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Interaction of Chemotactic Factors with Human Polymorphonuclear Leukocytes: Studies Using a Membrane Potential-Sensitive Cyanine Dye*

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Summary. Changes in the fluorescence intensity of the dye 3-3′dipentyloxacarbocyanine were measured in suspensions of purified human peripheral blood polymorphonuclear leukocytes (PMNs) during exposure to the chemotactic factors N-formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe) and partially purified C5a. Incubation of PMNs with dye resulted in a stable fluorescence reflecting the resting membrane potential of the cell. Exposure of PMNs to dye did not affect stimulated chemotaxis or secretion. The mechanism of cell-associated dye fluorescence involved solvent effects from partitioning of the dye between the aqueous incubation medium and the cell and not dye aggregation, Chemotactically active concentrations of f-met-leu-phe (5×10^{-9} M or greater) produced a biphasic response characterized as a decrease followed by an increase in fluorescence. No fluorescence response was seen in lysed PMNs, and no response was elicited by an inhibitor of f-met-leu-phe binding (carbobenzoxy-phenylalanyl-methionine). The ability of several other synthetic peptides to elicit a fluorescence response corresponded to their effectiveness as chemotactic agents. Although the first component of the response suggested a depolarization, it was not influenced by variation in the external concentration of sodium, potassium, chloride, or calcium, and could not be characterized as a membrane potential change. The second component of the response, which was inhibited by both Mg^{2+} (10 mM)-EGTA (10 mM) and high external potassium, was compatible with a membrane hyperpolarization. The data indicate that chemotactic factors produce changes in dye fluorescence which can, at least in part, be attributed to a hyperpolarizing membrane potential change occurring across the plasma membrane.

Polymorphonuclear leukocytes (PMNs) exposed to a gradient of chemoattractants exhibit directed migration. PMNs also release the content of their intracellular granules in response to these stimuli if the cells are simultaneously exposed to cytochalasin B [26, 27] or if they are adherent to a substratum [6]. Little is known about the mechanisms involved in these processes although recent reports have indicated that chemoattractants induce changes in calcium, sodium, and potassium ion fluxes and stimulate plasma membrane $Na^+ - K^+$ ATPase activity [9, 25, 41, 50]. In addition, extracellular calcium, sodium, and potassium are required for optimal PMN chemotaxis through micropore filter [5, 25, 48, 50]. These observations suggested that changes in membrane potential may be associated with exposure to chemoattractants. Recordings of membrane potential changes in macrophages using intracellular microelectrodes have indicated that chemotactically active molecules induce prolonged membrane hyperpolarizations [19, 20, 24] probably involving an increase in potassium permeability. Similar studies have not been possible in PMNs because of their small size. Therefore, we have chosen to monitor membrane potential indirectly using fluorescent probes that are sensitive to changes in membrane potential [29, 51, 55]. The cyanine dye 3-3′-dipentyloxacarbocyanine [di−O−C_5(3)] was selected as the fluorescent probe since this dye had previously been used to monitor membrane potential in a number of cell systems [39].

We have shown in previous studies employing di−O−C_5(3) [20] that chemotactic factors induce a potas-

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sium-dependent change in fluorescence in suspensions of monocytes similar to the electrophysiologically recorded hyperpolarizations obtained in cultured macrophages. In addition since our preliminary report indicating that chemotactic factors produced membrane potential changes in PMNs [47], Korchak and Weissman [33] have presented evidence using triphenylmethyl phosphonium ion, a radiolabeled indirect probe of membrane potential, suggesting that membrane potential changes also occur in PMNs following stimulation by the plant lectin concanavalin A and by immune complexes. The observations by Korchak and Weissman [33] substantiate the earlier work by Utsumi et al. [54]. Using the cyanine dye 3-3′ dipropylthiocarbocyanine iodide, these latter investigators presented evidence that concanavalin A produced changes in fluorescence compatible with a small hyperpolarization followed by a large depolarization. These changes in membrane potential were inhibited by temperature and several agents affecting membrane fluidity, leading Utsumi et al. to suggest that changes in membrane fluidity resulted in the membrane potential changes.

In the present report the effect of chemotactic factors on the fluorescence of di$-$O$-$$C_5$(3) in suspensions of PMNs is described along with the ion dependence of the resulting fluorescence changes. The results indicate that exposure of PMNs to chemotactic factors produces a complex change in fluorescence, a component of which is consistent with a membrane potential hyperpolarization occurring across the plasma membrane.

Materials and Methods

Buffers and Solutions

N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES, Sigma Chemical Co., St. Louis, Mo.) buffered Hanks' solution was used in all fluorescence experiments. Normal Hanks' contained 129 mM NaCl, 4.2 mM KCl, 0.9 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.2 mM $MgSo_4$, 0.1 mM $MgCl_2$, 11 mM glucose, and 10 mM HEPES (pH 7.3). In some experiments the concentration of potassium was varied from 1–122 mM at constant osmolarity and fixed concentrations of sodium (20 mM) and chloride (136.4 mM) by reciprocally adjusting the concentration of KCl and choline chloride. In other experiments the sodium concentration was varied from 1–122 mM in a similar manner, leaving potassium constant at its normal concentration of 4.6 mM. Chloride ion was varied by replacement of sodium chloride with sodium methylsulfonate. All inorganic salts were obtained from Fisher Scientific Co., Fair Lawn, N.J. The osmolarity (270 mmol) of all solutions was measured using a Fiske QF Osmometer (Uxbridge, Mass.).

The formylated peptides N-formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe), carbobenzoxy-phenlyalanyl-methionine (Z-phe-met), N-formyl-methionyl-methionyl-methionine (f-[met]$_4$), N-formyl-methionyl-leucyl-glutamate (f-met-leu-glu), and methionyl-leucyl-phenylalanine (des met-leu-phe) were generous gifts of Dr. Elliot Schiffmann (National Institute for Dental Research, NIH). Obtained as indicated were valinomycin, gramicidin S (Sigma Chemical Co.), cytochalasin B (Aldrich Chemical Co., Milwaukee, Wis.). These compounds were dissolved in dimethylsulfoxide (Fisher Scientific) or ethanol at the appropriate concentration, and 5-μl aliquots were added directly to the assay mixture, resulting in a 0.5% concentration of solvent. This amount of ethanol or dimethylsulfoxide had no effect on cell viability, function, or fluorescence responses. Neuraminidase was obtained from Boehringer Mannheim, West Germany.

Leukocyte Isolation

Peripheral blood PMNs from normal human subjects were prepared by Ficoll-Hypaque gradient centrifugation (Hypaque-M 90%, Winthrop Laboratories, N.Y.; Ficoll 10%, Pharmacia, Uppsula, Sweden) followed by dextran sedimentation (Dextran T 250, Pharmacia) [12]. This technique routinely resulted in cell populations containing over 95% PMNs. All experiments were completed within 6 hr of obtaining the cells unless otherwise indicated.

Glial Cells, Mast Cells, Lymphocytes, and Erythrocytes

Cells other than PMNs were used in some studies. Cultured glial cells (LRM 55) were cloned from a mixed glioma obtained from a rat spinal tumor induced by ethyl nitrosourea [37]. Rat mast cells were obtained from the peritoneal cavity by massage and saline washout [15]. Human nonadherent lymphocytes were collected 4 hr after plating the mononuclear cell band obtained from Ficoll-Hypaque gradients onto plastic petri dishes and incubating at 37 °C in RPMI. Red blood cells, also obtained from the Ficoll-Hypaque gradient, were separated from PMNs by dextran sedimentation and then washed twice in Hanks' media.

General Procedure for Fluorometric Assays

The cyanine dyes 3-3′-dipentyloxacarbocyanine, di$-$O$-$$C_5$(3), and 3-3′-dipropylthiocarbocyanine, di$-$S$-$$C_3$(5), were generous gifts from Dr. Alan Waggoner (Amherst College, Amherst, Mass.). Stock solutions of dye (10^{-3} M) were made in ethanol and kept in the dark at 4 °C. Prior to each set of experiments this solution was diluted 100-fold into distilled water to make a working solution; it was discarded after 4 hr of use. The addition of dye to the assay mixture lowered the osmolarity by 3% which, based on control studies, had no effect on dye fluorescence.

A standard assay volume of 1 ml HEPES-buffered Hanks' solution was used in a 1-cm pathlength cuvette. To this was added 50 μl of the working dye solution, giving an initial dye concentration of 5×10^{-7} M and less than 0.05% ethanol. The fluorescence was recorded using either an Aminco-Bowman SPF (Rockville, Md.) or a Farrand Mark I spectrofluorometer (Valhalla, N.Y.) with the excitation wavelength set at 460 nm and the fluorescence wavelength at 510 nm. The temperature of the cuvette was maintained at 35 °C, and the cell suspension was maintained by means of a magnetic flea and stirrer (300 rpm). As previously noted by others, di$-$O$-$$C_5$(3) adsorbed onto the cuvette and stirring flea [29], causing the fluorescence to decrease to a constant value within 4 min. Once the fluorescence had stabilized, 20 μl of a suspension of PMNs was added to give a final concentration of 5×10^5 cells/ml. The uptake of dye by the PMNs resulted in a change in fluorescence (Δf) as described in Results. Fluorescence changes are reported as a percentage of the total full-scale fluorescence. During these experiments the photomultiplier tube sensitivity was kept constant, permitting quantitative comparison

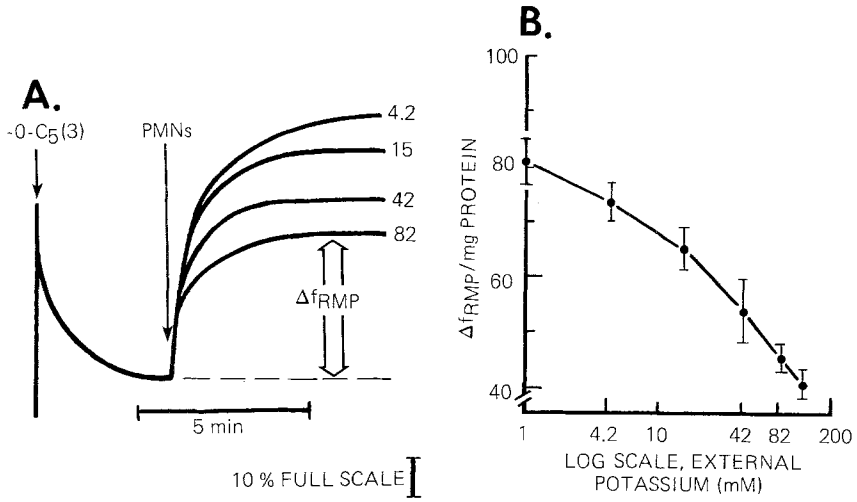

Fig. 1. Effect of varying the external concentration of potassium ion on Δf_{RMP}. (*A*): Recordings of fluorescence at various concentrations of potassium. Potassium concentrations (mM) are shown at the right of each tracing. Time scale, percent full-scale fluorescence and addition of $di-O-C_5(3)$ and PMNs are indicated. The steady-state basal fluorescence defined as Δf_{RMP} is indicated for 82 mM potassium. (*B*): The Δf_{RMP} values of fluorescence obtained at each of six different concentrations of external potassium are plotted as functions of the log potassium concentration. Each point represents the average of three experimental determinations corrected for protein and expressed as a percent of the full-scale fluorescence, ± SEM

of results. Further details of the assay method used and the mechanism by which $di-O-C_5(3)$ fluorescence is related to dye distribution in various compartments of the assay system and the nature of the interaction between the cyanine dye and PMNs are given in the Appendix.

Chemotactic Activity, Enzyme Determinations, Superoxide Generation, Surface Charge, Cell Aggregation, and Cell Viability

PMN chemotaxis was assessed with a previously described assay using ^{51}Cr-labeled PMNs and a double micropore chemotactic chamber [22]. Lysozyme activity, β-glucuronidase, and lactic dehydrogenase were assayed as described previously [10, 36, 53]. Superoxide ion was generated using xanthine (10^{-4} M), and xanthine oxidase (0.05 units/ml) (Sigma). The presence of superoxide was determined spectrophotometrically by monitoring the superoxide dismutase (3 units/ml, Miles Laboratories, Elkhart, Ind.) inhibitable reduction of cytochrome c (120 μg/ml, Sigma) [38]. Surface charge was measured using an electrophoretic mobility technique described previously [35]. Cell concentration, viability, and aggregation were determined microscopically at the end of each experiment using trypan blue and phase contrast microscopy. For some studies, cell aggregation was assessed by recording light scattering using an emission wavelength setting of 460 nm, the same as the excitation wavelength.

Results

Fluorescence Changes Following Addition of PMNs

Quenching of cyanine dye fluorescence by cells has been reported previously and attributed to the aggregation of dye molecules [29]. For our studies we elected to use concentrations of the dye less than that associated with quenching of fluorescence. A relatively low concentration of dye (5×10^{-7} M) was sufficient to detect responses and avoid problems encountered with high concentrations of the dye which include quenching, toxic effects of the dye, and interference with calcium-stimulated potassium fluxes [40, 51]. The accumulation of $di-O-C_5(3)$ by PMNs results in increased fluorescence. Addition of PMNs to $di-O-C_5(3)$ containing solution (5×10^{-7} M dye) resulted in a large increase in fluorescence which rapidly reached a stable value (Fig. 1 *A*). In this figure the change in full-scale fluorescence is shown beginning with the addition of $di-O-C_5(3)$ to a stirred cuvette. PMNs were added as indicated once dye had equilibrated with the cuvette. The fluorescence immediately increased to a stable value. As discussed below, tracings from four different experiments have been superimposed in this figure.

Effect of Potassium and Valinomycin on the Fluorescence of $di-O-C_5(3)$ with PMNs

To determine if the final stabilized level of fluorescence obtained after the addition of PMNs was a function of the resting membrane potential (RMP), the effect of independently varying potassium, sodium, and chloride was assessed. Increasing external potassium from 1–122 mM decreased the level at which the fluorescence stabilized following the addition of PMNs (Fig. 1 *A*). When the stabilized fluorescence (Δf_{RMP}) was plotted as a function of log external potassium, the greatest Δf_{RMP} was obtained with 1 mM and the lowest with 122 mM external potassium (Fig. 1 *B*). Extraction studies similar to those in Table 3 (Appendix) demonstrated that the difference in fluorescence could not be explained as a result of potassium ioninduced removal of dye from the cuvette or an effect of this ion on dye fluorescence before addition of cells.

The relationship between Δf_{RMP} and the log external potassium concentration is linear at high potas-

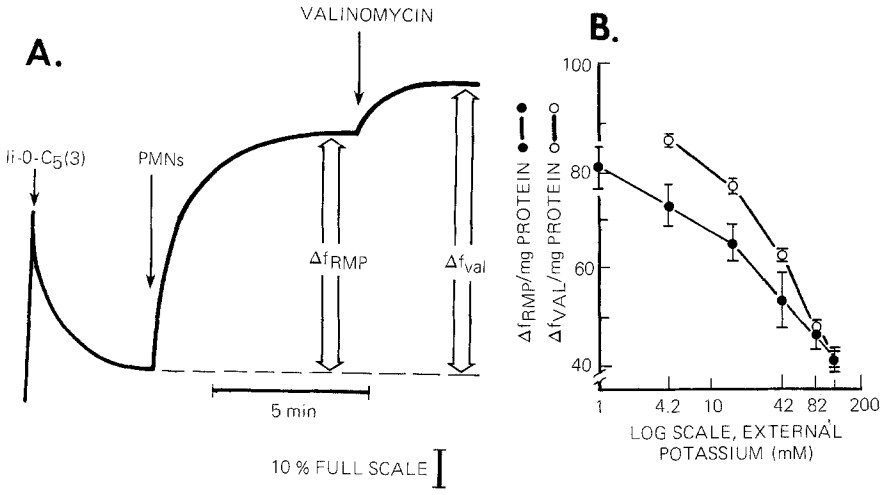

Fig. 2. Effect of varying the external concentration of potassium ion on the fluorescence response to valinomycin (Δf_{VAL}). (*A*): A typical recording is shown. Valinomycin (10^{-7} M) was added after the Δf_{RMP} had stabilized as indicated. The extracellular potassium concentration was 4.2 mM. Δf_{VAL} was measured as shown. (*B*): The effect of changing external potassium on Δf_{VAL} is shown (o——o) and compared to the effect of external potassium on Δf_{RMP} (●--●). All points represent the average of three observations and have been corrected for protein and expressed as percent full-scale fluorescence, ±SEM

Table 1. Effect of Di−O−C_5(3) on PMN chemotaxis and secretion

Chemoattractant	Chemotaxis[a]	
	No addition	di−O−C_5(3) (5×10^{-7} M)
Buffer	442± 45	395± 38
Casein (5 mg/ml)	2456± 70	2414± 99
Endotoxin-activated serum (5%)	2779±212	2455±263
f-met-leu-phe (1×10^{-8} M)	1311± 44	1784± 96

Secretagogue	Total lysozyme[b] released
None	2±0.5
di−O−C_5(3) (5×10^{-7} M)	2±0.3
Cytochalasin B (5 μg/ml)	5±1.0
f-met-leu-phe (10^{-6} M)	3±1.0
+cytochalasin B	46±4.0
+cytochalasin B+di−O−C_5(3)	41±5.0

[a] PMNs in lower filter, mean value of corrected cpm±SEM of four determinations. (*See* Materials and Methods.)

[b] Percent total lysozyme released, mean±SEM of six observations where total lysozyme was 29.3 μg egg white lysozyme equivalents per 10^7 PMNs.

sium concentration (Fig. 1*B*). This is similar to data obtained in a variety of cells using intracellular recording [30]. The deviation from linearity at low potassium concentrations is typical of cells in which potassium is the major, but not the exclusive, ion determining the membrane potential. When the potassium concentration was maintained at 4.6 mM, changing the sodium or chloride ion concentration in the external assay medium had no detectable effect on the stabilized fluorescence.

The potassium ionophore valinomycin (10^{-7} M) was used to study the effect of increasing potassium permeability on the membrane potential (Fig. 2*A*, 4.6 mM external potassium). Valinomycin increased fluorescence to a stable value (Δf_{VAL}). Increasing extracellular potassium decreased Δf_{VAL} (Fig. 2*B*). Valinomycin had no significant effect on fluorescence at 122 mM potassium ion. In control experiments, performed in the absence of cells, valinomycin had no effect on di−O−C_5(3) fluorescence.

Effect of di−O−C_5(3) on PMN Viability and Function

Incubation of PMNs for up to 2 hr in the concentration of di−O−C_5(3) used throughout our experiments had no effect on cell viability as monitored by trypan blue exclusion or release of the cytoplasmic enzyme LDH. In addition, as shown in Table 1, the dye had no effect on PMN random migration or chemotactic responsiveness to three different chemoattractants. In addition di−O−C_5(3) had no effect on the ability of PMNs to release lysozymal enzymes in response to the degranulating stimulus f-met-leu-phe plus cytochalasin B (Table 1).

Fluorescence Response Produced by Chemoattractants

Fluorescence Response to f-met-leu-phe. Addition of f-met-leu-phe (10^{-8} M) to PMNs resulted in a biphasic response consisting of a rapid decrease in fluorescence, measured quantitatively as Δf_1, followed by an increase in fluorescence to a maximum value (Δf_2) before decreasing to a stable value (Fig. 3*A*). The

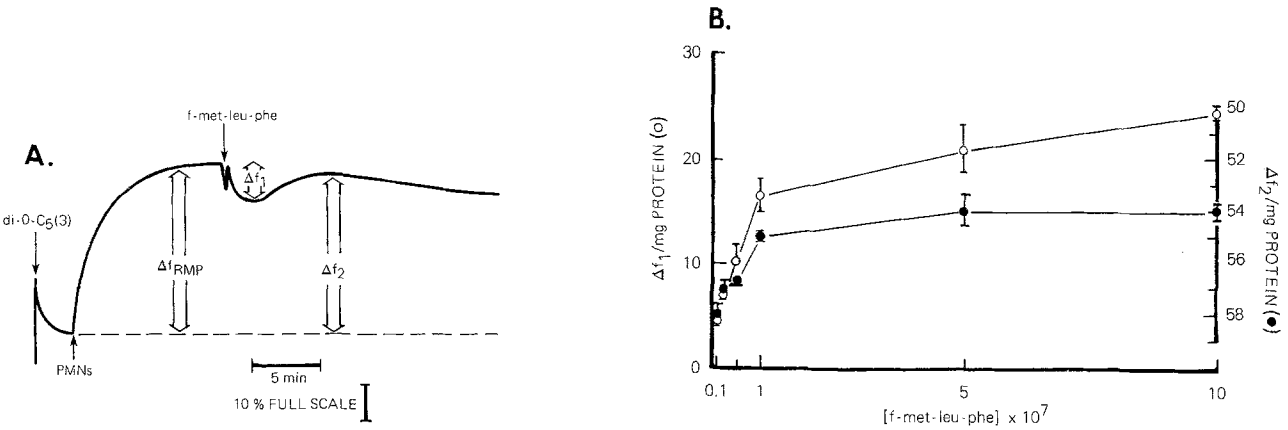

Fig. 3. (*A*): Time course of fluorescence response to stimulation by f-met-leu-phe. Fluorescence changes were measured as percent change of the full-scale fluorescence (*see* Materials and Methods). The addition of dye (5×10^{-7} M) to Hanks' solution was followed by addition of 20 µl of the PMN suspension (5×10^5 cells/ml, final concentration) followed by 10 µl of f-met-leu-phe (final concentration 5×10^{-8} M) as indicated. A biphasic response resulted with a decrease in fluorescence to a minimum (Δf_1) followed by an increase to a maximum (Δf_2). The spike seen immediately upon addition of f-met-leu-phe is an artifact produced upon the addition of stimulus due to the closing and opening of the shutter protecting the photomultiplier tube. (*B*): Cumulative data for Δf_1 (left axis, ○) and Δf_2 (right axis, ●) are plotted as a function of f-met-leu-phe concentration. Each point represents the average of three experiments, ± SEM

duration of the response was from 5–10 min depending on the particular cell donor and the concentration of peptide used. The rapid changes in fluorescence seen in Fig. 3 *A* upon addition of peptide is an artifact resulting from the momentary closing and opening of the shutter protecting the photomultiplier tube during addition of the stimulus. Increasing peptide concentrations produced larger and more prolonged responses until the response was saturated at approximately 5×10^{-7} M f-met-leu-phe (Fig. 3 *B*). Both Δf_1 and Δf_2 show similar saturation curves with respect to f-met-leu-phe concentrations, although the absolute changes in fluorescence of Δf_1 and Δf_2 are not necessarily similar (Fig. 3 *B*). The magnitude of Δf_1 changed with the age of the cells. Approximately 6 hr after drawing blood, this component began to increase (Fig. 4). For this reason all the results reported here were obtained within 6 hr of drawing the blood.

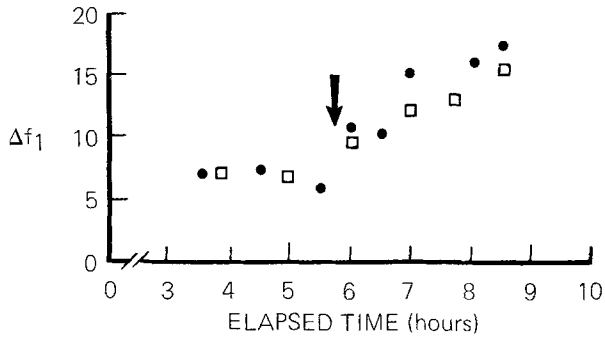

Fig. 4. Effect of PMN age on the Δf_1 component of the fluorescence response to f-met-leu-phe. The magnitude of Δf_1 is plotted as a function of the hours elapsed after blood was obtained from the donor. Measurements were made using f-met-leu-phe (5×10^{-8} M) as the stimulus. The two separate experiments shown (●, □) were performed on different days with cells obtained from different normal donors. The arrow indicates the approximate time when Δf_1 began to increase

Specificity of the f-met-leu-phe Fluorescence Response. The response to f-met-leu-phe required intact cells. It was not seen if PMNs were first sonicated or treated with a high concentration of gramicidin S (10^{-4} M) which lysed the cells (Fig. 5). Moreover, the response could not be related to cell aggregation. The specificity of the fluorescence response to f-met-leu-phe was characterized using an inhibitor of the f-met-leu-phe-induced chemotactic response, Z-phe-met [1, 44]. Z-phe-met inhibited the f-met-leu-phe-induced fluorescence response. When the data (Δf_1) obtained using Z-phe-met as the inhibitor were plotted as a double-reciprocal plot (Fig. 6), it was apparent that high concentrations of f-met-leu-phe overcame the inhibition by Z-phe-met in a manner suggesting the inhibition represents competition for f-met-leu-phe binding. At the concentrations used (and up to 10^{-4} M) Z-phe-met did not produce a fluorescence response. In other studies involving chemotactically unresponsive cells (mast cells, glial cells, nonadherent lymphocytes, and red blood cells) f-met-leu-phe did not produce a fluorescence response. In addition f-met-leu-phe did not affect fluorescence in the absence of cells (data not shown). The data therefore indicate that the f-met-leu-phe-induced response was not an artifact due to displacement of dye from the cuvette, nor due to nonspecific effects of peptide on cell-bound dye, but rather involved a specific effect of f-met-leu-phe on the cell. Moreover, the f-met-leu-phe response required viable, intact PMNs and was competitively inhibited by Z-

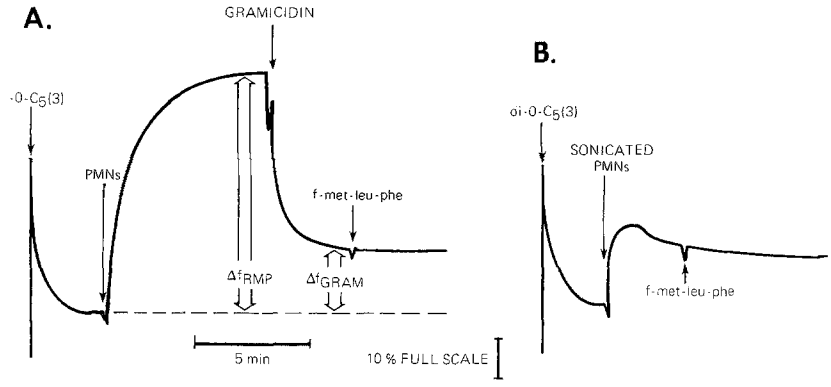

Fig. 5. Effect of cell lysis on Δf_{RMP} and the response to f-met-leu-phe. (*A*): Gramicidins (10^{-4} M) was added as indicated after PMN fluorescence had stabilized (Δf_{RMP}). Once fluorescence had stabilized again (Δf_{GRAM}) f-met-leu-phe (10^{-6} M) was added. When examined by phase contrast microscopy after gramicidin treatment, the cells appeared intact but did not exclude trypan blue. (*B*): The same number of cells used in *A* were sonicated before addition to the assay mixture. f-met-leu-phe (10^{-6} M) was then added. This preparation consisted largely of membrane fragments and vesicles permeable to trypan blue. Note decreased Δf_{RMP} after sonication

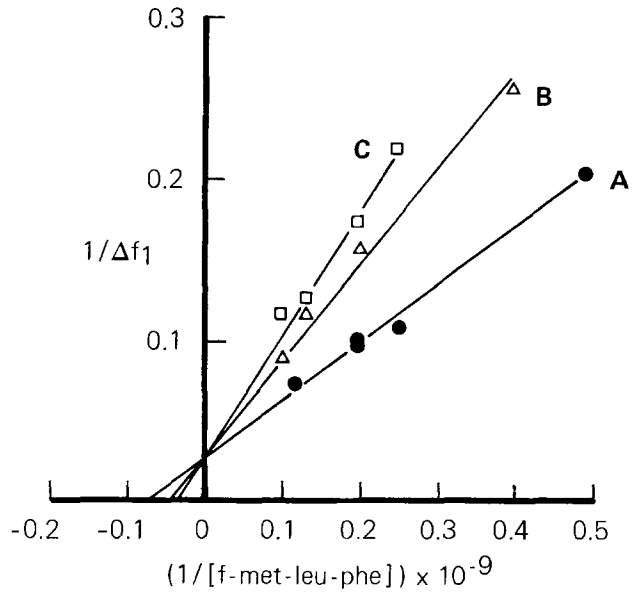

Fig. 6. Inhibition of the f-met-leu-phe response by Z-phe-met. Doublé reciprocal plot of Δf_1 as a function of the concentration of f-met-leu-phe in the presence of three concentrations of inhibitor Z-phe-met; 5×10^{-6} M (*A*), 2×10^{-6} M (*B*), and no Z-phe-met (*C*). Z-phe-met was added simultaneously with f-met-leu-phe

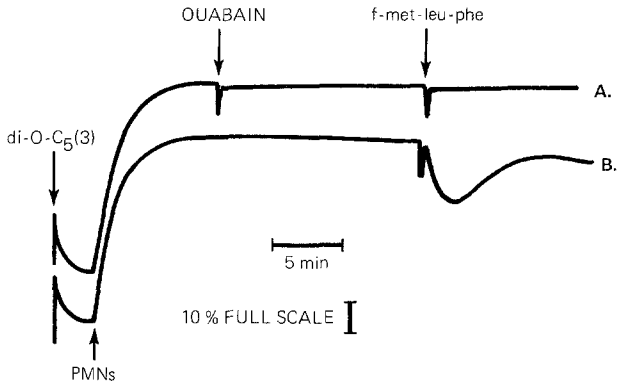

Fig. 7. Effect of Ouabain on the fluorescence response to f-met-leu-phe. PMNs (5×10^5 cells/ml) equilibrated with di$-$O$-$C$_5$(3) (5×10^{-7} M) were either exposed to Ouabain (10^{-4} M) as in tracing *A* or to buffer as in tracing *B* and allowed to incubate another 15 min. The chemoattractant f-met-leu-phe (10^{-7} M) was then added as indicated and the fluorescence recorded for an additional 10 min

phe-met, an inhibitor of f-met-leu-phe binding and chemotactic factor-induced chemotaxis [44].

Several other synthetic peptides with relatively different efficacy as chemoattractants [49] were studied. The response produced by each of these peptides was identical to that produced by f-met-leu-phe, and the concentration required to produce a half maximal fluorescence response (apparent K_m) corresponded to each peptide's effectiveness as a chemoattractant. The apparent K_m's were 3×10^{-8} M for both f-met-leu-phe and f-(met)$_4$, 10^{-5} M for des-met-leu-phe, and 5×10^{-5} M for f-met-leu-glu.

The response to f-met-leu-phe was inhibited by ouabain (10^{-4} M) in a time-dependent fashion. Figure 7 shows the effect a 15-min incubation with ouabain had on the fluorescence response produced by 10^{-7} M f-met-leu-phe (upper tracing *A*) compared to the response normally produced by this concentration of peptide (lower tracing *B*). Shorter incubation times with ouabain did not inhibit the response. Note that during the incubation with ouabain there was no change in Δf_{RMP}, inferring that the resting membrane potential of the PMNs was not altered during this time.

Ion Requirements for the f-met-leu-phe Response. The ionic dependence of each component of the f-met-leu-phe response was studied. Increasing extracellular potassium had no effect on Δf_1 but decreased Δf_2 (Fig. 8). Varying extracellular sodium or chloride had no effect on either Δf_1 or Δf_2. If both Δf_1 and Δf_2 were caused by changes in membrane potential across the plasma membrane, it would have been expected that they would both have been affected, albeit perhaps differently, by changes in the extracellular concentration of potassium. Therefore, the data strongly suggest that only part of the response, Δf_2, involves Δf_2, involves a potential change across the

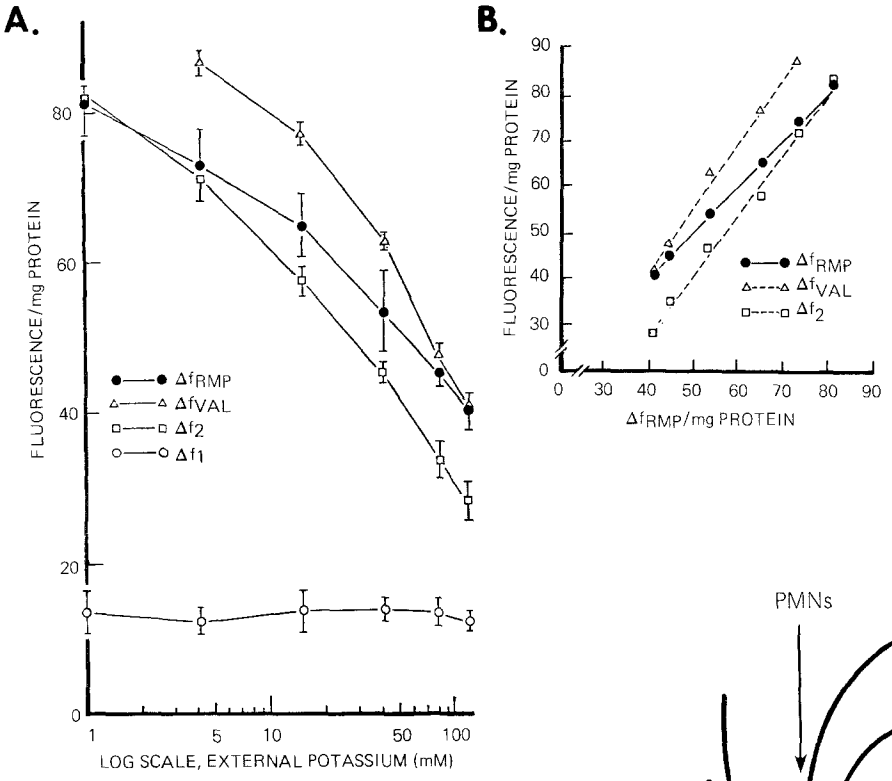

Fig. 8. Effect of varying the external concentration of potassium ion on Δf_{RMP}, Δf_{VAL}, Δf_1, and Δf_2. (*A*): Plots of Δf_{RMP} (●), Δf_{VAL} (△), Δf_2 (□), and Δf_1 (○) *vs.* the log external potassium concentration. Each point represents the average of three replicate determinations corrected for protein (1 mg/ml) ± SEM. (*B*): A replot of the data shown in *A*. Each plot consists of the variable component Δf_{RMP} (●), Δf_{VAL} (△), or Δf_2 (□) plotted on the vertical axis as a function of Δf_{RMP} plotted along the horizontal axis. The lines were fitted by means of linear regression. The slopes for each fitted line are 1.33 ± 0.02 (Δf_2), 1.44 ± 0.03 (Δf_{VAL}), and 1 (Δf_{RMP})

plasma membrane. In Fig. 8 *A* the curves for Δf_2 and Δf_{VAL} (derived from Fig. 2) appear parallel to each other and not parallel to Δf_{RMP} (derived from Fig. 1). We further compared the effect of varying extracellular potassium on each of these responses by replotting the data shown in Fig. 8 *A* as a function of Δf_{RMP} for each potassium concentration (Fig. 8 *B*). When plotted in this manner, Δf_2 and Δf_{VAL} produced lines with similar slopes (1.33 ± 0.02 *vs.* 1.44 ± 0.03, respectively, $P > 0.05$). These values are clearly different from the slope of one for Δf_{RMP} ($P < 0.01$). Thus the data demonstrate that high extracellular potassium affects Δf_2 similar to the way potassium affects the valinomycin response. The data therefore suggest that part of the fluorescence response to f-met-leu-phe, arbitrarily measured as Δf_2, represents a membrane hyperpolarization caused by increased potassium permeability.

The requirement of extracellular calcium for the fluorescence response to f-met-leu-phe was also assessed. Removal of calicum from the extracellular medium did not affect the fluorescence response (Fig. 9, response *A*). However, when 10 mM Mg^{2+} – 10 mM EGTA was present, Δf_2 was inhibited although Δf_1 was not affected (Fig. 9, response *B*). When Mg^{2+}-EGTA-treated PMNs were washed and resuspended in calcium-free Hanks', the normal response to f-met-leu-phe was restored (Fig. 9, response *C*).

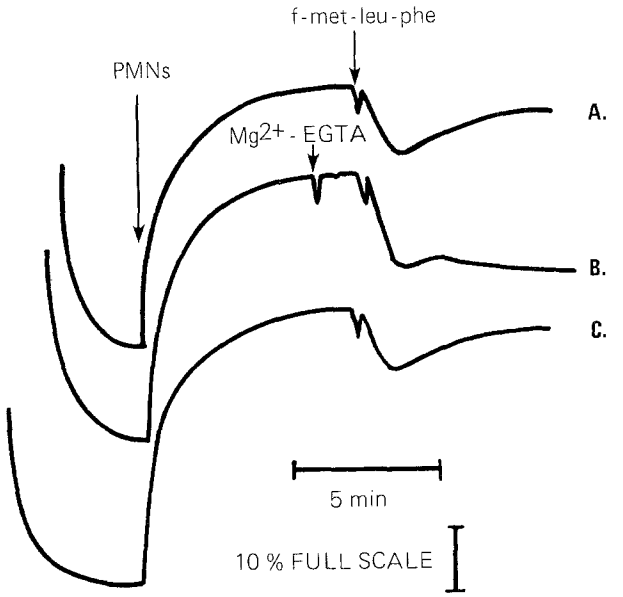

Fig. 9. Effect of calcium on the fluorescence response to f-met-leu-phe. f-met-leu-phe (5×10^{-7} M) was added as indicated under three conditions. (*A*): cells in calcium-free Hanks' containing magnesium. (*B*): Cells in calcium-free Hanks' containing magnesium, with 10 mM Mg^{2+} – 10 mM EGTA added as indicated 2 min prior to addition of f-met-leu-phe. (*C*): PMNs incubated with 15 mM Mg^{2+} – 10 mM EGTA for 15 min at 37 °C, pelleted, and resuspended in calcium-free Hanks' containing magnesium before being added to the assay solution. The same number of cells (5×10^5 PMNs/ml) were used in each case

Therefore, while the f-met-leu-phe induced changes in fluorescence suggested a depolarization (Δf_1) followed by a hyperpolarization (Δf_2), an ionic basis for Δf_1 could not be established. The possibility Δf_1 reflected dye aggregation was also considered, but this seemed unlikely since no shift in the absorbance spectra could be detected either at the minimum of Δf_1 or corresponding to Δf_2. In contrast to Δf_1, Δf_2 was affected by external potassium in a manner

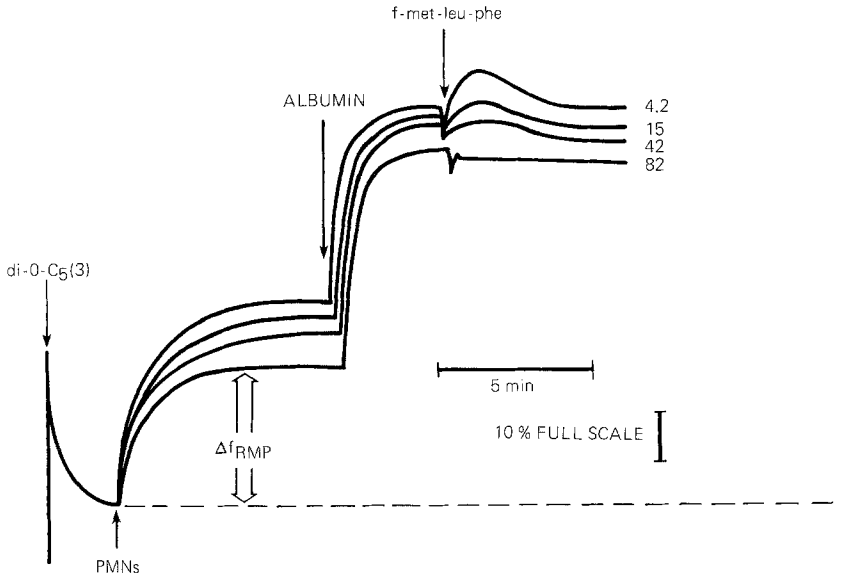

Fig. 10. Effect of albumin of the fluorescence response to f-met-leu-phe. After Δf_{RMP} stabilized albumin was added (0.5% final concentration) followed by f-met-leu-phe (2.5×10^{-8} M) as indicated. The responses obtained at the four different potassium concentrations (mM) indicated at the right of each tracing are also shown. The photomultiplier tube sensitivity was one-third that used in all other figures

suggesting that it represents a membrane hyperpolarization.

Effect of Superoxide on the f-met-leu-phe-Response. PMNs generate superoxide following stimulation by chemotactic factors [32]. To assess the effect of superoxide on di$-$O$-$C$_5$(3) fluorescence, two types of experiments were conducted. Superoxide was generated by the reaction of xanthine with xanthine oxidase either in the presence or absence of cells. The generation of significantly greater amounts of superoxide by this system than that produced by PMNs following stimulation with f-met-leu-phe (0.04 nmol/ml *vs.* 0.003 nmol/2.5×10^6 PMNs/ml, $p < 0.01$) had no effect on fluorescence or the ability of dye to equilibrate with cells. In related studies the effect scavengers of superoxide, hydrogen peroxide, and singlet oxygen had on the f-met-leu-phe-induced fluorescence response was assessed. Addition of superoxide dismutase (100 μg/ml) eliminated the accumulation of superoxide following stimulation of PMNs but had no effect on the fluorescence response. Catalase (10 μg/ml), a scavenger of hydrogen peroxide, or azide (2 mM), a scavenger of singlet oxygen, also did not affect the response. From these results we conclude that extracellular superoxide, singlet oxygen, or hydroxyl radical do not have any affect on di$-$O$-$C$_5$(3) fluorescence.

Effect of Agents that Modify Surface Charge. Di$-$O$-$C$_5$(3) is positively charged, and the binding of the dye to negatively charged membrane sites might affect fluorescence and the fluorescence changes produced by f-met-leu-phe. The effect of the dye on PMN surface charge (electrophoretic mobility) was therefore studied. Addition of the dye caused a small yet significant decrease in surface charge (2.10 ± 0.02 μm/sec/V cm to 1.85 ± 0.03 μm/sec/V cm, $P < 0.05$) that was less than the response to f-met-leu-phe alone (decreased to 1.79 ± 0.02, $P < 0.01$). Addition of f-met-leu-phe to cells equilibrated with di$-$O$-$C$_5$(3) caused a reduction of surface charge to 1.61 ± 0.01 μm/sec/V ($P < 0.01$). In related studies, neuraminidase (which removes sialic acid residues) was used to investigate the effect of decreasing the negative cell surface charge on dye fluorescence (Δf_{RMP}). Neuraminidase, at a concentration (5 μg/ml) causing a large decrease in surface charge, (decreased to 1.02 ± 0.02 μm/sec/V cm), had no effect on either Δf_{RMP} ($58 \pm 4\%$ of full-scale fluorescence in the presence of 5 μg/ml neuraminidase *vs.* $60 \pm 4\%$ of full-scale fluorescence in the absence of neuraminidase; mean ± SEM of three observations) or the fluorescence response produced by f-met-leu-phe. Similar conditions of neuraminidase treatment also did not inhibit chemotaxis [23]. In addition, when PMNs were equilibrated with dye and then were treated with neuraminidase, centrifuged, and resuspended, the same amount of cell fluorescence was retained ($83 \pm 5\%$ full-scale fluorescence) as in the control experiments ($85 \pm 6\%$ full-scale fluorescence) in which no neuraminidase was used. Thus cleavage of sialic acid groups did not result in the displacement of detectable amounts of cell-associated dye.

Effect of Albumin on PMN Fluorescence. Our initial experiments were performed in the absence of albumin because this protein caused an increase in fluorescence even in the absence of cells. The increase in fluorescence produced by albumin necessitated a reduction in photomultiplier sensitivity to a third of that used in experiments performed in the absence of albumin. We found, as did Burckhardt [13] studying Ehrlich ascites tumor cells, that once Δ_{RMP} stabilized following the addition of 0.15–0.3% albumin, further small increases in albumin had a negligible effect on Δf_{RMP}. When f-met-leu-phe was added to cells in the presence of 0.5% albumin, the response differed from that observed in the absence of albumin. Instead of the biphasic response produced in the absence of albumin, f-met-leu-phe produced a transient increase in fluorescence (Fig. 10). The apparent K_m (concentration producing a half maximal response) for the dose-response curve in the presence of albumin was lower (3×10^{-9} M) than that obtained for the biphasic response in its absence (3×10^{-8} M) (five experiments). Increasing the external potassium concentration (four experiments) reduced the response to f-met-leu-phe in a manner similar to that observed in the absence of albumin for the Δf_2 component of the response (Fig. 10). In three other studies the addition of valinomycin in the presence of albumin also caused an increase in fluorescence similar to that produced by valinomycin in the absence of albumin. Therefore albumin actually modified the response to f-met-leu-phe and did not just invert the fluorescence signal.

Since potassium-dependent fluorescence responses could be seen using f-met-leu-phe in the presence of albumin, experiments were performed to determine the PMN fluorescence response to partially purified C5a, which contained significant amounts of albumin [23]. The response produced by the C5a preparation, which contained 10 μg protein/ml, was similar to that produced by f-met-leu-phe in the presence of albumin (Fig. 11). No fluorescence response was seen in control experiments using chemotactically inactive proteins eluted from the G-75 Sephadex column used in isolating the C5a fraction.

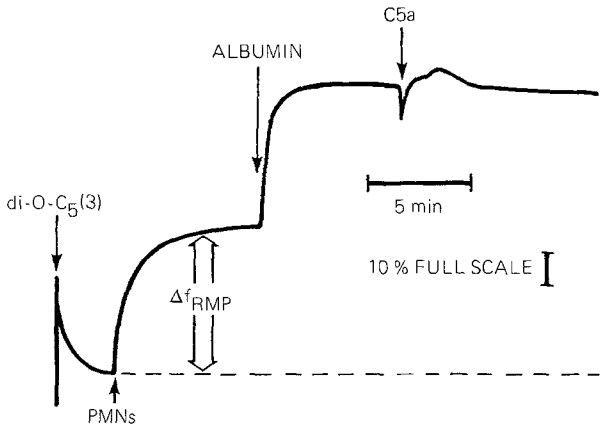

Fig. 11. Time course of fluorescence response to C5a. Fluorescence was measured as percent change in full-scale fluorescence (*see* Materials and Methods). The addition of dye was followed by 0.5% albumin. Once fluorescence stabilized, 100 μl of a partially purified and chemotactically active preparation of C5a was added as indicated. The photomultiplier tube sensitivity was as described in Fig. 10

Discussion

Cyanine dyes and radiolabeled ions have been used as indicators of membrane potential changes in a number of cell systems. The concentration of these probes within cells has been related to the magnitude of the membrane potential [29, 31, 34, 44, 55, 59]. The fluorescent dyes equilibrate rapidly (Fig. 1) and permit a continuous observation of membrane potential. Thus these dyes are suitable for qualitatively measuring relatively rapid changes in membrane potential [55]. In contrast, radiolabeled ions such as triphenyl methyl phosphonium ($TPMP^+$) [17, 33, 45] used recently to study potential changes in PMNs during exposure to concanavalin A and antigen-antibody complexes [33], equilibrate slowly and require that the cells be harvested for each time point of measurement. Because our studies involved rapid changes in membrane potential and it was necessary to avoid cell aging, we chose to use fluorescent probes.

When using fluorescent probes to study cells that contain numerous intracellular compartments such as PMNs, fluorescence changes cannot be simply equated with membrane potential changes. However, the demonstration that intact cells are required for fluorescence, that the intensity of fluorescence (Δf_{RMP}) was dependent on the extracellular potassium concentration (potassium itself had no effect on fluorescence) and that the potassium ionophore valinomycin induced predictable and consistent changes in fluorescence indicate that in PMNs the fluorescence is a qualitative measure of membrane potential. Values for the intracellular concentration of potassium in PMNs have been reported by a number of independent laboratories ranging from 120 to 137 mM [3, 14, 18]. Using a value of 120 mM for intracellular potassium, the equivlibrium potential for potassium (E_K) calculated using the Nernst equation, is approximately −85 mV. Assuming the PMN is at least partially permeable to potassium, raising the external concentration of potassium would shift E_K and depolarize the cells. Experimentally, we observed this effect as

a progressive decrease in fluorescence (Δf_{RMP}) as the external concentration of potassium was raised. However, the plot of log external potassium *vs.* fluorescence deviated from linearity at low potassium concentrations (Fig. 1 *B*), suggesting that at these concentrations of potassium either the membrane potential is not simply a potassium equilibrium potential and/or dye fluorescence is not a linear function of membrane potential.

Valinomycin, which selectively increases potassium permeability, caused a rapid increase in fluorescence at low external potassium concentrations. At the low concentration of valinomycin used in these experiments, the initial effect on cells is an immediate change in potassium permeability. The fact that after the initial change in fluorescence there was not a gradual loss of fluorescence infers that there was no significant change in the intracellular concentration of potassium within 10 min after addition of valinomycin. The effect of valinomycin indicates that the resting membrane potential of PMNs is not at the potassium equilibrium potential. The effect of potassium ion itself and of valinomycin on PMN di$-$O$-$$C_5$(3) fluorescence is consistent with results reported by other investigators using a variety of cell types using indirect probes [29, 31, 34, 43, 55, 59]. The studies involving extracellular potassium and valinomycin, together with studies in which extracellular sodium and chloride were varied independently of potassium, indicate that the PMN basal fluorescence, and hence the resting membrane potential, is primarily but not entirely a function of the permeability of potassium across the plasma membrane.

Di$-$O$-$$C_5$(3) had no adverse effect on PMN chemotaxis or release of intracellular granule contents. Furthermore, cell aggregation and decreases in surface charge, which occur during incubation of PMNs with chemotactic factors, had no effect on the intensity of dye fluorescence. Therefore, di$-$O$-$$C_5$(3) seemed an appropriate probe to monitor membrane potential changes during PMN exposure to chemotactic factors.

Addition of the chemotactic factor f-met-leu-phe to PMN suspensions in the presence of di$-$O$-$$C_5$(3) caused a reproducible fluorescence response. In the absence of albumin, the response was biphasic with a decrease in fluorescence (Δf_1) followed by an increase (Δf_2) (Fig. 3 *A*). These two components may overlap optically. The f-met-leu-phe elicited response could not be attributed to an effect of f-met-leu-phe on dye fluorescence or displacement of the dye from the cuvette. Furthermore it required viable PMNs and could not be attributed to the effects of extracellular superoxide, singlet oxygen, hydrogen peroxide, or hydroxyl radical, which are generated by incubation of PMNs with f-met-leu-phe [8].

The decrease in fluorescence, Δf_1, represented a change in fluorescence compatible with a depolarization. However, Δf_1 was not affected by changes in extracellular ion concentration, suggesting that it did not represent a change in potential across the plasma membrane. The PMN is a multicompartmental cell and changes in organelle membrane potential should not be directly affected by extracellular ion composition. Whether or not Δf_1 represents events occurring in subcellular organelles or solvent effects exerted on the dye within the cell in response to stimulation cannot be resolved with the available data. Mitochondria and presumably other subcellular organelles take up cyanine dyes as readily as intact cells [31, 34, 43, 55, 59]. To avoid effects of mitochondrial potentials in ascites tumor cells, Philo and Eddy [43] used oligomycin, antimycin, and 2,4-dinitrophenol to eliminate the potentials. We found that the protonophore carbonyl cyanide-*m*-chlorophenyl hydrazone, which uncouples oxidative phosphorylation leading to the depolarization of mitochondria, had no effect on the fluorescence response. Since PMNs contain few mitochondria compared to other cells this is not surprising. PMNs do contain numerous other subcellular organelles and membrane-bound granules and, therefore, the problem of subcellular organelle effects on di$-$O$-$$C_5$(3) fluorescence cannot be easily dealt with simply by poisoning the mitochondria.

Another possible explanation of the insensitivity of Δf_1 to ionic changes is that Δf_1 could reflect alteration of membrane or cytoplasmic fluidity by f-met-leu-phe. In recent studies of PMNs obtained from patients with the Chediak-Higashi syndrome, in which an abnormality of membrane fluidity has been described [28], the Δf_1 and Δf_2 components of the f-met-leu-phe response were normal, suggesting that the Δf_1 component is not a consequence of a change in membrane viscosity affecting dye fluorescence. However, the possibility that Δf_1 reflects changes in cytoplasmic microviscosity or membrane potential change occurring somewhere within the PMN other than across the plasma membrane awaits additional study.

In contrast to Δf_1, Δf_2 appears to represent a hyperpolarizing potential change across the plasma membrane. This component of the fluorescence response was dependent on the potassium concentration gradient across the plasma membrane in a manner similar to the response obtained with the potassium ionophore valinomycin.

In the presence of albumin, only a transient increase in fluorescence was observed in response to

f-met-leu-phe. This increase in fluorescence was inhibited by high extracellular potassium, and appeared to be a hyperpolarizing response. In the absence of albumin, the f-met-leu-phe response had an apparent K_m of 3×10^{-8} M, whereas in the presence of albumin, the apparent K_m was 3×10^{-9} M. These values are similar to concentrations of f-met-leu-phe that produce half-maximal chemotactic activity reported previously by our laboratory [16] for human peripheral blood PMNs in the presence of albumin and is consistent with the observation that chemotaxis is poor in the absence of albumin [57]. The mechanism for these albumin effects is unknown.

The specificity of the response to f-met-leu-phe was also demonstrated using various inhibitors of chemotaxis. The inhibition by Z-phe-met, which is reported to competively inhibit f-met-leu-phe binding [44], appeared to be competitive (Fig. 6). Therefore interaction of a ligand with a chemoattractant receptor does not appear to be sufficient to elicit a fluorescence response by itself. The effectiveness of four different synthetic peptides (*see* Results) as chemoattractants was directly correlated with the concentration of each required to elicit a fluorescence response. These results, plus the observations made using C5a and Z-phe-met, provide evidence that the response requires more than binding and implies a connection to some aspect of chemoattractant-induced function, either chemotaxis itself or another related event.

Inhibition of the chemoattractant elicited fluorescence response by ouabain (Fig. 7) is of particular interest. Ouabain has been used as a specific inhibitor of the $Na^+ - K^+$ATPase and effects are both time- and concentration-dependent. Becker et al. [7, 9] found that f-met-leu-phe stimulates a ouabain (10^{-4} M) inhibitable $Na^+ - K^+$ATPase activity in rabbit exudate PMNs concurrent with the influx of potassium. The activation of $Na^+ - K^+$ATPase activity was inhibited by Z-phe-met. The authors suggested that enhancement of $Na^+ - K^+$ATPase activity by f-met-leu-phe might be a consequence of the peptide acting directly on the $Na^+ - K^+$ATPase or indirectly through perturbation of the membrane. Our results corroborate these findings and extend them. The fluorescence response to f-met-leu-phe is inhibited by both ouabain and Z-phe-met. Further, our data indicate that the effect of ouabain on the fluorescence response is a direct result of inhibition of the $Na^+ - K^+$ATPase and not a consequence of a general depolarization of the resting membrane potential since the baseline fluorescence did not change. It seems likely that the fluorescence changes and therefore the potential changes result from changes in $Na^+ - K^+$ ATPase activity consequent to the effect of f-met-leu-phe on the $Na^+ - K^+$ ATPase.

There is considerable evidence that calcium is involved in initiation of chemotaxis. The optimal chemotactic response through micropore filters requires extracellular calcium, and chemoattractant-induced transmembrane calcium fluxes have been demonstrated [5, 11, 25, 41]. In addition, in macrophages the chemoattractant-induced transmembrane potential changes [19] were similar to those induced by the calcium ionophore A23187 [20, 21]. We have recently shown [46] that the calcium ionophore A23187 causes changes in di$-$O$-$C$_5$(3) fluorescence. Low concentrations of ionophore stimulate a transient potassium-dependent increase in fluorescence (hyperpolarization), while high concentrations of ionophore (10^{-7} M) cause a large and permanent decrease in fluorescence(depolarization). This is consistent with the observations made directly with intracellular recording from macrophages [21]. In the studies reported here, $Mg^{2+}$$-$EGTA blocked they hyperpolarizing fluorescence response, although cell responsiveness recovered when the cells were washed and resuspended in calcium-free media. It is possible that only trace levels of extracellular calcium are required for the response. Alternatively, cell-associated calcium may be available to activate the response.

The use of the fluorescent dye di$-$O$-$C$_5$(3) to monitor membrane potential in human PMNs has provided evidence supporting the view that chemotactic factors (f-met-leu-phe and C5a) initiate hyperpolarizing potential changes similar to those noted earlier in macrophages using direct intracellular recording techniques [19] and in monocytes using the cyanine dye [20]. The current studies also demonstrate that the data obtained using membrane potential-sensitive probes to study responses to substances such as chemotactic factors in multicompartmental cells such as PMNs must be interpreted with caution; i.e., that changes in fluorescence occur which are not necessarily related to alteration of potential across the plasma membrane.

Appendix

Distribution of di$-$O$-$C$_5$(3) in the Assay System and Nature of the Interaction between the Cyanine Dye and PMNs.

The change in fluorescence of probes such as di$-$O$-$C$_5$(3) may result from one of two possible properties of the dye [29]. First, cyanine dyes typically exhibit enhanced fluorescence in less polar solvents in addition to a shift in fluorescence maximum toward longer wavelengths [52]. Second, cyanine dyes demonstrate

Table 2. Fluorescence in Hanks' solution of di−O−C_5(3) equilibrated with supernatant and PMN fractions

Preparation	Fluorescence[a]
1) Complete system[b]	1.60 ± 0.02
2) Complete system before addition of PMNs	0.30 ± 0.03
3) Media from the complete system after removal of PMNs	0.28 ± 0.03
4) Media (# 3) plus fresh PMNs	0.81 ± 0.05
5) Rinsed cuvette from # 2 plus PMNs and media	0.40 ± 0.06
6) Rinsed cuvette from # 1 plus fresh PMNs and media	0.21 ± 0.04
7) Rinsed cells from # 1 plus fresh cuvette and media	1.08 ± 0.05

[a] Maximum fluorescence obtained, measured as described in Materials and Methods; mean of three observations ± SEM.

[b] Complete system contained di−O−C_5(3) (5×10^{-7} M, 2-min incubation) plus PMNs (5×10^5 cells/ml, additional 10-min incubation) in a total volume of 1.5 ml Hanks' solution.

Table 3. Partitioning of di−O−C_5(3) in various compartments of the assay system: Butanol extraction of cuvette and cell pellet[a]

Conditions of butanol extraction	Extracted di−O−C_5(3) (fluorescence)
Extraction of rinsed cuvette following[b]	
1) 5 sec with di−O−C_5(3)	0.02 ± 0.02
2) 2 min with di−O−C_5(3)	0.37 ± 0.04
3) 2 min with di−O−C_5(3) then 10 min with PMNs	0.20 ± 0.04
Extraction of PMN Pellet[c]	
4) PMNs from condition # 3	1.80 ± 0.12
5) PMNs from condition # 3 lysed with gramicidin (10^{-4} M)	0.10 ± 0.05

[a] Partitioning of di−O−C_5(3) among PMNs, cuvette surface, and extracellular milieu as assessed by butanol extraction and expressed as the mean of three observations ± SEM (*see* Materials and Methods). Total fluorescence of 5×10^{-7} M di−O−C_5(3) in butanol was 6.15 ± 0.13 units of fluorescence. In each experiment the initial dye concentration was 5×10^{-7} M and cell concentration was 5×10^5 cells/ml.

[b] Cuvette decanted, rinsed with 3.0 ml H_2O, and decanted again prior to addition of butanol.

[c] PMNs pelleted by centrifugation, washed with Hanks' solution, then resuspended in butanol to extract dye.

the property of aggregation above a critical concentration. The resulting aggregates are nonfluorescent and characterized by a shift in absorbance maximum to shorter wavelengths [29, 42, 45, 55]. Either of these phenomena could occur as the dye becomes concentrated inside the cell. The experiments reported in the Appendix were designed to assess the distribution and redistribution of di−O−C_5(3) in the assay system and to assess the fluorescent spectra of the dye during the assay conditions.

Distribution of di−O−C_5(3) in the assay. For these studies, the dye was extracted from the cuvette or cells with butanol. Following equilibration of dye with the cuvette (and where appropriate with cells) the medium was removed, centrifuged for 1 min to pellet the cells, and the supernate placed in a fresh dye-free cuvette. The pellet and original cuvette were rinsed rapidly and either soaked for 15 min with 1.5 ml butanol to extract residual dye or used in additional experiments (as described in Tables 2 and 3). In all instances fluorescence was recorded immediately after each manipulation and the final maximum fluorescence noted. Fluorescence was recorded at a constant sensitivity setting using a gain of 1 and an arbitrary full-scale reading at 2.0 fluorescence units.

As shown in Table 2, addition of PMNs caused a fivefold enhancement of fluorescence (preparations *1* and *2*, $P < 0.01$). There was sufficient dye left in the supernatant to enable a enhancement (nearly fourfold) by fresh cells, as demonstrated in preparations *3* and *4* (Table 2). The large enhancement of fluorescence by PMNs was not obtained when only cuvette absorbed dye was available to equilibrate with the PMNs (preparations *5* and *6;* Table 2). The amount of dye adsorbed to the cuvette surfaces or associated with cells was measured by extracting dye from the cuvette or cell pellet with a common solvent, butanol (Table 3). Extraction into butanol made possible the measurement of the actual amount of dye independent of any solvent effects. Dye adsorbed to the cuvette and magnetic flea accounted for the decrease in fluorescence between the measurements taken at 0 time and after 2 min (Fig. 12). Evidence for this are the measurements reported in Table 3 (preparations *1* and *2*). The amount of dye adsorbed onto the cuvette after a brief (5 sec) exposure to dye-containing medium represents the control situation (preparation *1*), while that recorded at 2 min represents adsorbed dye present which can exchange with free dye in solution (preparation *2*). Unless the cuvette-adsorbed dye is bound irreversibly the addition of cells necessitates the displacement of dye from the cuvette and establishment of a new steady state between the cells, cuvette, and supernatant. A small yet significant amount of dye was displaced upon addition of cells (Table 3; preparation *3 vs. 2* $P < 0.01$). The amount of dye partitioned into the cells was measured by recording the fluorescence after extracting the cell pellets with butanol (preparations *4*

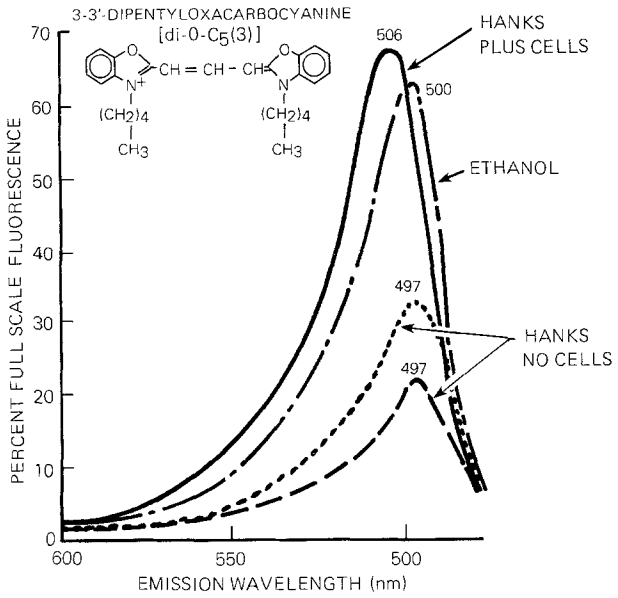

Fig. 12. Emission spectra of Di−O−C_5(3). The cyanine dye di−O−C_5(3) was dissolved in Hanks' solution (----,--), absolute ethanol (----), or Hanks' plus 5×10^5 PMNs per ml (—) and the emission spectra scanned from 400–600 nm with excitation at 460 nm. The maxima recorded were 497, 500, and 506 nm, respectively. The spectra shown for dye in Hanks' solution were obtained immediately after addition of dye (-----) and 2 min (---). The structure of di−O−C_5(3) is shown

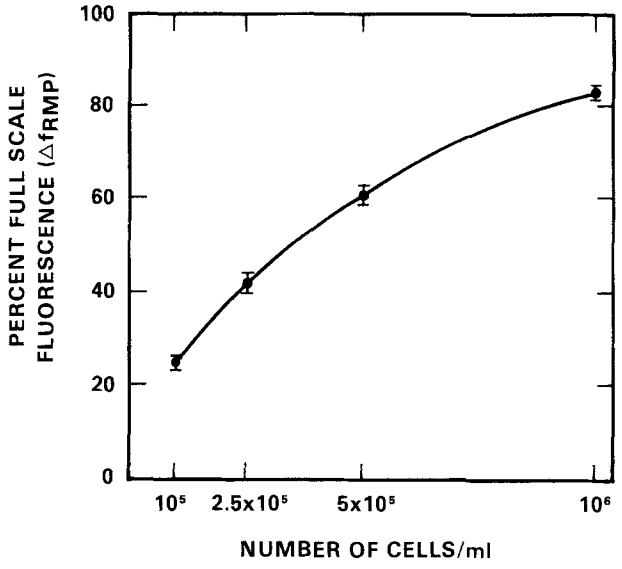

Fig. 13. Δf_{RMP} as a function of cell number. The cell concentration was varied from 10^5 to 10^6 cells/ml and the value of Δf_{RMP} recorded using the same dye concentration [5×10^{-7} M di−O−C_5(3)]. Each point represents the mean of three observations ± SEM and is expressed as a percent of the full-scale fluorescence

and *5;* Table 3). The amount of dye associated with cells after equilibration (preparation *4*; Table 3) was much greater than the decrease in dye adsorbed to the cuvette (preparations *2* and *3,* Table 3); the dye adsorbed to the cuvette accounting for only 6% of the total cell-associated dye. Based on the butanol extraction studies, it appears that although the equilibration of dye with PMNs causes some movement of cuvette-bound dye into the supernate this cannot account for the large increase in fluorescence seen upon addition of PMNs. Instead the increased fluorescence represents uptake of the dye by the cells. Furthermore, the cell-associated enhanced dye fluorescence requires intact cells since cell lysis by sonication (*see* text, Fig. 5) or gramicidin S decreases fluorescence (preparation *5;* Table 3). The gramacidin effect was due to actual loss of dye and not to alteration of the solvent environment (preparations *4* and *5;* Table 3).

Cell concentration is another variable which could affect the results. At the dye concentration we used the fluorescence increased with cell concentrations from 10^5 to 10^6 cells/ml (Fig. 13). A cell concentration of 5×10^5 cells/ml was therefore used for all experiments.

Fluorescence Spectra. Uncorrected fluorescence spectra of di−O−C_5(3) (5×10^{-7} M) obtained with excitation at 460 nm in various solvents are shown in Fig. 12. A dye concentration of 5×10^{-7} M was chosen following preliminary studies which demonstrated that this dye concentration did not cause cell death nor impair PMN function (*see* Results) and was sufficient to enable fluorescence to be recorded accurately. Two spectra are shown with only di−O−C_5(3) dissolved in Hanks' (Hanks', no cells; Fig. 12). One was obtained immediately following addition of dye to the solution and the other was recorded 2 min later. The fluorescence maximum (497 nm) was decreased in the latter but not shifted. This decrement in fluorescence may have resulted from dye adsorbing to the cuvette and magnetic flea, a phenomenon observed by others using the cyanine dyes [29]. When dye was dissolved in the less polar solvent ethanol, the fluorescence maximum was enhanced and shifted toward the red by 3 nm. This solvent effect is characteristic of cyanine dyes [52].

The spectrum obtained after a 5-min incubation of dye with PMNs in Hanks' is also shown (Hanks' plus cells; Fig. 12). In this case the fluorescence maximum was enhanced and shifted 9 nm toward the red compared to the spectra of dye in Hanks' without cells. We observed a similar enhancement of fluorescence with glial cells, mast cells, and nonadherent lymphocytes, although quenching was observed with erythrocytes. The enhancement of fluorescence by

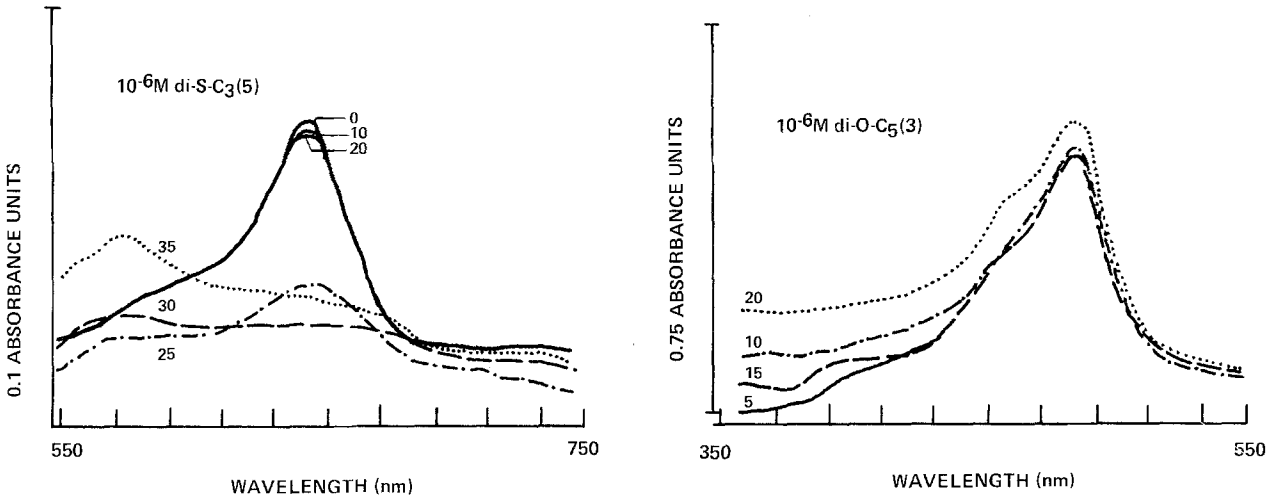

Fig. 14. Absorbance spectra of di−S−$C_3(5)$ and di−O−$C_5(3)$ with and without cells. *Left panel:* The absorbance spectra of di−S−$C_3(5)$ (10^{-6} M) in HEPES Hanks' solution was obtained at 0, 10, and 20 min after addition of dye to an unstirred solution as indicated by the solid lines. Cells (5×10^5/ml) were added at minute 24 and spectra were obtained every 5 min thereafter (broken lines). Scan rate was 120 nm/min. *Right panel:* Absorbance spectra fo di−O−$C_3(5)$ (10^6 M) is shown, recorded at minute 5 (solid line). Cells were then added at minute 9 and consecutive recordings obtained at 10, 15, and 20 min (broken lines)

cells was similar to the enhancement seen in ethanol. The similarities between the shift in fluorescence spectrum of di−O−$C_5(3)$ occurring upon addition of PMNs and that of dye in ethanol provide evidence that solvent effects may account for fluorescence changes in the PMN system.

To determine if the enhanced fluorescence we noted with di−O−$C_5(3)$ was unique to the cells we studied and/or a special property of this dye, a second cyanine dye was employed, di−S−$C_3(5)$, at a concentration comparable to that used with di−O$C_5(3)$. The absorbance spectra obtained for both dyes are shown in Fig. 14. Aggregation of cyanine dye molecules results in the formation of nonfluorescent aggregates with an absorbance maximum at shorter wavelength [42, 52]. The spectra obtained with di−S−$C_3(5)$ after addition of cells is typical of that seen when aggregation occurs (Fig. 14, left panel). Spectra were run before addition of cells, immediately after the addition of di−S−$C_3(5)$ (0 time), as well as 10 and 20 min later. PMNs were added at minute 24 and consecutive spectra obtained at 25, 30, and 35 min. The addition of cells caused a decrease in the absorption maximum at $\simeq$595 nm and the appearance of a new maximum at a shorter wavelength ($\simeq$560 nm). This is consistent with the formation of dye aggregates. However, when a similar series of spectra was obtained using di−O−$C_5(3)$ (Fig. 14, right panel) no such shift was observed. The first spectrum was obtained 5 min after addition of dye, cells were added at 9 min and consecutive spectra obtained at 10, 15, and 20 min. The absorbance maximum did not decrease or shift. When the concentration of di−O−$C_5(3)$ was raised 10-fold or greater, both quenching of fluorescence and a change in the absorbance maximum typical for dye aggregation was seen upon addition of PMNs. A similar effect of dye concentration on fluorescence in the presence of cells was reported by Sims et al. [52]. These results are consistent with the hypothesis that aggregation occurs when the dye concentration becomes greater than a critical value characteristic for each particular dye. The concentration of dye used throughout these experiments (5×10^{-7} M) is less then this critical value and therefore enhanced fluorescence is seen with the addition of PMNs resulting from interaction of dye with the microenvironment of the cell.

In summary, on the basis of the observations reported in the Appendix, it appears that once di−O−$C_5(3)$ partitions into cells it experiences a solvent environment causing enhanced fluorescence. Fluorescence correlates with the actual accumulation or loss of dye molecules. Furthermore, cell-associated dye is apparently not tightly bound.

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