*Biochimica et Biophysica Acta*, 512 (1978) 525—538


BBA 78145

# $Mg^{2+}$-ATPase AS A MEMBRANE ECTO-ENZYME OF HUMAN GRANULOCYTES

## INHIBITORS, ACTIVATORS AND RESPONSE TO PHAGOCYTOSIS

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(Received February 27th, 1978)

## Summary

(1) The $Mg^{2+}$-ATPase of purified human granulocytes is located at the plasma membrane. Thus, no additional enzyme activity was detected when the cells were disrupted. Moreover, the $Mg^{2+}$-ATPase activity of intact cells was inhibited by such poorly permeant reagents as diazotized sulfanilic acid and suramin. Finally, the enzyme activity of cell homogenates was recovered in particulate fractions.

(2) The surface $Mg^{2+}$-ATPase of human granulocytes had an apparent $K_m$ of 50 $\mu$M for ATP and displayed substrate inhibition.

(3) The enzyme was not affected by ouabain, but was inhibited by *N*-ethyl maleimide, sodium *meta*-periodate, suramin and diazotized sulfanilic acid. The enzyme was activated by cytochalasins B and D and by UDP. Activation by UDP was characterized by changes in the enzyme's apparent $K_m$ and $V$ and by relief of substrate inhibition.

(4) Internalization of surface membranes subsequent to phagocytosis of suitable particles did not result in depletion of $Mg^{2+}$-ATPase from the cell surface. The enzyme activity did not decrease after exposure to several varieties of paraffin oil emulsion particles, even if the challenged cells had been pretreated with colchicine or cytochalasin B.

(5) Since suramin, which inhibited $Mg^{2+}$-ATPase, had no effect upon other granulocyte functions such as chemotaxis, superoxide anion generation, or phagocytosis, it is unlikely that the enzyme plays a major role in these functions.

## Introduction

Direct investigation of the human granulocyte plasma membrane has been hampered by lack of suitable membrane markers. Cell fractionation studies,

particularly those leading to isolation of plasma membrane preparations similar to those reported for rabbit granulocytes [1], have been most affected by this limitation. However, two recent reports have suggested that magnesium-dependent adenosine triphosphatase ($Mg^{2+}$-ATPase) constitutes a membrane marker for human granulocytes [2,3]. At least 90% of the cellular $Mg^{2+}$-ATPase activity appears to be membrane-associated and can be assayed using intact cells [2]. Inorganic phosphate resulting from ATP hydrolysis was localized outside the cells [2], suggesting that $Mg^{2+}$-ATPase is a true ecto-enzyme (i.e., its active site faces the extracellular space). Enzyme activity is not ouabain-sensitive and does not appear to be due to non-specific neutral phosphatase [2]. In addition, there is indirect evidence that $Mg^{2+}$-ATPase might be involved in phagocytosis [3].

Studies of the incorporation of surface markers into phagocytic vacuoles [1,4—8] have indicated that microtubules or other colchicine-sensitive mechanisms are responsible for the selective inclusion [1] or exclusion [4,5] of membrane components from nascent phagosomes. The dispositions of some human granulocyte membrane components have already been examined [8]. As an enzymatic membrane marker, $Mg^{2+}$-ATPase should therefore be well suited for similar studies of transmembrane controls in phagocytosis.

In this report, we provide evidence that $Mg^{2+}$-ATPase is a reliable membrane marker for human granulocytes. The effects of phagocytosis and a variety of biochemical reagents on enzyme activity have also been examined. Finally, experiments are detailed which examine the role of $Mg^{2+}$-ATPase in such processes as chemotaxis, superoxide anion generation, and phagocytosis.

## Materials and Methods

*Reagents.* The following materials were purchased from Sigma Chemical Co.: colchicine, cytochalasin D, adenosine 5′-monophosphate, adenosine 5′-triphosphate, guanosine 5′-monophosphate, inosine 5′-diphosphate, cytidine 5′-triphosphate, cytidine 5′-diphosphate, uridine 5′-monophosphate, uridine 5′-diphosphate, uridine 5′-triphosphate, ouabain, quercetin, rutin, kaempferol, epinephrine, sodium *meta*-periodate, lipase, phospholipase A, papain, neuraminidase, 2-deoxyglucose, tetracaine, iodoacetamide, *N*-ethyl maleimide, dibutyryl-cyclic AMP, dibutyryl-cyclic GMP and concanavalin A.

*Ricinus communis* agglutinin, *Lens culinaris* hemagglutinins A and B, and peanut agglutinin were purchased from Miles Laboratories, A23187 from Eli Lilly and Co., hydrocortisone hemisuccinate from Upjohn Co., phospholipase C from Calbiochem, trypsin from Worthington Biochemicals, and *N*-formyl methionyl leucylphenylalanine from Peninsular Laboratories. Suramin was a generous gift of Dr. P. D'Arcy Hart. All other materials were reagent grade.

*Preparation of cell suspensions.* Heparizined (10 units/ml) venous blood was obtained from healthy adult donors. Purified preparations of granulocytes were isolated from this blood by means of Hypaque/Ficoll gradients [9] followed by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes [10]. This allowed studies of cell suspensions containing 98 ± 1% granulocytes with few contaminating platelets, lymphocytes or erythrocytes. The cells were suspended in a buffered salt solution consisting of 138 mM NaCl/2.7 mM

KCl/8.1 mM $Na_2HPO_4$/1.5 mM $KH_2PO_4$/1.0 mM $MgCl_2$/0.6 mM $CaCl_2$, pH 7.4 (hereafter called "phosphate-buffered saline"). Platelet-rich fractions were prepared by the method of Hamberg et al. [11].

*Phagocytosis.* Emulsions of di-*iso*-decylphthalate (in place of paraffin oil) containing oil red O were prepared in phosphate-buffered saline according to the method outlined by Stossel et al. [12], using *Escherichia coli* lipopolysaccharide B (Difco Labs, Detroit, Mich.) as an emulsifier. The "paraffin oil" emulsion was either used directly or treated with serum before addition to cell suspensions. Treatment of emulsion with fresh human serum or heat-inactivated serum (preincubated at 56° C for 30 min to destroy complement components) was conducted according to Stossel et al. [13].

Suspensions of granulocytes containing $5 \cdot 10^7$ cells/ml were preincubated for 10 min at 37° C with cytochalasin B (5 μg/ml; Aldrich Chemical Co.), colchicine (10 μM; Sigma), cytochalasin D (5 μg/ml; Sigma) or with phosphate-buffered saline alone. Phagocytosis was initiated by the addition of 0.2 vols. of serum-treated paraffin oil emulsion or a 1 : 1 dilution of untreated emulsion. Incubation proceeded for 5 s (control sample) or for other specified lengths of time, after which phagocytosis was terminated by the addition of 3 vols. of ice-cold phosphate-buffered saline and subsequent maintenance of the samples at 4° C. The cells were centrifuged and washed twice with ice-cold phosphate-buffered saline to remove uningested emulsion.

Uptake of paraffin oil emulsion was determined spectrophotometrically by dioxane extraction of oil red O dye from suitable portions of the cell suspension [14]. The amount of emulsion adsorbed to the cell surface, usually minimal, was assessed qualitatively using light microscopy and quantitatively by the oil red O content of control samples.

For experiments in which release of cellular enzymes was studied, the cells were harvested by centrifugation and the supernatant fluid was saved. This emulsion-containing medium was partially clarified by centrifugation at 100000 × *g* for 60 min and then assayed for enzyme activity.

*$Mg^{2+}$-ATPase assay.* The procedure for measuring $Mg^{2+}$-ATPase activity was a slight modification of that reported by DePierre and Karnovsky [15]. In brief, $5—10 \cdot 10^6$ cells were incubated at 37° C for 30 min in 1.0 ml phosphate-buffered saline containing 1 mM [$\gamma$-$^{32}$P]ATP (New England Nuclear) and 5 mM *p*-nitrophenyl phosphate (Sigma). Incubation was terminated by the addition of 1 ml of 10% (w/v) acid-washed charcoal (Norit A, Fisher Scientific) in 10% (w/v) trichloroacetic acid. Unhydrolyzed ATP adhered to the charcoal, which was removed by filtration, leaving inorganic [$^{32}$P]phosphate to be measured by liquid scintillation counting.

For enzyme kinetic studies, in which [$^{32}$P]ATP concentrations exceeded 1 mM, the content of $MgCl_2$ in the assay medium was increased to maintain an equimolar ratio of $Mg^{2+}$ to ATP.

*Miscellaneous procedures.* Cell-free supernatants were assayed for beta-glucuronidase [16] and lactate dehydrogenase [17]. Enzyme activities are expressed as percentages of total activity released from duplicate reaction mixtures by 0.2% Triton X-100 (Rohm and Haas, Co.). Superoxide generation was measured by cytochrome *c* reduction [18].

Cell protein was determined by the method of Lowry et al. [19] using egg

white lysozyme (Worthington Biochemical Corp.) as a standard.

Diazotized sulfanilic acid was prepared as outlined by DePierre and Karnovsky [15].

Chemotaxis of granulocytes in response to 2% zymosan-activated serum was performed by the "leading front" method of Zigmond and Hirsch [20].

Radioactivity was measured in Bray's Solution (New England Nuclear) using a Beckman LS-100 scintillation counter.

## Results

*$Mg^{2+}$-ATPase as a membrane marker for human granulocytes.* Intact human granulocytes hydrolyze extracellular [$\gamma$-$^{32}$P]ATP at a rate of 14 ± 2 nmol/min per mg cell protein ($n$ = 20). The divalent cation specificities reported by Harlan et al. [2], which showed that the enzyme was maximally stimulated by magnesium, have been verified by this laboratory. Harlan et al. also provided two important pieces of evidence which suggested that $Mg^{2+}$-ATPase was an ecto-enzyme localized exclusively on the granulocyte cell surface: (1) Enzyme activity was not latent (not increased by disruption of the cells) and so all activity could be attributed to that which was measurable on intact cell surfaces; (2) inorganic [$^{32}$P]phosphate liberated from [$\gamma$-$^{32}$P]ATP by action of the intact cell enzyme was localized in the extracellular space.

The first line of evidence, namely that no latent $Mg^{2+}$-ATPase activity could be detected, was also investigated in this laboratory and the results are shown in Table I. Under no condition of cell disruption was additional enzyme activity observed (these conditions were sufficient to increase measurable cytoplasmic lactate dehydrogenase and lysosomal beta-glucoronidase activities at least 20-fold over intact cell levels). The variable losses of $Mg^{2+}$-ATPase activity

TABLE I

EFFECT OF GRANULOCYTE DISRUPTION ON $Mg^{2+}$-ATPase

Aliquots of purified granulocytes ($3 \cdot 10^7$ cells/ml) in phosphate-buffered saline were subjected to sonication (approximately 20 W; sonicator manufactured by Kontes Co.), dounce homogenization, or freeze/thaw cycles (using a dry ice/acetone bath). $Mg^{2+}$-ATPase activities of the disrupted cell samples were measured and are expressed as percentages of that activity (14 ± 2 nmol/min per mg protein) found for undisrupted (control) cells. Lactate dehydrogenase and beta-glucuronidase activities were measured in cell-free supernatants and are similarly expressed. Undisrupted cell supernatants contained 0.8% the total cellular lactate dehydrogenase and 0.2% of the total beta-glucuronidase activities.

| Mode of disruption | % Enzyme activity | | |
|---|---|---|---|
| | ATPase | Lactate dehydrogenase | Beta-glucuronidase |
| Sonication | | | |
| 20 s | 79 | 14000 | 17000 |
| 60 s | 67 | 13000 | 25000 |
| Dounce homogenization | | | |
| 2 min | 81 | 2000 | 3000 |
| 5 min | 65 | 4000 | 12000 |
| Freeze/thaw | | | |
| 2 cycles | 70 | 10000 | 27000 |
| 5 cycles | 42 | 12000 | 35000 |

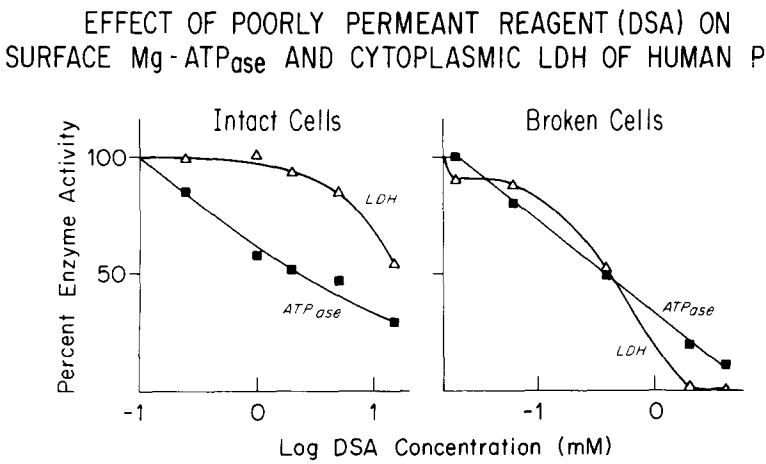


Fig. 1. Left. Purified granulocytes of polymorphonuclear leukocytes ($5 \cdot 10^7$ in 2 ml phosphate-buffered saline) were incubated for 15 min at 37°C with various concentrations of diazotized sulfanilic acid (DSA). The cells were then washed twice with ice-cold phosphate-buffered saline, sonicated, and finally assayed for lactate dehydrogenase (LDH) and $Mg^{2+}$-ATPase. Enzyme activities are expressed as percentages of those measured for untreated cells. Right. Granulocytes ($3 \cdot 10^7$ in 2 ml phosphate-buffered saline) were sonicated for 30 s and then incubated for 15 min at 37°C with the indicated concentrations of diazotized sulfanilic acid. The treated sonicates were then assayed for lactate dehydrogenase and $Mg^{2+}$-ATPase; enzyme activities are expressed as above.

might be due to lability of the enzyme or to the formation of "inside-out" vesicles. In any case, no enzyme activity which might conceivably reside within the cell was detected.

The poorly permeant reagent diazotized sulfanilic acid [21] has been used to selectively inactivate ecto-enzymes [15]; those enzymes exposed on the cell surface should be more susceptible than intracellular enzymes to diazotized sulfanilic acid present in the medium. As can be seen in Fig. 1 (left), intact cell $Mg^{2+}$-ATPase is irreversibly inhibited by this reagent more readily than lactate dehydrogenase. With sonicated cells (Fig. 1, right) the two enzymes are equally susceptible to diazotized sulfanilic acid. Thus, the greater sensitivity of $Mg^{2+}$-ATPase in the intact cell system is consistent with the existence of a permeability barrier (most likely the plasma membrane) between this enzyme and cytoplasmic lactate dehydrogenase.

$Mg^{2+}$-ATPase can be reversibly inhibited by the poorly penetrating reagent suramin [22]. While suramin can eventually penetrate cells [23], it is most likely impermeant during the time span of these experiments [22]. Intact cell $Mg^{2+}$-ATPase is inhibited 50% by 30 $\mu$M suramin; maximal inhibition (70%) is obtained at 100 $\mu$M. These data further suggest that $Mg^{2+}$-ATPase is an ecto-enzyme.

Additional data consistent with a surface membrane localization of $Mg^{2+}$-ATPase is that the enzyme is sedimentable. Human granulocytes were homogenized by the method of DePierre and Karnovsky [24] and post-nuclear supernatants (containing 80% of the enzyme activity) were prepared. Centrifugation of these supernatants resulted in pellet fractions, wherein most of the $Mg^{2+}$-ATPase was found (62% after 10000 $\times$ $g$ for 30 min and 79% after 100000 $\times$ $g$ for 60 min). These results demonstrate a particulate localization for this

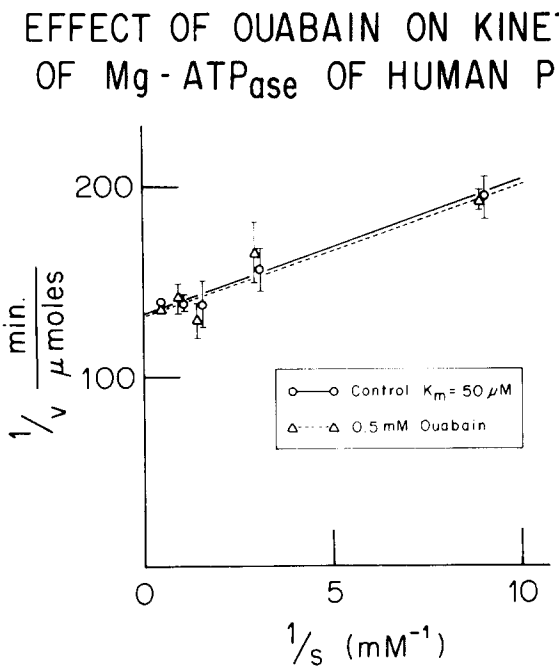


Fig. 2. Purified granulocytes or polymorphonuclear leukocytes in phosphate-buffered saline were sonicated for 30 s and then assayed for $Mg^{2+}$-ATPase in the presence or absence of 0.5 mM ouabain at various substrate concentrations. The Lineweaver-Burk plots shown were calculated using linear regression analysis. Correlation coefficients were 0.953 for "control" (o———o) and 0.889 for "ouabain" (Δ- - - - -Δ).

enzyme and suggest that $Mg^{2+}$-ATPase is membrane associated.

In order to ascertain whether or not observed enzyme activity was due to contaminating cells in granulocyte preparations, lymphocytes and platelets (the most common contaminants) were assayed for $Mg^{2+}$-ATPase. Lymphocytes, isolated by the Hypaque/Ficoll technique [9], proved to have intact cell enzyme activity comparable to that of granulocytes, namely 8 nmol/min per $10^7$ cells. Consequently, all other experiments on granulocytes have used purified preparations (98 ± 1% granulocytes). Platelets, which are a variable contaminant even in purified granulocyte suspensions, had negligible ATPase activity (0.003 nmol/min per $10^7$ cells).

*Properties of human granulocyte $Mg^{2+}$-ATPase.* The $Mg^{2+}$-ATPase of granulocyte homogenates had an apparent $K_m$ of approximately 50 μM for ATP and was not affected by ouabain (Fig. 2). Intact cell $Mg^{2+}$-ATPase had the same $K_m$ and lack of ouabain sensitivity. The whole cell enzyme also displayed substrate inhibition at ATP concentrations of 1 mM and higher (see later), a phenomenon which was rarely seen with sonicated cells (e.g., Fig. 2).

A variety of biological and chemical reagents were tested for their effects on $Mg^{2+}$-ATPase. Most of these materials did not affect enzyme activity at any concentrations tested (Table II). Known inhibitors were *N*-ethyl maleimide, sodium *meta*-periodate, and *R. communis* agglutinin, as well as diazotized sulfanilic acid and suramin. 50% of the $Mg^{2+}$-ATPase activity was inhibited by 0.2 mM *N*-ethyl maleimide. Periodate inhibited up to 70% of the enzyme with

TABLE II

MATERIALS HAVING NO EFFECT ON $Mg^{2+}$-ATPase

The materials below were included in ATPase assay media containing $10^7$ cells at several concentrations within the ranges indicated. These agents were considered to be without effect on $Mg^{2+}$-ATPase as they caused no more than 20% enzyme inhibition or 15% activation.

| | | |
|---|---|---|
| Lectins | Hydrolases | Agents affecting metabolism |
| Concanavalin A (0.1—1 mg/ml) | Trypsin (10—100 μg/ml) | Iodoacetamide (0.2—2 mM) |
| Peanut Agglutinin (40—200 μg/ml) | Papain (10—100 μg/ml) | 2-Deoxyglucose (0.5—5 mM) |
| Soybean Agglutinin (40—200 μg/ml) | Neuraminidase (2—20 munits/ml) | Epinephrine (0.1—1 mM) |
| *Lens culinaris* hemagglutinins | Lipase (0.1—1 mg/ml) | Dibutyryl cyclicAMP (0.1—1 mM) |
| A (20—500 μg/ml) | Phospholipase A (10—100 munits/ml) | Dibutyryl cyclicGMP (5—50 μM) |
| B (40—200 μg/ml) | Phospholipase C (10—100 μg/ml) | |
| ATPase "Inhibitors" | Nucleotide phosphates | Miscellaneous |
| Ethacrynic Acid (0.1—1 mM) | AMP (0.3—1 mM) | Colchicine (5—20 μM) |
| Rutin (30—300 μM) | GMP (0.3—1 mM) | Dimethyl sulfoxide (0.02—2%) |
| Quercetin (30—300 μM) | IDP (0.3—1 mM) | Ethanol (2—5%) |
| Kaempferol (30—300 μg/ml) | CDP (0.3—1 mM) | Heparin (3—50 units/ml) |
| Agents interacting with membranes | CTP (0.3—1 mM) | Immunoglobulin G (30—300 μg/ml) |
| Tetracaine (0.3—1 mM) | UMP (0.3—1 mM) | Aggregated immunoglobulin G (300—300 μg/ml) |
| A23187 (0.1—1 μM) | UTP (0.3—1 mM) | Tuftsin (0.1—1 mg/ml) |
| Melittin (0.1—1 μM) | | *N*-formyl methionyl leucyl phenylalanine (1 pM—100 nM) |

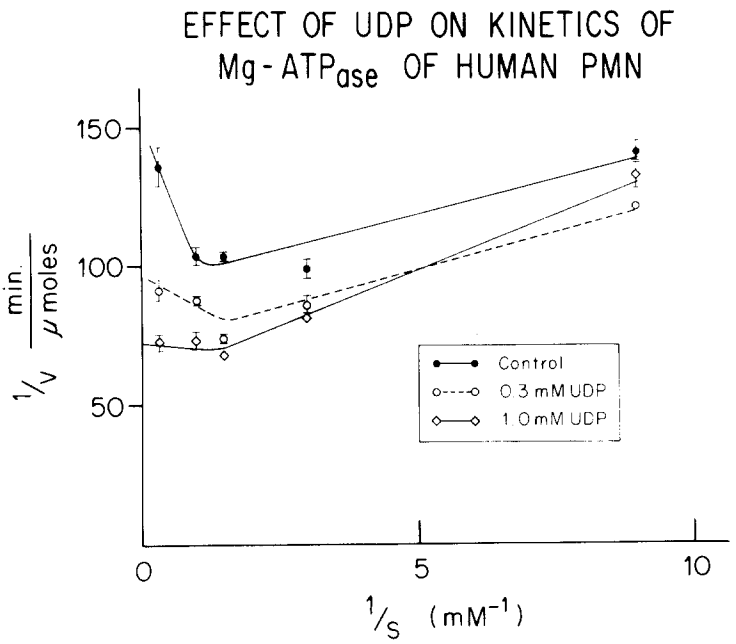


Fig. 3. Intact granulocytes or polymorphonuclear leukocytes ($1 \cdot 10^7$) were assayed for $Mg^{2+}$-ATPase at various substrate concentrations in the presence of freshly prepared 1 mM UDP (◇———◇), 0.3 mM UDP (○- - - - -○), or phosphate-buffered saline alone ("control", ●———●). The lines shown were calculated by linear regression analysis using the points in the "linear" parts of the curve (the 3 mM ATP data for control and 0.3 mM UDP-treated cells were omitted from these calculations). Correlation coefficients are 0.929 (control), 0.933 (0.3 mM UDP), and 0.973 (1 mM UDP). Extrapolated values for $K_m$ (μM) are 50 (control), 70 (0.3 mM UDP), and 120 (1 mM UDP) and for $V$ (nmol/min) are 10.6 (control), 13.6 (0.3 mM UDP), and 15.7 (1 mM UDP).

50% inhibition at 0.2 mM. While this result suggested that $Mg^{2+}$-ATPase might be a glycoprotein, this notion was not supported by the inability of a wide variety of lectins to modify enzyme activity (Table II). The only lectin having any effect on the enzyme was *R. communis* agglutinin, which could inhibit up to 40% of its activity (using 20—500 μg/ml). However, inhibition by this lectin was most likely an artifact resulting from agglutination of cells during the assay procedure.

Cytochalasins B and D (5 μg/ml) each activated $Mg^{2+}$-ATPase up to 40%. UDP also increased enzyme activity; the effect of this agent on the kinetics of $Mg^{2+}$-ATPase is shown in Fig. 3. The control sample displayed the prominent substrate inhibition often seen with intact cells; an apparent $K_m$ of 50 μM was calculated by extrapolation from the linear portion of the curve. As UDP concentration increased from 0.3 to 1 mM, so did the extrapolated $K_m$ (from 70 to 120 μM) and $V$ (up to 48% above control levels). In addition, UDP tended to abolish the pronounced substrate inhibition observed at high ATP concentrations.

*Effect of phagocytic stimuli on $Mg^{2+}$-ATPase.* As an ecto-enzyme, $Mg^{2+}$-ATPase might be interiorized along with plasma membrane during the formation of phagocytic vesicles. The enzyme might also function in such processes as the recognition or adhesion of particles. These phenomena were tested by allowing cell-particle interactions to take place during the enzyme assay (Table

TABLE III

EFFECT OF PHAGOCYTIC STIMULI ON $Mg^{2+}$-ATPase

Cell samples containing $10^7$ granulocytes were preincubated with 5 μg/ml cytochalasin B ("+cytochalasin B") or phosphate-buffered saline alone ("—cytochalasin") for 10 min at 37°C. The indicated phagocytic stimulus was added and the cells were incubated for 5 additional min after which the enzyme assay was initiated by the addition of concentrated substrate stocks. Enzyme activities (average of two experiments) are expressed as percentages of those activities determined for control cells which were incubated with buffer (13 ± 2 nmol/mg per min) or cytochalasin B (18 ± 2 nmol/mg per min) alone.

| Stimulus | % Enzyme activity | |
|---|---|---|
| | — Cytochalasin B | + Cytochalasin B |
| Polystyrene Latex [a] | 93 | 99 |
| Zymosan [b] | 98 | 90 |
| Zymosan (opsonized) [b] | 70 | 60 |
| Immune complex (bovine serum albumin · anti-bovine serum albumin) [c] | 81 | 79 |
| Paraffin oil emulsion I [d] | 101 | 92 |
| Paraffin oil emulsion I (serum-treated) [d] | 110 | — |
| Paraffin oil emulsion II [e] | 104 | 104 |
| Paraffin oil emulsion II (opsonized) [e] | 90 | 91 |
| Paraffin oil emulsion II (treated with heat-inactivated serum) [e] | 107 | 103 |

[a] Dialyzed latex beads (1.1 μm diameter, Dow Chemical) were added to a final 100 : 1 particle : cell ratio.
[b] 2 mg zymosan, untreated or opsonized (ICN Pharmaceuticals).
[c] Immune complex (80 μg) prepared according to Ward and Zvaifler [25].
[d] 50 μl emulsion prepared according to Stossel et al. [14], using bovine serum albumin as an emulsifier.
[e] 50 μl emulsion prepared according to Materials and Methods using lipopolysaccharide as an emulsifier.

III). The most pronounced effect was a 30% decrease in enzyme activity resulting from exposure to opsonized zymosan. This decrease, however, was also observed in the presence of cytochalasin B, which inhibited phagocytosis. Cells preincubated with cytochalasin D (which blocked phagocytosis of paraffin oil emulsion more effectively than cytochalasin B; see below) showed only a 7% decrease in enzyme activity following exposure to opsonized zymosan. Slight decreases in activity were observed with paraffin oil emulsion II and immune complexes, but not with paraffin oil emulsion I.

The effect of phagocytosis on $Mg^{2+}$-ATPase was investigated more directly as follows: cells were first exposed to paraffin oil emulsion (lipopolysaccharide coated) for various lengths of time, washed free of uningested particles, and then assayed for residual surface enzyme activity. The effects of surface adhesion of emulsion were thus minimized by washing and by use of a control population which was briefly exposed to particles at 37°C (see Materials and Methods). The apparent $K_m$ of the enzyme was not affected by particle ingestion (data not shown). As illustrated in Fig. 4 (left), ingestion of paraffin oil emulsion was accompanied by a slight increase in beta-glucuronidase secretion, but not by release of cytoplasmic lactate dehydrogenase. Furthermore, the amount of cell surface $Mg^{2+}$-ATPase decreased only slightly, and that decrease did not correlate with continuing particle ingestion. Similar observations were made with cytochalasin D-treated cells, which cannot readily ingest particles (Fig. 4, right). Lactate dehydrogenase release and $Mg^{2+}$-ATPase activity did not change. Beta-glucuronidase release was greater and far more rapid in cytochalasin D-treated cells; enzyme secretion was virtually complete following a brief exposure to paraffin oil emulsion. Cytochalasin B did not inhibit particle

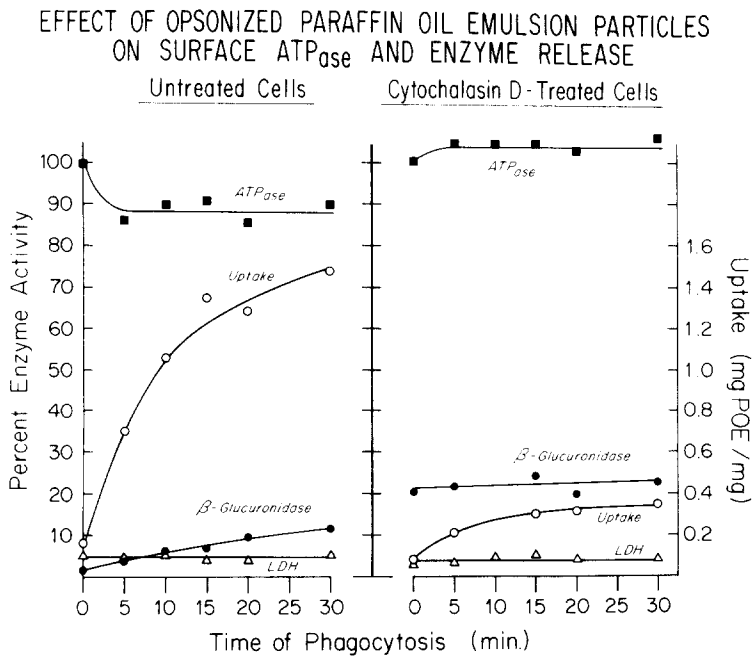


**Fig. 4. Washed cells ($5 \cdot 10^7$/ml) were preincubated with (right) and without (left) 5 μg/ml cytochalasin D for 10 min at 37° C, after which phagocytosis was initiated by the addition of paraffin oil emulsion. Incubation proceeded for the indicated periods of time in the manner outlined in Materials and Methods. The amounts of extracellular lactate dehydrogenase (LDH) or beta-glucuronidase are expressed as percentages of the total cellular content. Phagocytic uptake is expressed as mg paraffin oil (POE) ingested/mg cell protein. ATPase specific activities (based on cell protein) were determined and are reported as percentages of those measured for control cells.**

uptake as efficiently as did cytochalasin D; for cytochalasin B-treated cells, the extent of phagocytosis also did not correlate with lactate dehydrogenase release and $Mg^{2+}$-ATPase activity (data not shown).

Table IV shows the effects of 20 min exposure to different types of paraffin oil emulsion particles on $Mg^{2+}$-ATPase activity. None of the three particle types resulted in a significant diminution of residual cell surface ATPase; this lack of response persisted despite substantial particle ingestion (opsonized and untreated emulsions) and, presumably, membrane internalization. Colchicine, which did not inhibit phagocytosis, also did not alter this behavior. Finally, cytochalasin B, which was used at a concentration sufficient to inhibit some microfilament functions, only slightly inhibited particle uptake; no significant loss of surface $Mg^{2+}$-ATPase was seen in the presence of this drug.

*Effect of suramin on $Mg^{2+}$-ATPase and other granulocyte functions.* To obtain information about the physiological role of $Mg^{2+}$-ATPase, the effect was tested of the relatively specific inhibitor, suramin, on granulocyte function. Since suramin is also an inhibitor of transport ATPases [22] and other enzymes, its inhibitory effects cannot, therefore, serve as unambiguous indications of $Mg^{2+}$-ATPase involvement. Nevertheless, under conditions when $Mg^{2+}$-ATPase activity was inhibited by 65% (intact cells exposed to 100 μM suramin) the

TABLE IV

EFFECT OF PHAGOCYTOSIS ON $Mg^{2+}$-ATPase

Aliquots of cells were preincubated for 10 min at 37°C with 10 μM colchichine, 5 μg/ml cytochalasin B, or phosphate-buffered saline alone ("None"). These cells were then incubated with the specified particle for 20 min at 37°C and finally washed free of uningested paraffin oil emulsion. $Mg^{2+}$-ATPase is expressed as a percentage of that activity determined for control cells (briefly exposed to emulsion) and uptake is expressed as mg cell-associated paraffin oil/mg cell protein (from which that value obtained for control cells has been subtracted). Control $Mg^{2+}$-ATPase values (nmol/mg per min) were 12.1 ± 1.8 (no pretreatment), 12.5 ± 2.2 (colchicine), and 14.0 ± 1.8 (cytochalasin B).

| Particle type | Pretreatment | | |
|---|---|---|---|
| | None | Colchicine | Cytochalasin B |
| Paraffin oil emulsion (opsonized with fresh serum) | | | |
| ATPase | 101 ± 13 | 96 ± 12 | 86 ± 14 |
| Uptake | 1.44 ± 0.19 | 1.53 ± 0.09 | 1.16 ± 0.33 |
| *n* | 10 | 8 | 8 |
| Paraffin oil emulsion (treated with heat-inactivated serum) | | | |
| ATPase | 115 ± 8 | 99 ± 8 | 96 ± 7 |
| Uptake | 0.13 ± 0.08 | 0.12 ± 0.04 | 0.09 ± 0.06 |
| *n* | 8 | 8 | 6 |
| Paraffin oil emulsion (no serum) | | | |
| ATPase | 100 ± 7 | 105 ± 21 | 100 ± 6 |
| Uptake | 0.81 ± 0.39 | 0.64 ± 0.23 | 0.66 ± 0.25 |
| *n* | 8 | 7 | 8 |

cells' capacity to react to a chemotactic stimulus, to generate superoxide anion, and to phagocytize, were not significantly impaired: phagocytosis of opsonized paraffin oil emulsion was 86 ± 10% of control (1.4 ± 0.2 mg oil/mg cell protein per 20 min; $n = 4$), superoxide generation by cytochalasin B-treated cells in response to opsonized zymosan was 84 ± 16% of control (23 ± nmol cytochrome *c* reduced/15 min per $10^6$ cells; $n = 5$), and chemotaxis in response to zymosan-activated serum was 102 ± 1% of control (126 ± 1 μm/45 min; $n = 2$).

## Discussion

5′-Nucleotidase, an enzyme usually considered to be associated with plasma membranes, has been convincingly shown to be a surface membrane marker for guinea-pig [26] and rabbit granulocytes [1]. Human granulocytes apparently lack this enzyme [27,28], but the work reported here and in ref. 2 strongly suggest that $Mg^{2+}$-ATPase is a suitable marker ecto-enzyme for these cells. The evidence, which is based upon the biochemical criteria first used by DePierre and Karnovsky [15,26], is summarized as follows: (1) Additional enzyme activity cannot be detected within the cells; (2) inorganic phosphate released by hydrolysis of ATP is located extracellularly; (3) $Mg^{2+}$-ATPase is especially susceptible to inhibition by diazotized sulfanilic acid; (4) the enzyme can be inhibited by the poorly permeant reagent suramin; (5) the enzyme activity of homogenates is associated with particulate fractions.

$Mg^{2+}$-ATPase is also associated with the plasma membranes of rat [29] and

guinea pig granulocytes [26]. The guinea pig $Mg^{2+}$-ATPase, while not localized exclusively on the plasma membrane, is otherwise similar to the human enzyme; it is ouabain-insensitive and has an apparent $K_m$ of 30 $\mu$M. A ouabain-inhibitable ATPase has been reported for human granulocytes [30]. These researchers found a ouabain-sensitive ATPase activity of approximately 1 nmol/min per mg protein in homogenates of normal cells, an enzymatic component which would not be detected in our system against a background of 14 nmol/min per mg protein. Values for apparent $K_m$ of 600 $\mu$M [2] and 330 $\mu$M [3] have been reported for the human $Mg^{2+}$-ATPase. The reason our value is considerably lower than these is not clear. One difference which might explain this discrepancy is that our assay medium was based upon phosphate-buffered saline (instead of 100 mM Tris · HCl, as in ref. 2) in order to maintain intact cell viability, and routinely contained *p*-nitrophenyl phosphate to inhibit nonspecific phosphatases. All other properties of the enzyme which we have examined are in accordance with those reported by Harlan et al. [2]. Human granulocyte $Mg^{2+}$-ATPase does resemble myosin $Mg^{2+}$-ATPase in some respects and the latter is, in fact, an occasional component of surface membranes [31]. However, the relationship (if any) between these two enzymes is currently unknown.

As a surface membrane component, $Mg^{2+}$-ATPase is a likely constituent of phagosomes following particle ingestion. Such internalization of $Mg^{2+}$-ATPase along with plasma membrane would be detected as a loss of enzyme activity from intact cell surfaces. The data in Fig. 4 and Table IV, however, show no correlation between phagocytic uptake and enzyme activity. It is unlikely that those $Mg^{2+}$-ATPase molecules internalized during phagocytosis were quantitatively replaced upon the cell surface, as no intracellular pool of enzyme can be detected (the possibility of an inactive precursor form of $Mg^{2+}$-ATPase being activated cannot, however, be excluded. The data, therefore, suggest that $Mg^{2+}$-ATPase is preserved on the cell surface despite substantial internalization of plasma membrane.

Previous investigators have shown that microtubules or other colchicine-sensitive mechanisms were responsible for such directed movement of membrane components [1,5,32]. The exclusion of $Mg^{2+}$-ATPase from forming phagosomes was not affected by the presence of 10 $\mu$M colchicine nor by pretreatment with 5 $\mu$g/ml cytochalasin B (Table IV). The simplest interpretation of these results is that the exclusion process is not dependent upon unimpaired microtubule and microfilament function. However, this conclusion must be tempered with the knowledge that these drugs have specific and non-specific effects other than the disruption of cytoskeletal structures and that concentrations of reagents sufficient to interfere with some processes might not be sufficient for others.

The above conclusions apply strictly to lipopolysaccharide-coated paraffin oil emulsion, but might also be relevant to albumin-coated paraffin oil emulsion, polystyrene latex, and zymosan particles, all of which produce little or no decrease in $Mg^{2+}$-ATPase activity during the enzyme assay (Table III). The slight decrease seen in the presence of immune complex and the far greater decrease observed in the presence of opsonized zymosan suggested that internalization of $Mg^{2+}$-ATPase was taking place. Such an interpretation suggests

that phagocytosis of opsonized zymosan and opsonized paraffin oil emulsion are substantially different processes, although both particles ostensibly present the same ligand (C3b) to cell surface receptors. However, the ingestion of zymosan, unlike that of paraffin oil emulsion, cannot be accurately measured. Finally, it should be emphasized that for all of the data in Table III, phagocytic stimuli were presented to the cells during the ATPase assay; we do not know what effects (if any) ATP and *p*-nitrophenyl phosphate have on the various endocytic processes.

The biochemical role of $Mg^{2+}$-ATPase is not known and the pharmacological studies reported in this work provided little additional information. However, the fact that $Mg^{2+}$-ATPase exhibits substrate inhibition and that this inhibition can be relieved by UDP suggests that the enzyme is capable of allosteric regulation. The role of UDP, which also alters the $K_m$ and $V$ of $Mg^{2+}$-ATPase, in regulating the enzyme is unclear, but the activation phenomenon is quite striking.

The experiments using suramin as a "specific" inhibitor of $Mg^{2+}$-ATPase have been somewhat more informative. It is not safe to conclude that an observed effect of suramin upon some phenomenon is a direct result of the drug's action on $Mg^{2+}$-ATPase, since other ATPases of low specific activity might also be affected. However, the lack of an effect of suramin on a cellular function constitutes strong evidence against the direct involvement of $Mg^{2+}$-ATPase. In particular, since 100 $\mu$M suramin did not substantially affect superoxide generation, chemotaxis or phagocytosis by human granulocytes, it is unlikely that $Mg^{2+}$-ATPase is directly involved in these processes. Although it is conceivable that the $Mg^{2+}$-ATPase activity remaining after suramin treatment (35%) is sufficient for these functions, such a model is unlikely for functions directly linked to $Mg^{2+}$-ATPase. Thus, we find no evidence to support the circumstantial correlation between $Mg^{2+}$-ATPase and phagocytosis previously reported [3], a result perhaps due to non-specific action of the drugs employed in that study.

## Acknowledgments

The authors wish to thank Dr. H. Daniel Perez for performing the chemotaxis experiments. This work was supported by a grant AM-07176 from the National Institutes of Health and also aided by grant (AM-11949, GM-23211, HL-19072, HL-19721) from the National Institutes of Health, the National Foundation-March of Dimes, the National Science Foundation (76-05621), the Whitehall Foundation and the New York Heart Association.

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