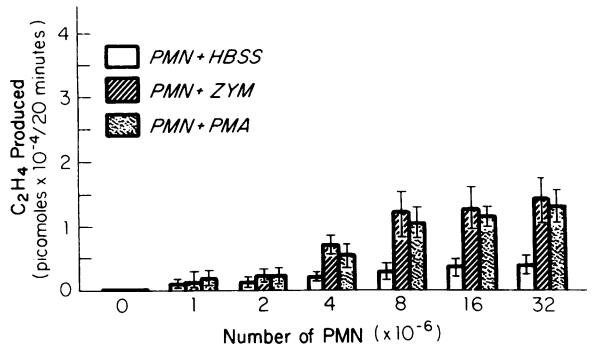

FIGURE 1 Generation of C_2H_4 by human PMN as a function of the number of PMN. C_2H_4 production progressively increased as the number of PMN was increased from 1 to 8 × 10^6, but did not increase as the number of PMN was increased from 8 to 32 × 10^6.

(29, 30). The mass spectrometer operating parameters were adjusted to yield maximal signal and resolution. Samples were introduced via a gas sampling value equiped with a 0.7 ml sampling loop. Spectra were recorded over the range mass/charge (m/e) 15–19 before and during elution of the CH_4 peak. The relative abundance of ions at m/e 16–19 were calculated using m/e 19 (d_3-CH_4) as the base peak and the background values subtracted from those recorded during elution of the CH_4 peak to yield the mass spectrum of d_3-CH_4. Ions at m/e 18 were not included because of the large background contribution of water.

Measurement of O_2^-. Production of O_2^- was determiend by measuring O_2^--dependent reduction of horse heart ferricytochrome *c* spectrophotometrically (31).

Measurement of H_2O_2. H_2O_2 was measured using the standard fluorometric scopoletin assay (32).

Measurement of O_2 consumption. O_2 uptake by PMN was performed using a biologic O_2 probe attached to an oxygen analyzer and Beckman recorder (15).

Measurement of [1-^{14}C]glucose oxidation. [1-^{14}C]Glucose oxidation was determined by measuring the amount of $^{14}CO_2$ produced from incubations of PMN and opsonized zymosan (15).

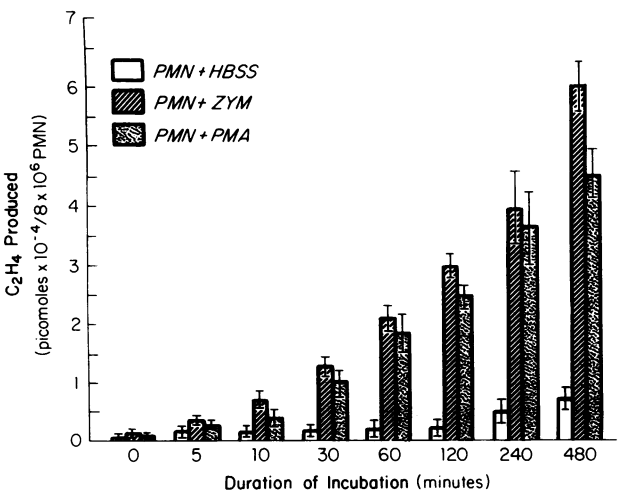

FIGURE 2 Generation of C_2H_4 by human PMN as a function of the duration of incubation. C_2H_4 production by stimulated human PMN continues for as long as 480 min.

RESULTS

Our initial goal was to compare the production of C_2H_4 from KMB by PMN stimulated with opsonized zymosan or PMA. Treatment of PMN with zymosan or PMA stimulated production of C_2H_4 from KMB. But doubling the number of PMN did not proportionately increase C_2H_4 generation (Fig. 1). This was not caused by a limitation of substrate because doubling the amount of KMB, zymosan, or PMA did not increase production of C_2H_4 (data not shown). Furthermore, prolonged incubation of stimulated PMN led to increased production of C_2H_4 from KMB, a process which continued for at least 8 h (Fig. 2). This differs from the patterns of O_2 consumption, [1-^{14}C]glucose oxidation, H_2O_2 generation and chemiluminescence at 37°C by stimulated PMN, all of which usually cease within 30 min (data not shown, 15, 33–35). These findings suggested that C_2H_4 arising from KMB and stimulated PMN might reflect processes in addition to the active cellular generation of ·OH.

During the above investigations, we observed that PMN in the presence of PMA, but not zymosan, also produced CH_4 (Table I). PMA preparations contained DMSO, which was used as a solvent for PMA (15). After addition of pure DMSO, zymosan-stimulated PMN also synthesized CH_4 in a dose-dependent manner. Maximal CH_4 production occurred over the range of 13–130 mM DMSO. CH_4 production from mixtures of zymosan- or PMA-treated PMN and DMSO

TABLE I
Effect of DMSO on the Production of CH_4 by Human PMN Stimulated by Opsonized Zymosan or PMA

Test conditions*	CH_4 produced
	pmol/20 min
PMN + PMA (dissolved in 2.6 mM DMSO)	320±41 (5)‡
PMN + zymosan	0 (11)
PMN + zymosan + DMSO (1.3 mM)	120±110 (8)
PMN + zymosan + DMSO (13 mM)	990±180 (8)
PMN + zymosan + DMSO (26 mM)	1,080±175 (16)
PMN + zymosan + DMSO (130 mM)	1,190±210 (19)
PMN + PMA + DMSO (13 mM)	1,020±240 (8)
PMN + PMA + DMSO (130 mM)	1,040±119 (15)
PMN + DMSO (13 mM)	240±42 (12)
PMN + HBSS	0 (15)
Zymosan + DMSO (13 mM)	4.1±0.9 (6)
PMA + DMSO (13 mM)	2.6±1.3 (8)

* Each 3-ml tube contained in a final vol of 1 ml, 8 × 10^6 PMN, 15 mg opsonized Zymosan or 0.5 μg PMA, and HBSS with or without DMSO and was incubated at 37°C.
‡ Mean±SE (number of determinations).

was approximately four times greater than from untreated PMN and DMSO. In addition, and in striking contrast to C_2H_4 production from KMB, increasing the number of zymosan- or PMA-stimulated PMN incubated with DMSO progressively increased CH_4 production (Fig. 3). Moreover, CH_4 generation by mixtures of stimulated PMN and DMSO plateaued in about 30 min (Fig. 4), a pattern in accord with the other metabolic responses of stimulated PMN.

Reactions of DMSO or KMB with chemically or enzymatically generated forms of activated O_2

Because our goal was to determine the nature of the production of CH_4 in systems containing phagocytes, we investigated CH_4 production from chemical reactions known to produce activated forms of O_2. These studies were performed in complete HBSS, at or near physiologic pH and using previously identified scavengers in concentrations that did not decrease cell viability or phagocytosis (6–10, 36–39). Additional studies showed that DMSO, thiourea and urea did not inhibit phagocytosis of radiolabeled bacteria by PMN (40) or decrease the ability of phagocytes to exclude trypan blue.

Xanthine/xanthine oxidase. It has been assumed that ·OH is generated by xanthine and xanthine oxidase through reaction of O_2^- and H_2O_2 and that this reaction is catalyzed by contaminating or enzyme-associated trace metals (4, 5, 28):

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH$$

In the presence of DMSO, the complete xanthine oxidase system produced large quantities of CH_4 (Table II). CH_4 production was decreased by SOD and nearly eliminated by catalase. Thiourea, a potent

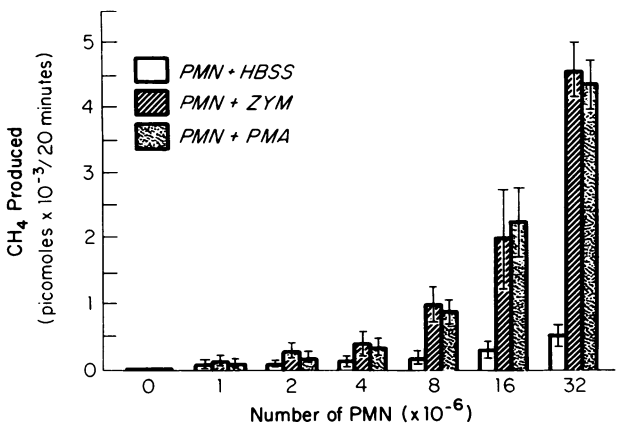

FIGURE 3 Generation of CH_4 from 13 mM DMSO by human PMN as a function of the number of PMN. CH_4 production progressively increases as the number of PMN are increased from 1 to 32 × 10^6.

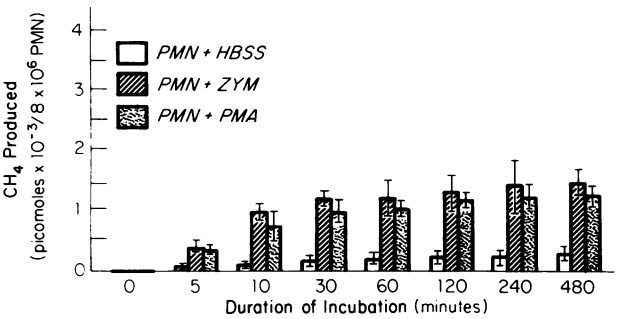

FIGURE 4 Generation of CH_4 from 13 mM DMSO by human PMN as a function of the duration of incubation. CH_4 production by stimulated PMN reaches maximal levels after 30–60 min of incubation.

scavenger of ·OH, also decreased CH_4 generation, whereas urea, an ineffective scavenger, had no effect. C_2H_4 was also formed from KMB by the xanthine/

TABLE II
Production of CH_4 from DMSO or C_2H_4 from KMB during Reaction of Xanthine with Xanthine Oxidase

Test conditions*	CH_4 produced from DMSO (13 mM)	C_2H_4 produced from KMB (20 mM)
	pmol/20 min	
Xanthine + XO	2,029±182 (21)‡	1,877±250 (17)
Xanthine + XO + SOD	1,389±65 (8)§	702±100 (9)§
Xanthine + XO + catalase	108±20 (8)§	72±44 (7)§
Xanthine + XO + thiourea	648±87 (9)§	12±15 (8)§
Xanthine + XO + urea	2,196±200 (9)	1,710±52 (9)
Xanthine + XO + DMSO	ND‖	24 (3)§
Xanthine + XO KMB	1676 (3)	ND
Xanthine	12±14 (9)	18±21 (9)§
XO	24±19 (6)	9±5.2 (9)§
HBSS	1.4±18 (32)	2.2±0.4 (21)

XO, xanthine oxidase.

* For assay of CH_4 or C_2H_4 production, each 3-ml tube contained in a final vol of 1 ml; 4 mM xanthine, 0.1 mM EDTA, 0.025 U xanthine oxidase, 100 μg SOD/ml, 500 μg catalase/ml, 150 mM thiourea, 150 mM urea, 13 mM DMSO, 20 mM KMB and/or phosphate-buffered HBSS, pH 7.8. In the absence of DMSO or KMB, xanthine, EDTA and xanthine oxidase did not make appreciable amounts of CH_4 or C_2H_4. Acetaldehyde and xanthine oxidase produced CH_4 in the absence of DMSO and was not used. Heat-inactivated, dialyzed SOD or heat-inactivated catalase did not significantly inhibit CH_4 or C_2H_4.

‡ Mean±SE (number of determinations).

§ Value significantly ($P < 0.05$) different from value with no inhibitor added.

‖ ND, not done.

xanthine oxidase system and the above inhibitors had similar effects. When both DMSO and KMB were added, considerable CH_4 was produced but only negligible amounts of C_2H_4 appeared. Thus, reactions with DMSO which produce CH_4 take precedence over reactions with KMB which generate C_2H_4.

Because O_2^- and H_2O_2 are also produced by the xanthine/xanthine oxidase system, additional experiments were performed to determine if O_2^- or H_2O_2 reacted directly with DMSO or KMB to cause liberation of CH_4 or C_2H_4. First, neither DMSO (13–130 mM) nor KMB (2–20 mM) inhibited O_2^--dependent reduction of cytochrome *c* by the complete xanthine oxidase system (41), whereas SOD nearly blocked this reaction (data not shown). Second, in the presence of DMSO or KMB, H_2O_2 (0.003–30 μM) failed to generate detectable amounts of CH_4 or C_2H_4 (Table III). Thus, neither O_2^- nor H_2O_2 reacts directly with DMSO or KMB to form CH_4 or C_2H_4.

Fe/EDTA/H_2O_2. Another system which generates ·OH involves the reaction of ferrous (Fe^{++}) sulfate/EDTA with H_2O_2 (42):

$$Fe^{++}/EDTA + H_2O_2 \rightarrow Fe^{+++}/EDTA + OH^- + \cdot OH$$

This reaction produced large quantities of CH_4 from DMSO (Table III). CH_4 production was not diminished by SOD but was greatly decreased by catalase, implicating an involvement of H_2O_2. However, as previously shown, H_2O_2 and DMSO alone did not produce CH_4.

TABLE III
Production of CH_4 during Reaction of Fe^{++} with H_2O_2

Test conditions*	CH_4 produced	
	pmol/20 min	
$Fe^{++} + H_2O_2$	11±1.3	(38)‡
$Fe^{++} + H_2O_2$ + DMSO	36,101±1,885	(35)
$Fe^{++} + H_2O_2$ + SOD	ND‖	
$Fe^{++} + H_2O_2$ + SOD + DMSO	38,528±2,540	(12)
$Fe^{++} + H_2O_2$ + catalase	ND	
$Fe^{++} + H_2O_2$ + catalase + DMSO	12,428±1,286	(12)§
$Fe^{++} + H_2O_2$ + KMB	ND	
Fe^{++} + DMSO	18±9	(24)
H_2O_2 + DMSO	1.4±1.8	(32)
HBSS + DMSO	1.8±1.3	(26)

* For assay of CH_4 production, each 3-ml tube contained in a final vol of 1 ml; 11 mM ferrous sulfate, 1 mM EDTA, 30 μM H_2O_2, 100 μg SOD/ml, 500 μg catalase/ml 13 mM DMSO, 20 mM KMB and/or phosphate-buffered HBSS, pH 7.8. $FeSO_4$ reacted directly with KMB to form C_2H_4, so studies of C_2H_4 production were not performed. Autoclaved, dialyzed SOD or heat-inactivated catalase did not significantly inhibit CH_4 production.

‡ Mean±SE (number of determinations).

§ Value significantly ($P < 0.05$) different from value with no inhibitor.

‖ ND, not done.

Generation of CH_4 from DMSO or C_2H_4 from KMB by stimulated phagocytes

CH_4 produced by the above chemical reactions was almost certainly derived from DMSO. To determine that this was also true for complex cellular systems, we performed preliminary studies with stimulated PMN using d_6-DMSO. These studies confirmed that the CH_4 produced did indeed come from DMSO. The mass spectrum of the CH_4 formed showed a mol wt of 19, which corresponds to d_3-CH_4. This agrees with the published mass spectrum of d_3-CH_4 (base peak = m/e 19, m/e 17 = 51.1% [29]).

We also performed studies that indicated that intact PMN with normal oxidative metabolic activities were needed for the generation of CH_4 from DMSO. These studies showed that appreciable amounts of CH_4 were not produced from mixtures of DMSO and zymosan- or PMA-stimulated, heat-killed or CGD PMN (data not shown).

Studies with scavengers of O_2^- or H_2O_2 confirmed that both species contributed to the production of CH_4 from DMSO or C_2H_4 from KMB by stimulated PMN (Table IV). Furthermore, thiourea, a purported ·OH scavenger, but not urea, practically abolished CH_4 generation, while decreasing C_2H_4 production ~70–80%. Another presumed scavenger of ·OH, tryptophane, also eliminated CH_4 or C_2H_4 production. other suspected scavengers of ·OH, sodium benzoate, mannitol, or ethanol, although not as effective as thiourea or tryptophane, were more efficient in systems containing DMSO (50–60% inhibition) that KMB (20–35%). Azide or histidine also decreased CH_4 or C_2H_4 production by stimulated PMN.

Zymosan- or PMA-treated human MN also produced CH_4 from DMSO (Table V). SOD or catalase inhibited CH_4 production by ~25–45%. The production of CH_4 by zymosan- or PMA-stimulated MN was approximately one-half as much per cell as the CH_4 generation by stimulated PMN. As expected, stimulated MN from patients with CGD did not produce increased amounts of CH_4.

Zymosan- or PMA-treated human AM also generated CH_4 from DMSO, a process inhibited by SOD, catalase, or thiourea (Table V). Generation of CH_4 by AM was approximately one-sixth as great per cell as PMN and about one-third that of peripheral blood MN.

DISCUSSION

Considerable interest has focused on ·OH production by phagocytes because this may be important in their bactericidal or cytotoxic activities (43, 44). However, the techniques for measurement of ·OH production

TABLE IV

Effect of Scavengers on the Production of CH_4 from DMSO or C_2H_4 from KMB by PMN Stimulated by Opsonized Zymosan or PMA

Test conditions: inhibitor added*	CH_4 production		C_2H_4 production	
	Zymosan-stimulated PMN	PMA-stimulated PMN	Zymosan-stimulated PMN	PMA-stimulated PMN
	% inhibition			
SOD, 100 μg/ml	84±12 (8)‡	91±9.1 (8)	ND‖	ND
SOD, 10 μg/ml	82±9.6 (8)	88±12 (8)	92±8.4 (6)	90±6.4 (6)
SOD, 1.0 μg/ml	79±13 (8)	78±10 (8)	ND	ND
SOD, 10 μg/ml, inactivated	12±0.9 (6)	21±2.1 (8)	ND	ND
Catalase, 1000 μg/ml	51±19 (8)	52±8.2 (8)	ND	ND
Catalase, 250 μg/ml	48±9.2 (8)	57±16 (8)	74±11 (6)	71±9.3 (6)
Catalase, 100 μg/ml	36±12 (8)	42±19 (8)	ND	ND
Catalase, 500 μg/ml, heat inactivated	0 (8)	4.2±3.8 (8)	ND	ND
Albumin 1%	2.6±0.8 (8)	3.9±1.4 (4)	1.4±0.9 (5)	2.9±1.6 (4)
Thiourea, 15 mM	102±4.1 (5)	96±5.8 (7)	72±15 (6)	81±11 (5)
Thiourea, 1.5 mM	90±9.8 (5)	92±9.8 (5)	ND	ND
Urea, 15 mM	4.2±3.6 (7)	3.1±2.6 (4)	9.1±3.1 (6)	3.6±1.8 (5)
Urea, 1.5 mM	0.4±0.6 (5)	6.4±10 (4)	ND	ND
Tryptophane, 1.0 mM	108±14 (4)	100±12 (6)	94±14 (8)	98±26 (4)
Tryptophane, 0.5 mM	52±11 (5)	42±9.1 (6)	ND	ND
Sodium benzoate, 20 mM	46±9.1 (5)	42±11 (5)	26±9.2 (5)	34±16 (5)
Sodium benzoate, 2 mM	36±16 (5)	42±11 (5)	ND	ND
Mannitol, 50 mM	74±14 (5)	59±12 (5)	34±6.8 (5)	26±9.4 (7)
Mannitol, 5 mM	52±6.4 (5)	47±11 (4)	ND	ND
Ethanol, 40 mM	64±14 (5)	59±14 (5)	ND	ND
Ethanol, 20 mM	46±11 (5)	ND	34±15 (5)	35±11 (6)
Histidine, 0.1 mM	84±26 (4)	84±15 (4)	94±15 (6)	ND
Azide, 1.0 mM	89±15 (6)	66±14 (5)	94±14 (6)	92±14 (7)
Azide, 0.1 mM	78±11 (4)	ND	ND	ND

* Each 3-ml tube contained in a final vol of 1 ml; 8×10^6 PMN with 15 mg Zymosan or 0.5 μg PMA, the inhibitors and/or HBSS with 13 mM DMSO or 2 mM KMB. Base line gas production in the absence of inhibitors was ~1,000 pmol CH_4 from DMSO/20 min or ~10,000 pmol C_2H_4 from KMB/20 min for Zymosan or PMA-stimulated PMN.

‡ Mean±SE (number of determinations).

§ Results of mean inhibition calculated from % inhibition = (CH_4 without inhibitor − CH_4 with inhibitor/CH_4 without inhibitor) × 100.

‖ ND, not done.

by phagocytes have not been totally satisfactory because of vagaries in specificity or incompatibilities with biological substances. We are reporting a new approach for the measurement of ·OH generation by phagocytes, namely the generation of CH_4 from DMSO.

Previous investigations suggested that DMSO might be a potent scavenger of ·OH (16–24), that reaction of DMSO with titanium chloride and H_2O_2 produces methyl radical (22), and that CH_4 might be produced by reaction of ·OH with DMSO (20, 21, 25). However, the specificity of the reaction of ·OH with DMSO to produce CH_4 and its potential as a detection system for ·OH have not previously been determined. The rate constant for reaction of ·OH with DMSO (5.8×10^9 M^{-1} s^{-1}) indicates rapid reaction of the two compounds (16, 45).

$$(CH_3)_2SO + \cdot OH \rightarrow (CH_3)_2S\begin{matrix}OH\\ \diagup\\ \diagdown\\ O\cdot\end{matrix} \rightarrow \cdot CH_3 + CH_3SO_2H$$

$$\cdot CH_3 + RH \rightarrow CH_4 + R\cdot$$

This reaction is consistent with the observation that ·OH attacks dialkyl sulfoxides at sulfur rather than by hydrogen abstraction (46). Furthermore, our mass spectral studies of the CH_4 formed by stimulated

TABLE V

Production of CH_4 from DMSO by Normal or CGD MN and Normal AM Stimulated by Opsonized Zymosan or PMA

Test conditions*	CH_4 produced from DMSO		
	Normal MN	CGD MN	Normal AM
		pmol/20 min	
Cells + HBSS	150±43 (12)‡	88±28 (6)	120±28 (8)‡
Cells + zymosan	1,080±290 (8)§	120±42 (6)	320±85 (6)§
Cells + SOD + zymosan	420 (3)—61%¶	ND‖	110 (3)—66%¶
Cells + catalase + zymosan	510 (3)—53%	ND	180 (3)—44%
Cells + thiourea + zymosan	150 (3)—86%	ND	135 (3)—58%
Cells + tryptophan + zymosan	160 (3)—85%	ND	ND
Cells + sodium benzoate + zymosan	540 (3)—50%	ND	ND
Cells + ethanol + zymosan	560 (3)—48%	ND	ND
Cells + PMA	920±106 (8)§	125±38 (6)	280±60 (6)
Cells + SOD + PMA	440 (3)—52%	ND	120 (3)—57%
Cells + catalase + PMA	385 (3)—58%	ND	160 (3)—43%
Cells + thiourea + PMA	190 (3)—79%	ND	100 (2)—64%
Cells + tryptophane + PMA	180 (3)—80%	ND	ND
Cells + sodium benzoate + PMA	410 (3)—55%	ND	ND
Cells + ethanol + PMA	490 (3)—47%	ND	ND

* Each 3-ml tube contained a final vol of 1 ml, which included 20×10^6 MN, 15 mg zymosan or 0.5 μg PMA, and 100 μg SOD, 500 μg catalase, 15 mM thiourea, 1 mM tryptophan, 20 mM sodium benzoate, 40 mM ethanol and/or HBSS with 13 mM DMSO.

‡ Mean±SE (number of determinations).

§ Value significantly different ($P < 0.05$) from value with HBSS alone.

‖ ND, not done.

¶ % inhibition from value without inhibitor.

phagocytes (and by Fe^{++}/EDTA/H_2O_2, data not presented) showed that $\underline{d}_3$-CH_4 was produced from $\underline{d}_6$-DMSO. This is the expected finding because it is known that methyl radicals do not abstract hydrogen from DMSO in solution (47); abstraction of hydrogen from $\underline{d}_6$-DMSO would yield $\underline{d}_4$-CH_4. This was not observed.

The results of the present investigation indicate that generation of CH_4 from DMSO is a relatively specific result of reaction with ·OH and that this approach may prove useful for evaluation of the production of ·OH in biological systems. DMSO does not spontaneously form CH_4 (Table I) and chemical systems (xanthine and xanthine oxidase or Fe^{++}/EDTA and H_2O_2) known to give rise to ·OH produced large amounts of CH_4 from DMSO (Tables II and III). Whereas production of CH_4 from DMSO by xanthine oxidase is decreased by SOD or catalase (implicating participation of O_2^- and H_2O_2), O_2^- or H_2O_2 alone do not appear to produce CH_4 from DMSO. If O_2^- reacted directly with DMSO to make CH_4, catalase would not inhibit the generation of CH_4 by xanthine and xanthine oxidase (28). In addition, DMSO does not inhibit xanthine/xanthine oxidase-mediated reduction of ferricytochrome *c*, a reaction that is dependent on O_2^- (41). Furthermore, multiple concentrations of H_2O_2 and DMSO do not form CH_4. Thus, whereas large quantities of CH_4 are produced from DMSO by ·OH generating systems, there is probably little or no production of CH_4 from DMSO in the presence of O_2^- or H_2O_2 alone. Although technical difficulties with the generation and measurement of pure $^1O_2$ make it very difficult to determine if reaction of $^1O_2$ with DMSO to produce CH_4 or with KMB to generate C_2H_4, preliminary studies addressing this question (employing several $^1O_2$ generators including rose bengal) indicte that DMSO is unreactive with $^1O_2$. Thus, the evidence presented favors the proposition that CH_4 production arises predominantly from reaction of ·OH with DMSO.

Comparison of the production of CH_4 from DMSO or C_2H_4 from KMB further supports the possibility that reaction of ·OH with DMSO might be a more effective approach for evaluation of the generation of ·OH. First, DMSO appears to be a more effective scavenger of ·OH than KMB. In mixtures containing the complete xanthine oxidase system with both DMSO and KMB the production of CH_4 from DMSO was nearly normal, while there was no appreciable generation of C_2H_4 from KMB. Second, production of CH_4 from DMSO by stimulated phagocytic cells

increases according to the number of PMN added and the kinetics of CH_4 production reflect those of other O_2-dependent metabolic events. In contrast, the generation of C_2H_4 from KMB does not increase with the number of PMN present or follow expected patterns, producing considerable amounts of C_2H_4 for as much as 8 h, long after other metabolic activities of the cells have ceased. A self-propagating event, known to occur between certain oxygen intermediates ($^1O_2$) and lipids might account for this prolonged production of C_2H_4 from KMB by metabolically exhausted PMN. KMB may also react with cell-associated Fe to produce C_2H_4. We find that large amounts of C_2H_4 are generated from KMB by the EDTA chelate of Fe^{++} (data not presented). Third, the results show that intact cells or cells with normal oxidative metabolic capabilities are necessary for CH_4 generation from DMSO. Heat-killed cells and PMN or MN from patients with CGD did not make appreciable amounts of CH_4 from DMSO. Fourth, DMSO was not toxic to the cells and did not inhibit uptake of bacteria by PMN. In addition, the results of investigations with purported scavengers of various O_2 metabolites support the relative specificity of the reaction of DMSO with ·OH. However, it should be noted that, as with most inhibitor studies, the specificity of these agents is not absolute and, the mechanism of their action is open to question in many cases.

Hydroxyl radical is considered a part of the mechanism which phagocytic cells use to kill ingested microorganisms (2). The assumed role of ·OH in the microbicidal processes of phagocytes is supported by the decreased bactericidal activities of PMN that have been treated with sodium benzoate, a scavenger of ·OH (3), and the amelioration by ·OH scavengers of the bactericidal actions of chemical systems that generate activated forms of O_2 (48–50). Hydroxyl radical may also participate in phagocyte-mediated cytotoxicity.

In addition to its presumed role in the bactericidal and cytotoxic activities of phagocytes, ·OH may participate in many other important biological reactions (43, 44). The basis for implicating ·OH in these diverse processes has often been that DMSO, as well as other scavengers of ·OH, alter expected responses. For example, DMSO decreases radiation injury (23), alloxan-induced diabetes (19), arthritis (51), contact dermatitis (52), and noninfectious or neoplastic inflammation of the bladder (53). DMSO has also been used frequently as a solvent (15, 52), and as a cryopreservative for leukocytes (54), platelets (55), erythrocytes (56), and other mammalian cells (57). Whereas the precise mechanism underlying these and many other actions of DMSO are unknown, many may involve the scavenging ·OH by DMSO. This is a particularly appealing possibility in the case of the anti-inflammatory effects of DMSO. Although most organisms have very active enzymes for the catabolism of H_2O_2 and O_2^- (precursors of ·OH), there are no direct enzymatic mechanisms for clearance of highly reactive ·OH. Thus, the reaction of DMSO with ·OH may explain the potent anti-inflammatory action of DMSO. DMSO has an extremely low toxicity, can be easily administered by oral, topical, and other routes, is rapidly distributed throughout the body, and readily penetrates cells (58–61). Thus, the generation of CH_4 by reaction of ·OH with DMSO should be useful for detecting and measuring the production of ·OH both in vitro and in vivo and determining its biological effects.

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REFERENCES

1. Babior, B. M. 1978. Oxygen-dependent microbial killing by phagocytes. *N. Engl. J. Med.* **298:** 659–668, 721–725.
2. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* **12:** 117–142.
3. Johnston, R. B., Jr., B. B. Keele, Jr., H. P. Misra, J. E. Lehmeyer, L. S. Webb, R. L. Baehner, and K. V. Rajagopolan. 1975. The role of superoxide anion generation in phagocytic bactericidal activity. Studies with normal and chronic granulomatous disease leukocytes. *J. Clin. Invest.* **55:** 1357–1372.
4. McCord, J. M., and E. D. Day, Jr. 1978. Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *Febs. (Fed. Eur. Biochem. Soc.) Lett.* **86:** 139–142.
5. Cohen, G. 1977. In defense of Haber-Weiss. *In* Superoxide and Superoxide Dismutases. J. M. McCord and I. Fridovich, editors. Academic Press, Inc. New York. 317–321.
6. Tauber, A. I., and B. M. Babior. 1977. Evidence for hydroxyl radical production by human neutrophils. *J. Clin. Invest.* **60:** 374–381.
7. Weiss, S. J., G. W. King, and A. F. LoBuglio. 1977. Evidence for hydroxyl radical generation by human monocytes. *J. Clin. Invest.* **60:** 370–373.
8. Weiss, S. J., P. K. Rustagi, and A. F. LoBuglio. 1978. Human granulocyte generation of hydroxyl radical. *J. Exp. Med.* **147:** 316–323.
9. Hoidal, J. R., G. D. Beall, and J. E. Repine. 1979. Production of hydroxyl radical by human alveolar macrophages. *Infect. Immun.* In press.
10. Klebanoff, S. J., and H. Rosen. 1978. Ethylene formation by polymorphonuclear leukocytes. Role of myeloperoxidase. *J. Exp. Med.* **148:** 490–506.
11. Pryor, W. A., and R. H. Tang. 1978. Ethylene formation from methional. *Biochem. Biophys. Res. Commun.* **81:** 498–503.
12. Yang, S. F. 1969. Further studies on ethylene formation from α-keto-methylthiobutyric acid or β-methylthiopropionaldehyde by peroxidase in the presence of sulfite and oxygen. *J. Biol. Chem.* **244:** 4360–4365.

13. Yang, S. F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. *Arch. Biochem. Biophys.* **122:** 481–486.
14. Bors, W., E. Lengfelder, M. Saran, C. Fuchs, and C. Michel. 1976. Reactions of oxygen radical species with methional: a pulse radiolysis study. *Biochem. Biophys. Res. Commun.* **70:** 81–87.
15. Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes. 1974. The influence of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. *J. Clin. Invest.* **54:** 83–90.
16. Reuvers, A. P., C. L. Greenstock, J. Borsa, and J. D. Chapman. 1973. Studies on the mechanisms of chemical radioprotection by dimethyl sulfoxide. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **24:** 533–536.
17. Panganamala, R. V., H. M. Sharma, R. E. Heikkila, J. C. Geer, and D. G. Cornwell. 1976. Role of hydroxyl radical scavengers dimethyl sulfoxide, alcohols, and methional in the inhibition of prostaglandin synthesis. *Prostaglandins.* **11:** 599–607.
18. Cederbaum, A. I., E. Dicker, E. Rubin, and G. Cohen. 1977. The effect of dimethylsulfoxide and other hydroxyl radical scavengers on the oxidation of ethanol by rat liver microsomes. *Biochem. Biophys. Res. Commun.* **78:** 1254–1262.
19. Heikkila, R. E., B. Winston, G. Cohen, and H. Barden. 1976. Alloxan-induced diabetes—evidence for hydroxyl radical as a cytotoxic intermediate. *Biochem. Pharmacol.* **25:** 1085–1093.
20. Miller, J. S., and D. G. Cornwell. 1978. The role of cryoprotective agents as hydroxyl radical scavengers. *Cryobiology.* **15:** 585–588.
21. Brownlee, N. R., J. Huttner, R. V. Panganamala, and D. G. Cornwell. 1977. Role of vitamin E in glutathione-induced oxidant stress: methemoglobin, lipid peroxidation, and hemolysis. *J. Lipid Res.* **18:** 635–644.
22. Damerau, W., G. Labmann, and K. Lohs. 1969. Zur Einwirk ung von hydroxylradikalen auf dimethylsulfoxid. *Z. Chem.* **9:** 343–344.
23. Ashwood-Smith, M. J. 1975. Current concepts concerning radioprotective and cryoprotective properties of dimethylsulfoxide in cellular systems. *Ann. N. Y. Acad. Sci.* **243:** 246–256.
24. Chapman, J. D., A. P. Reuvers, J. Borsa, and C. L. Greenstock. 1973. Chemical radioprotection and radiosensitization of mammalian cells growing in vitro. *Radiat. Res.* **56:** 291–306.
25. Cohen, G., and A. Cederbaum. 1979. Chemical evidence for production of hydroxyl radicals during microsomal electron transfer. *Science (Wash, D. C.)* **204:** 66–68.
26. Repine, J. E., and C. C. Clawson. 1978. Influence of surface proteins and separation techniques on neutrophil unstimulated and stimulated locomotion in vitro. *J. Reticuloendothel. Soc.* **24:** 217–224.
27. Repine, J. E., C. C. Clawson, and P. S. Friend. 1977. Influence of a deficiency of the second component of complement of the bactericidal activity of neutrophils in vitro. *J. Clin. Invest.* **52:** 802–809.
28. Beauchamp, C., and I. Fridovich. 1970. A mechanism for the production of ethylene from methional. The generation of the hydroxyl radical by xanthine oxidase. *J. Biol. Chem.* **245:** 4641–4646.
29. Cornu, A., and R. Massot. 1966. Compilation of Mass Spectral Data. Heyden & Son Ltd, London. 1B.
30. McFadden, W. H. 1973. Techniques of Combined Gas Chromatography/Mass Spectrometry: Applications in Organic Analysis. John Wiley & Sons, Inc., New York, 48.
31. Curnutte, J. T., D. M. Whitten, and B. M. Babior. 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *N. Engl. J. Med.* **290:** 593–597.
32. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* **52:** 741–754.
33. Cheson, B. D., R. L. Christensen, R. Speiling, B. E. Kohler, and B. M. Babior. 1976. The origin of the chemiluminescence of phagocytosing granulocytes. *J. Clin. Invest.* **58:** 789–796.
34. Anderson, B. R., and A. M. Brendzel. 1978. Use of a unique chemiluminescence spectrometer in a study of factors influencing granulocyte light emission. *J. Immunol. Methods.* **19:** 279–287.
35. Sagone, A. L., D. S. Mendelson, and E. N. Metz. 1977. The effect of sodium azide on the chemiluminescence of granulocytes—evidence for the generation of multiple oxygen radicals. *J. Lab. Clin. Med.* **89:** 1333–1340.
36. Dorfman, L. M., and G. E. Adams. 1973. Reactivity of the hydroxyl radical in aqueous solutions. U. S. Department of Commerce, National Bureau of Standards. NSRDS-NBS no 46. 1–59.
37. Neta, P., and L. M. Dorfman. 1968. Pulse radiolysis studies. XIII. Rate constants for the reaction of hydroxyl radical with aromatic compounds in aqueous solutions. *Adv. Chem. Sci.* **81:** 222–230.
38. Cederbaum, A. I., E. Dicker, and G. Cohen. 1978. Effect of hydroxyl radical scavengers on microsomal oxidation of alcohols and on associated microsomal reactions. *Biochemistry.* **17:** 3058–3064.
39. Stossel, T. P. 1973. Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. *Blood.* **42:** 121–130.
40. Verhoef, J., P. K. Peterson, and P. G. Quie. 1977. Kinetics of staphylococcal opsonization, attachment, ingestion, and killing by human polymorphonuclear leukocytes: a quantitative assay using ^{3}H-thymidine labeled bacteria. *J. Immunol. Methods.* **14:** 303–311.
41. McCord, J. M., and I. Fridovich. 1968. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* **243:** 5753–60.
42. Walling, C. 1975. Fenton's reagent revisited. *Accounts Chem. Res.* **8:** 125–131.
43. Cohen, G. 1978. The generation of hydroxyl radicals in biologic systems: toxicological aspects. *Photochem. Photobiol.* **28:** 669–675.
44. Fridovich, I. 1978. The biology of oxygen radicals. *Science (Wash. D. C.).* **201:** 875–880.
45. Gilbert, B. C., R. O. C. Norman, and R. C. Sealy. 1975. Electron spin resonance studies. Part XLIII. Reaction of dimethyl sulphoxide with hydroxyl radical. *J. Chem. Soc. Perkin Trans. II.* 303–308.
46. Lagercrantz, C., and S. Forshult. 1969. Trapping of short-lived free radicals as nitroxide radicals detectable by ESR spectroscopy. The radicals formed in the reaction between OH radicals and some sulphoxides and sulphones. *Acta. Chem. Scand.* **23:** 811–817.
47. Carton, P. M., B. C. Gilbert, H. A. H. Lane, R. O. C. Norman, and R. C. Sealy. 1975. Electron spin resonance studies. Part XLVII. Sulphenyl- and sulphonyl-substituted aliphatic radicals. *J. Chem. Soc. Perkin Trans. II.* 1245–1249.
48. Rosen, H., and S. J. Klebanoff. 1979. Bactericidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leukocyte. *J. Exp. Med.* **149:** 27–39.

49. Gregory, E. M., and I. Fridovich. 1974. Oxygen metabolism in *Lactobacillus plantarum*. *J. Bacteriol.* **117**: 166–169.
50. Klebanoff, S. J. 1974. Role of the superoxide anion in the myeloperoxidase mediated antimicrobial system. *J. Biol. Chem.* **249**: 3724–3728.
51. Rosenbaum, E. E., and S. W. Jacob. 1964. Dimethyl sulfoxide (DMSO) in musculoskeletal injuries and inflammations. II. Dimethyl sulfoxide in rheumatoid arthritis, degenerative arthritis. *Northwest Med.* **63**: 227–229.
52. Wood, D. C., and J. Wood. 1975. Pharmacologic and biochemical considerations of dimethyl-sulfoxide. *Ann. N. Y. Acad. Sci.* **243**: 7–19.
53. Shirley, S. W., B. H. Stewart, and S. Mirelman. 1978. Dimethyl sulfoxide in treatment of inflammatory genitourinary disorders. *Urology.* **XI**: 215–220.
54. French, J. E., W. J. Flor, M. P. Grissom, J. L. Parker, G. Sajko, and W. G. Ewald. 1977. Recovery, structure, and function of dog granulocytes after freeze-preservation with dimethylsulfoxide. *Cryobiology.* **14**: 1–14.
55. Spector, J. I., J. A. Yarmala, L. D. Marchionni, C. P. Emerson, and C. R. Valeri. 1977. Viability and function of platelets frozen at 2 to 3C per minute with 4 or 5 per cent DMSO and stored at −80C for 8 months. *Transfusion (Phila.).* **17**: 8–15.
56. Farrant, J., and A. E. Woolgar. 1972. Human red cells under hypertonic conditions; a model system for investigating freezing damage. 3. dimethylsulfoxide. *Cryobiology.* **9**: 131–136.
57. Mironescu, S. 1978. Hyperosmotic injury in mammalian cells. 3. volume and alkali cation alteration of CHO cells in unprotected and DMSO-treated cultures. *Cryobiology.* **15**: 178–191.
58. Rubin, L. F. 1975. Toxicity of dimethyl sulfoxide alone and in combination. *Ann. N. Y. Acad. Sci.* **243**: 98–103.
59. Pribor, D. B. 1975. Biological interactions between cell membranes and glycerol or DMSO. *Cryobiology.* **12**: 309–320.
60. Leake, C. D. 1966. Dimethyl sulfoxide. *Science (Wash. D. C.).* **152**: 1646–1649.
61. Jacob, S. W., M. Bischel, and R. J. Herschler. 1964. Dimethyl sulfoxide (DMSO): a new concept in pharmacotherapy. *Curr. Ther. Res. Clin. Exp.* **6**: 134–148.