Plasma membrane receptors for the Fc portion of IgG and for the major fragment of the third component of complement (C3b) play an essential role in the function of macrophages as phagocytic cells. Fc receptors mediate both attachment and phagocytosis of particles coated with IgG antibody (1–3). Receptors for C3b on normal (resident) mouse peritoneal macrophages mediate only the attachment of particles to the cell (1, 3, 4), whereas C3b receptors on macrophages activated by infection or macrophages elicited by injection of inflammatory agents mediate both attachment and ingestion (3, 5–7).

During phagocytosis, or as a consequence of perturbation of the phagocyte plasma membrane by soluble stimuli such as phorbol myristate acetate (PMA), the cell undergoes a “respiratory burst,” in which it consumes oxygen and converts it to the potent microbicidal agents superoxide anion (O$_2^-$), hydrogen peroxide, hydroxyl radical, and perhaps, singlet oxygen. The role of phagocytosis through Fc or C3b receptors in stimulation of this oxidative burst has not been defined in macrophages. Studies with neutrophils in this regard suggest that the C3b receptor permits the attachment of C3b-coated particles to the cell; however, additional interaction with a second receptor (e.g., Fc receptor [8, 9] or receptor for a component of fungal cell wall [9], perhaps conjugated mannose [10]) appears to be required for both phagocytosis and stimulation of the respiratory burst. In these systems, ingestion has invariably been associated with stimulation of oxidative metabolism.

We report here that phagocytosis of IgG-coated sheep erythrocytes (E) by macrophages elicited a vigorous oxidative response. In contrast, phagocytosis by macrophages of E coated with IgM and complement or E treated with glutaraldehyde was not accompanied by stimulation of the respiratory burst. This dissociation could protect the host from oxidative tissue damage.
Materials and Methods

Macrophages. Mouse peritoneal macrophages were harvested, washed, and suspended in medium supplemented with 20% heat-inactivated fetal calf serum (Sterile Systems, Inc., Logan, UT) (11); approximately $1 \times 10^6$ cells in a total volume of 1 ml were plated on 16-mm diam tissue culture dishes or 13-mm diam glass coverslips (12). After incubation for 120 min at 37°C in 5% CO$_2$-95% air, plated cells were washed vigorously with medium twice, then cultured overnight (11). The cells adherent after overnight culture were washed with Krebs-Ringer phosphate buffer with 11 mM dextrose (KRPD) before assay. Peritoneal macrophages were elicited with lipopolysaccharide (LPS) or obtained from bacille Calmette-Guérin (BCG)-infected mice as previously described (11).

With each type of peritoneal exudate, cells adherent after overnight culture were $>97\%$ macrophages and $<1\%$ granulocytes, as estimated by differential counts of stained cells (11) and by phagocytic capacity for candida (12). The protein content of the dishes at the time of assay was 15–40 $\mu g$/dish (11).

Erythrocytes. Antibody-coated or antibody plus complement-coated E were prepared as described by Bianco et al. (3) and Griffin et al. (4), with slight modification. IgG and IgM made from rabbit anti-E antiserum were obtained from Cappel Laboratories, Cochranville, PA. The IgG (33 mg/ml) agglutinated E at dilutions of up to 1:10, 240. Trace amounts of IgG were removed from the IgM preparation by adsorption with Sepharose CL 4B-protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) (13). The adsorbed IgM preparation (350 $\mu g$/ml) agglutinated E at dilutions of up to 1:160. E in Alsever's solution (Colorado Serum Co., Denver, CO) were washed three times in phosphate-buffered saline (PBS), pH 7.3, and were suspended in KRPD. When E were opsonized by incubation with increasing amounts of IgG, release of O$_2$ (see below) by resident or LPS-elicited macrophages varied directly with the amount of Ig used, over a range of 6.6 $\mu g$/ml to 3.3 mg/ml ($n = 5$). A wide range of antibody concentrations was used to sensitize E, as described in Results; concentrations of IgM of 200 or 400 $\mu g$/ml agglutinated the E in the tube. Unless otherwise stated, in the experiments reported here, 10$^8$ E in 1 ml KRPD were incubated with 330 $\mu g$ of rabbit anti-E IgG or 8.8 $\mu g$ of rabbit anti-E IgM. After incubation for 15 min at 37°C, the suspensions were centrifuged and washed and resuspended with KRPD. These preparations are designated E(IgG) or E(IgM). Freshly thawed C5-deficient DBA/2J mouse serum, used as a source of complement, was diluted 1:10 with veronal-buffered saline containing 1 mM Mg$^{2+}$, 0.15 mM Ca$^{2+}$, and 0.1% gelatin (14) and mixed with an equal volume of suspension of E(IgM), 1 $\times 10^9$/ml, in this buffer. After incubation for 10 min at 37°C, cold KRPD was added to the reaction mixture and the suspension was centrifuged for 5 min at 750 g. The pelleted E were resuspended in KRPD. This preparation is designated E(IgM)C. With the IgM preparation used for most of the experiments reported here, optimal or near optimal ingestion was achieved with a final concentration of mouse serum of 5%.

Glutaraldehyde-treated E(GE) were prepared as described by Capo et al. (15). Three ml of 25% E (vol/vol) in PBS were incubated with 1% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) for 12 h on a rotating wheel at room temperature (15). The suspension was centrifuged and washed six times with PBS and resuspended in KRPD.

Phagocytosis. Ingestion of E(IgG) or E(IgM)C by macrophages was studied as described by Bianco et al. (3) and Griffin et al. (4). The assay was started by the addition of suspensions of $1 \times 10^7$ red blood cells (RBC) in 1 ml KRPD to macrophages cultivated overnight on coverslips; the ratio of RBC to macrophages was ~25:1. After incubation for 60 min at 37°C, coverslips were washed with cold KRPD twice, dipped into water for 5 sec to lyse uningested RBC, fixed with 1.25% glutaraldehyde, and examined with phase-contrast microscopy. Each variable was studied with duplicate slides, and at least 100 macrophages were observed with each slide. Results were expressed as the mean number of RBC ingested per macrophage.

Ingestion of GE was studied by a modification of the method of Loike and Silverstein (16). A suspension of $1 \times 10^7$ GE in 1 ml KRPD was added to cultured macrophages. After incubation for 60 min at 37°C, the coverslips were washed twice with cold KRPD and inverted over one drop of 0.8 mg/ml trypan blue on a microscope slide. The slides
were immediately observed with a fluorescence microscope with the excitation wavelength set at 490 nm and the emission spectrum set above 515 nm. The discrimination between extracellular GE (which cannot be easily lysed) was based on the observation that extracellular GE fluoresced with a relatively weak red color, whereas intracellular particles maintained a chartreuse color like that of fluorescein (16). The number of ingested RBC was determined for at least 100 macrophages using an oil immersion lens. All preparations were also examined by bright-field and phase-contrast microscopy, and viability was determined by exclusion of trypan blue.

Release of Superoxide Anion. The release of $O_2^-$ was quantitated as the superoxide dismutase-inhibitable reduction of ferricytochrome $c$ (11). The particle-to-macrophage ratio was ~25:1. Each reaction was run in triplicate. Controls contained the same reaction mixture without macrophages. The extent of $O_2^-$ release was expressed in relationship to the mean protein content of three culture plates incubated without E, using the method of Lowry et al. (17) with albumin as standard (11). Macrophage viability was >90% after incubation with E(IgM)C or GE for 90 min at 37°C and >80% after incubation with E(IgG).

Oxygen Consumption. Oxygen consumption during phagocytosis by cells in suspension was measured at 37°C by a polarographic technique using a Clark membrane electrode (12). LPS-elicited peritoneal cells were washed with PBS twice after contaminating E were eliminated by hypotonic lysis (12). Granulocyte-rich preparations were obtained by harvesting ~36 h after intraperitoneal injection of LPS; macrophage-rich preparations were harvested 4 d after injection of LPS (12). The 3 ml reaction mixture contained $1 \times 10^7$ peritoneal cells, $2 \times 10^8$ RBC, and 2 mM KCN in KRPD. The polarographic tracings were recorded for 10 min after the addition of RBC, and initial rates were measured. Differential counts of exudate cells were performed using Wright's and Giemsa stains.

Results

A comparison of the extent of phagocytosis of E(IgG), E(IgM)C, or GE, and the amount of $O_2^-$ release stimulated by ingestion of these particles, is summarized in Table I. Resident, LPS-elicited, and BCG-activated peritoneal macrophages all ingested large numbers of E(IgG) and released $O_2^-$ actively during phagocytosis.

Release of $O_2^-$ during phagocytosis of E(IgG) was greater in LPS-elicited and BCG-activated macrophages than in resident macrophages (Table I), as shown previously with PMA, candida, or opsonized zymosan as stimulus (11, 12). This relationship was explored further through the comparison of resident and LPS-elicited macrophages. The latter released a greater amount of $O_2^-$ than did resident macrophages at each of eight concentrations of IgG used to make E(IgG) (range, 33 µg/ml to 3.3 mg/ml; $n = 2$), at each of the incubation times of 30, 60, 90, and 120 min ($n = 2$), and with ratios of E(IgG)/macrophages of 10:1–100:1 ($n = 3$).

There was only slight phagocytosis of E(IgM)C by resident macrophages, but LPS-elicited and BCG-activated macrophages ingested E(IgM)C effectively (Table I). In contrast, none of the macrophage types released $O_2^-$ during phagocytosis of E(IgM)C (Table I). The same finding was observed when the ratio of E(IgM)C to macrophages was increased to levels of 60:1–150:1 ($n = 3$ with each cell type).

It has been reported (15, 16, 18) that macrophages bind and ingest aldehyde-fixed E in the absence of opsonins, perhaps because cross-linking of red cell membrane components increases local rigidity and modifies hydrophobicity of the red cell surface (15). We studied phagocytosis and $O_2^-$ release stimulated by
TABLE I

Comparison of O$_2^-$ Release and Phagocytosis by Murine Peritoneal Macrophages*

| Type of macrophage | Stimulus | O$_2^-$ released (nmol/mg protein) | Phagocytosis (No. RBC/macrophage) |
|--------------------|----------|----------------------------------|----------------------------------|
| Resident           | E        | 3 ± 1 (10)                       | 0.01 ± 0 (8)                     |
|                    | E(IgG)   | 43 ± 5 (9)                       | 8.80 ± 0.87 (6)                  |
|                    | E(IgM)C  | 4 ± 2 (6)                        | 0.64 ± 0.17 (8)                  |
|                    | GE       | 3 ± 3 (4)                        | 1.48 ± 0.11 (5)                  |
| LPS-elicited       | None     | 4 ± 2 (5)                        | —                                |
|                    | E        | 3 ± 1 (15)                       | 0.13 ± 0.03 (5)                  |
|                    | E(IgG)   | 88 ± 7 (15)                      | 17.40 ± 0.81 (6)                 |
|                    | E(IgM)   | 2 ± 1 (6)                        | 0.10 ± 0.02 (7)                  |
|                    | E(IgM)C  | 3 ± 1 (12)                       | 2.33 ± 0.30 (7)                  |
|                    | GE       | 2 ± 1 (6)                        | 4.50 ± 0.50 (4)                  |
| BCG-activated      | E        | 7 ± 1 (6)                        | 0.03 ± 0.01 (5)                  |
|                    | E(IgG)   | 103 ± 9 (6)                      | 11.80 ± 0.81 (6)                 |
|                    | E(IgM)C  | 4 ± 1(6)                         | 1.42 ± 0.25 (5)                  |
|                    | GE       | 3 ± 2 (5)                        | 2.19 ± 0.28 (4)                  |

* The extent of ingestion and O$_2^-$ release with each of three macrophage types is compared using E(IgG), E(IgM)C, or GE as stimulus. Incubation was for 60 min for ingestion or 90 min for O$_2^-$ release. The RBC/macrophage ratio was ~25:1. Values represent mean ± SEM (n). When PMA, 500 ng/ml, was used as a stimulus, resident, LPS-elicited, and BCG-activated macrophages released 83 ± 11 (14), 261 ± 14 (18), and 421 ± 26 (6) nmol O$_2^-$/mg/90 min, respectively.

GE as a possible model for macrophage clearance of autologous cells. As summarized in Table I, we found that all macrophage types ingested GE relatively effectively, but that ingestion did not stimulate release of O$_2^-$.

The dissociation between C3 receptor-mediated O$_2^-$ release and ingestion was emphasized by experiments in which E(IgM)C were prepared with different dilutions of fresh, C5-deficient serum used as complement source (Fig. 1). E(IgM) not further opsonized by incubation with fresh serum stimulated no appreciable ingestion or release of O$_2^-$, as illustrated with LPS-elicited macrophages in Table I. When E(IgM)C were prepared using final concentrations of fresh serum of 2.5–25%, ingestion of the E(IgM)C increased in proportion to the concentration of serum used. However, release of O$_2^-$ in the presence of the E(IgM)C remained at the level obtained with unopsonized E. The amount of IgM used to make E(IgM)C had no effect on either ingestion or O$_2^-$ release over the range of 1–400 μg/ml, using 5 or 10% C5-deficient serum as complement source, presumably because optimal fixation of the relevant opsonin, cleaved C3, was achieved in this system with the lower concentrations of IgM (n = 2, data not shown).

Because ingestion of E(IgM)C was lower than that of E(IgG) when the same numbers of RBC were added (Table 1), a reduced number of E(IgG) was incubated with LPS-elicited macrophages so that ingestion of E(IgG) was equivalent to or less than ingestion of E(IgM)C. As shown in Fig. 2, release of O$_2^-$ stimulated by ingestion of decreased numbers of E(IgG) was still at least fivefold
FIGURE 1. Effect of the concentration of the serum used as complement source on the extent of ingestion of E(IgM)C and on E(IgM)C-stimulated O$_2^-$ release by LPS-elicited macrophages. Macrophages were incubated with E(IgM)C, $1 \times 10^7$, prepared with different concentrations of serum. The points indicate the means and the bars the SEM of three paired experiments.

FIGURE 2. Comparison of O$_2^-$ release and ingestion using E(IgM)C and reduced numbers of E(IgG) as stimuli. The number of RBC added to cultured LPS-elicited macrophages is given in parentheses. The columns indicate the means and the bars the SEM of five experiments for O$_2^-$ release or three for ingestion.
DISSOCIATION OF PHAGOCYTOSIS FROM THE OXIDATIVE BURST

FIGURE 3. Effect of E(IgM)C on macrophage release of $O_2^-$ stimulated by E(IgG) or PMA. (A) E(IgG) or a mixture of E(IgG) and E(IgM)C were added as indicated. (B) PMA, 500 ng/ml, was used as stimulus in the presence or absence of E(IgM)C. The columns indicate the means and the bars the SEM; the number of experiments was three with $1 \times 10^7$ RBC, six with $5 \times 10^6$ RBC, and five with PMA.

greater than that stimulated by phagocytosis of E(IgM)C, which was equivalent to the baseline value obtained in the presence of unopsonized E. The relationship between the ingestion of E(IgG) and release of $O_2^-$ was also tested in five experiments with LPS-elicited macrophages by opsonizing E with lower concentrations of IgG (2.2, 3.3, 6.6, 13, 26, and 33 µg/ml). At no concentration tested could ingestion be detected without the concomitant release of $O_2^-$. For example, with E(IgG) made with 6.6 µg/ml IgG, 1.1 RBC were ingested per macrophage and 29 nmol $O_2^-$ were released per mg cell protein (means of two experiments).

We explored the possibility that the difference in $O_2^-$ release by macrophages exposed to E(IgG) or E(IgM)C might be caused by greater removal of $O_2^-$ by the latter. A mixture of E(IgM)C and E(IgG), or of E(IgM)C and PMA, was added to LPS-elicited macrophages as a stimulus. When exposed to a mixture of E(IgG) and E(IgM)C, either $1 \times 10^7$ or $5 \times 10^6$ each, macrophages released an equivalent amount of $O_2^-$ to that stimulated by the same number of E(IgG) alone (Fig. 3A). When LPS-elicited macrophages were exposed to PMA and E(IgM)C at the same time, even more $O_2^-$ release was observed than with PMA alone (Fig. 3B). Additional evidence that E(IgM)C or GE did not scavenge $O_2^-$ or inhibit function of the enzyme responsible for the respiratory burst in macrophages was obtained by studying $O_2^-$ release by LPS-elicited macrophages exposed to these E further coated with IgG. In four paired experiments with LPS-elicited macrophages, $O_2^-$ release stimulated by E(IgM)C-IgG (99.2 ± 16.4 nmol/mg) was equivalent to that obtained with E(IgG) (107 ± 12.7 nmol/mg). In contrast to the lack of stimulation of $O_2^-$ release seen with GE (Table I), GE coated with IgG stimulated the release of $O_2^-$ effectively (69.1 ± 8.9 nmol/mg, $n = 5$).
TABLE II
Comparison of the Capacity of E(IgG), E(IgM)C, and GE to Stimulate Macrophage Oxygen Uptake*

| Stimulus | Oxygen consumed | Macrophage preparations | Granulocyte preparations |
|----------|-----------------|--------------------------|--------------------------|
|          | nmol/10^7 cells/min |                        |                          |
| E        | 0.7 ± 0.2 (4) | 0.6 ± 0.1 (3)           |                          |
| E(IgG)   | 6.8 ± 0.3 (5) | 10.5 ± 1.1 (3)          |                          |
| E(IgM)C  | 1.0 ± 0.2 (6) | 4.5 ± 0.5 (3)           |                          |
| GE       | 1.2 ± 0.4 (5) | 1.2 ± 0.1 (4)           |                          |

* LPS-elicited peritoneal cells, 1 x 10^7, were tested in suspension, as required for this assay. In the macrophage preparations, 81% of the phagocytes were macrophages and 19% were granulocytes (means, n = 5; range, 74–86%). In the granulocyte preparations, a mean of 70% of the phagocytes were granulocytes (range, 61–82%, n = 3). A mean of 42% of the cells in macrophage preparations and a mean of 31% in neutrophil preparations were lymphocytes. KCN, 2 mM, and 1 x 10^6 RBC were added to begin the reaction. Oxygen consumption by E or GE was negligible in the presence of 2 mM KCN. When stimulated with PMA, 0.5 μg/ml, macrophage preparations consumed 17.7 ± 0.5 nmol oxygen/10^7 cells/min (n = 4).

Discussion

Previous studies (8, 9, 21, 22) with neutrophils have indicated that ingestion of particles through Fc or C3b receptors (or both) is invariably associated with the stimulation of oxidative metabolism. Moreover, the extent of the oxidative response has varied directly with the extent of ingestion (8, 21, 22). Phagocytosis of unopsonized candida and certain protozoa, bacteria, and inert particles by neutrophils or mononuclear phagocytes has also been shown to stimulate oxidative metabolism vigorously (12, 23–29), although the involvement of a receptor in the process has not been clearly defined.
Studies in other systems, however, suggest that under certain circumstances phagocytosis is not associated with an equivalent stimulation of the respiratory burst. For example, a dissociation between phagocytosis of complement-coated particles and the oxidative response has been shown to exist in cells of a continuous tissue culture line derived from a patient with acute myeloblastic leukemia (30). A similar dissociation was created pharmacologically by incubating normal neutrophils with a relatively small concentration of cytochalasin B (1 μg/ml), which inhibited the ingestion of serum-treated yeasts by only 5% but decreased the concurrent release of $O_2^-$ by 75% (31). A relative dissociation has been shown between the ingestion of two species of candida and their capacity to stimulate the respiratory burst in peritoneal macrophages (12). Although the ingestion of unopsonized *C. albicans* and *C. parapsilosis* was equivalent, *C. albicans*, which was killed relatively poorly by macrophages, stimulated the respiratory burst much more weakly than did *C. parapsilosis*. Preincubation of gelatin-coated latex or zymosan in plasma fibronectin and heparin improved phagocytosis of these particles by elicited rat neutrophils, as estimated by light and electron microscopy, but enhanced only slightly the hexose monophosphate shunt and chemiluminescence responses to these particles, suggesting that heparin/fibronectin-mediated ingestion could be dissociated from the respiratory burst (32).

Although treatment of phagocytes with partially purified neuraminidase was reported to inhibit the release of $O_2^-$ (33) or $H_2O_2$ (34) without decreasing phagocytosis, this effect was not achieved with purified neuraminidase (35) or when the enzyme was removed before assay of oxidative metabolism (25).

The results reported here indicate that stimulation of macrophages through Fc receptor-mediated phagocytosis of E(IgG) triggers the expected oxidative response, manifested as vigorous consumption of oxygen and elaboration of $O_2^-$. In contrast, phagocytosis of approximately equivalent numbers of E(IgM)C or E treated with glutaraldehyde (GE) failed to stimulate the respiratory burst. An inability of C3b or C3bi attached to surfaces to stimulate release of hydrogen peroxide has been found recently with human neutrophils, monocytes, and monococyte-derived macrophages (36).

The biochemical events that link receptor-ligand binding at the membrane of a phagocytic cell and the subsequent processes of endocytosis, degranulation, and oxidative metabolism are not known. The complete dissociation between phagocytosis and the oxidative burst shown here with C3b-coated or glutaraldehyde-treated E suggests that the pathway linking membrane binding to phagocytosis is different from that linking binding to stimulation of the enzyme responsible for the respiratory burst. Binding through the Fc receptor would appear to stimulate both pathways in both neutrophils and macrophages (although phagocytosis is not required to induce the release of oxygen metabolites by phagocytes bound to IgG-coated surfaces (36–38)). In contrast, attachment to macrophages through the receptor for C3b or through nonspecific chemical bonds appears to stimulate the cell's contractile machinery without triggering the respiratory burst. Electron microscopic analysis of the mechanism of internalization of E(IgG) and E(IgM)C by rat Kupffer cells and mouse peritoneal macrophages suggests clearcut differences that may relate to our findings, including a paucity of cytoplasmic extensions and much less contact between the
macrophage plasma membrane and the erythrocyte during ingestion of E(IgM)C (39, 40). Study of the biochemical mechanisms by which macrophages respond to the ingestion of E opsonized with IgG or C3b could provide a basis for further elucidation of the molecular events that mediate the microbicidal activity of phagocytic cells.

Macrophages elicited by LPS or activated by BCG infection released over twice as much O$_2^-$ as did resident cells on exposure to E(IgG) (Table I). Thus, stimulation of oxidative metabolism through the Fc receptor, like stimulation by PMA, zymosan, bacteria, candida, or toxoplasma (12, 28, reviewed in 41), is greater with elicited or activated macrophages than with resident macrophages.

The generation of highly reactive oxygen intermediates during phagocytosis is believed to be essential for phagocytic microbicidal activity. However, toxic oxygen metabolites are released to the outside during this process (11, 12, 19, 20, 29). Therefore, the potential exists for oxidative damage to surrounding tissues (37, 38), and such has been demonstrated to occur (reviewed in 42). Moreover, the oxidative metabolic response has been shown to markedly reduce the chemotactic, phagocytic, and metabolic capacities of the phagocytes themselves (43, 44). The data reported here illustrate clearly that phagocytosis by macrophages is not always accompanied by a respiratory burst. Whether removal of some or all effete cells by macrophages in vivo requires opsonization by autoantibody is not clear (45–47). Thus, it is possible that phagocytic removal of particles not "foreign" enough to elicit an IgG antibody response, including damaged or aged cells, might occur in tissues or in the mononuclear phagocyte system without inflicting unnecessary oxidative damage to surrounding cells or to the phagocyte itself.

Summary

We explored the relationship between phagocytosis and the triggering of oxidative metabolism using resident, lipopolysaccharide (LPS)-elicited, and bacille Calmette-Guérin (BCG)-activated murine peritoneal macrophages. Sheep erythrocytes (E) coated with IgG [E(IgG)], E coated with IgM and complement [E(IgM)C], and E treated with 1% glutaraldehyde (GE) were used as stimuli. All three types of macrophages released superoxide anion (O$_2^-$) during phagocytosis of E(IgG). All macrophage types phagocytosed E(IgM)C and GE but none were stimulated to release O$_2^-$ during phagocytosis of these particles. Vigorous consumption of oxygen was also stimulated by the ingestion of E(IgG) but not by ingestion of E(IgM)C or GE. E(IgM)C did not scavenge the O$_2^-$ released from macrophages during phagocytosis of E(IgG) or during exposure to phorbol myristate acetate, and further addition of IgG anti-E antibody to E(IgM)C or GE permitted optimal stimulation of macrophage O$_2^-$ release by these particles. The capacity of macrophages to ingest E(IgM)C and GE without stimulating the respiratory burst raises the possibility that clearance of particulate matter not opsonized with specific IgG might be achieved without stimulation of the release of toxic oxygen metabolites, and, therefore, without the risk of oxidative damage to the phagocytic cell or surrounding tissue.
We thank Lindsay A. Guthrie for expert technical assistance.

Received for publication 14 February 1983 and in revised form 19 October 1983.

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