QUANTITATIVE STUDIES OF PHAGOCYTOSIS

Kinetic Effects of Cations and Heat-Labile Opsonin

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ABSTRACT

Kinetic analysis of the initial ingestion rate of albumin-coated paraffin oil particles by human granulocytes and rabbit alveolar macrophages was undertaken to study the mechanism of action of cations and of heat-labile opsonin on engulfment. The rate of uptake of the particles was stimulated by Ca++, Mg++, Mn++, or Co++. At high concentrations (> 20 mM) Ca++ and Mg++ inhibited the rate of ingestion. Treatment of the particles with fresh serum (heat-labile opsonin) also stimulated the rate of ingestion. 125I-labeled C3 was bound to the particles during opsonization. C3-deficient human serum lacked opsonic activity, which was restored by addition of purified C3. Normal, C2-deficient, and hereditary angioneurotic edema sera had equivalent opsonic activity. The serum opsonic activity thus involved C3 fixation to the particles by means of the properdin system. Although Mg++ and heat-labile opsonin both accelerated the maximal rates of ingestion of the particles, neither altered the particle concentrations associated with one-half maximal ingestion rates. Opsonization of the particles markedly diminished the concentrations of divalent cations causing both stimulatory and inhibitory effects on ingestion rates and altered the shapes of the cation activation curves. 43Ca was not bound to the particles during opsonization. The results are consistent with a mechanism whereby divalent cations and heat-labile opsonin activate ingestion by stimulating the work of engulfment rather than by merely enhancing cell-particle affinity, and whereby heat-labile opsonin acts by potentiating the effects of divalent cations.

Early in this century it was appreciated that fresh serum (1) and divalent cations (2) enhanced the ingestion of particles by mammalian phagocytes. Observations on the morphology of ingestion revealed that the phagocytes spread over the surfaces of particles, maintaining intimate contact with them during engulfment (3). Explanation for the action of opsonins and of divalent cations on this spreading process was sought in their effects on particle surface wettability and charge, and alterations in particle surfaces that facilitated spreading and deformability by the phagocytes were predicted to accelerate phagocytosis (4–7). These early theories considered the phagocytes as relatively passive subjects of physical forces. More recent work has shown that phagocytosis requires energy expenditure by the cell (8), and that contractile activity, dependent upon hydrolysis of ATP, may regulate mobility of single cells in general (9). Therefore, it is possible that particles may elicit spreading by acting on a contractile mechanism, and that opsonins and divalent cations stimulate this process.

Ingestion must be studied with intact cells, thus complicating characterization of its basis. Kinetic studies of particle uptake represent one approach to this problem. This report presents kinetic characteristics concerning the interactions of particles, cations, and heat-labile serum opsonin on ingestion by two types of phagocytes from two
species. Heat-labile opsonin appears to accelerate the rate of ingestion by potentiating the effects of divalent cations.

MATERIALS AND METHODS

Preparation of Phagocytic Cells

Human peripheral blood was collected in acid citrate dextrose, and erythrocytes were removed by sedimentation in 3% dextran and lysis with 0.87% ammonium chloride. Leukocyte* suspensions containing 60–95% neutrophilic polymorphonuclear leukocytes, band forms, and 3–10% monocytes were obtained. Suspensions containing over 90% alveolar macrophages were collected from rabbits by tracheal lavage with 0.15 M NaCl (10). To increase cell yields, the rabbits received Freund's adjuvant intravenously 4 wk before sacrifice (11). Cells were washed twice with 0.15 M NaCl at 4°C and suspended in appropriate incubation media. Cellular protein was measured by the Folin method (12), and cell counts were determined with an electronic counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla). Polymorphonuclear leukocytes, band forms, and monocytes enumerated on Wright's-stained smears were counted as phagocytes.

Medium

For experiments investigating the effect of serum concentration on opsonization or the effect of particle concentrations on the rate of uptake of unopsonized and opsonized particles, the cells were suspended in Krebs-Ringer phosphate medium, pH 7.4, containing one-third the recommended amount of calcium. Choline was used to replace sodium, and sodium to replace potassium when effects of monovalent cations were analyzed. Tris-HCl buffer was substituted for phosphate buffer for most experiments in which effects of divalent cations were examined. These buffers could, however, be interchanged without alteration in the rates of ingestion, although phosphate buffer was not tested when very high concentrations of divalent cations were employed. All solutions were prepared with deionized water.

Preparation of Paraffin Oil Particles

Particles of paraffin oil stabilized with albumin and containing oil red 0 were prepared as previously described (13). 1 ml of heavy paraffin oil (Fischer Scientific Co., Pittsburgh, Pa.) containing oil red 0, 30 mg/ml, (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) was sonicated with 15 mg of human albumin (Worthington Biochemical Corp., Freehold, N. J.) in 5 ml of 0.15 M NaCl.

Sera and Serum Proteins

Serum was obtained from normal humans and rabbits. Serum from patients genetically deficient in C2 (14) or C3 (15), from patients with hereditary angioneurotic edema during acute attacks and purified human C3 (16) were gifts from Dr. Chester A. Alper and Dr. Fred S. Rosen (Immunology Division, Children's Hospital Medical Center, Boston, Mass.). The sera were used immediately or else stored at −70°C. Serum was heated or incubated with zymosan as previously described (17).

Opsonization

The particles were suspended in an equal volume of fresh serum or else in fresh serum diluted with Krebs-Ringer phosphate medium containing human serum albumin, 5 mg/ml. The particles and serum were incubated for 15 min at 37°C. Particles suspended with albumin solution not containing fresh serum constituted unopsonized particles. In most experiments the particles and serum were added directly to cells after opsonization. In some cases, the particles were washed twice with 10 vol of albumin solution (centrifugation at 40,000 g for 15 min), the pellicle finally being restored to its original volume with albumin solution. Divalent cations which were present during opsonization were removed in certain experiments before adding the opsonized particles to the cells by dialyzing the particles against 0.15 M NaCl containing 1 mM EDTA, pH 7.0, and subsequently against 0.15 M NaCl containing 15 mM Tris-HCl, pH 7.4. These reagents were prepared with deionized water.

Assay of Ingestion

Cells were suspended in 4 ml of medium containing various ions or ouabain (S. B. Penick and Co., Division of CPC International Inc., New York) and incubated in siliconized glass flasks or plastic vials in a shaking water bath at 37°C for 10 min. Cell protein concentrations of rabbit macrophages were 1–4 mg/ml, and human leukocyte suspensions contained 0.4–1 × 10⁹ phagocytes/ml. Particles (1 ml) prewarmed to 37°C were added (zero time), and samples (0.5 ml) of cells plus medium were removed at this time and at 3, 5, and 7 min thereafter to determine the initial rate of ingestion. These samples were added to 6 ml of ice-cold 0.15 M NaCl containing 0.1 M NaN₃ and 0.2 M N-ethylmaleimide. Uningested particles were removed from the cells by differential
centrifugation. Ingested paraffin oil was extracted from the washed cell pellets with dioxane. Oil red 0 was spectrophotometrically quantified at 524 nm as described previously (13). In the presence of an excess (saturating) quantity of particles (0.2 ml/ml of cells), the rate of ingestion of unopsonized albumin particles by rabbit macrophages and human leukocytes was constant for 30 min. The rate of engulfment of opsonized albumin particles by human leukocytes was constant for 5 min, and by rabbit macrophages for 10 min. Unless otherwise specified, all incubation flasks contained a saturating concentration of particles (25 mg of paraffin oil/ml), such that the initial ingestion rate was independent of particle concentration (13).

Binding of Calcium to Albumin Particles

Albumin-paraffin oil particles were dialyzed against 0.1 M EDTA, pH 7.0, and subsequently against 0.15 M NaCl. Fresh serum or serum heated at 56°C for 30 min was dialyzed against 0.13 M NaCl, 15 mM sodium phosphate buffer, pH 7.4, and 5 mM EDTA for 2 h at 4°C and then against the same solution minus EDTA. To 0.5 ml of dialyzed particles was added 0.5 ml of dialyzed fresh or heated serum, and the mixtures were incubated for 15 min at 37°C in the presence of 1 mM MgCl₂, 1 mM CaCl₂, and 4CaCl₂, 10⁶ dpm (1.84 Ci/mmol, Amersham/Searle Corp., Arlington Heights, Ill.). The particles were then dialyzed against 2 liters of 0.1 M EDTA and later against 1 liter of 0.15 M NaCl. Samples of the dialyzed particles were tested for ingestibility by human leukocytes. Samples of 0.1 ml of the particles were digested overnight with 2 ml of Protocol (New England Nuclear Corp., Boston, Mass.), neutralized with 0.5 ml of glacial acetic acid, and then added to 15 ml of Aquasol (New England Nuclear Corp.) containing 1 mM EDTA. Radioactivity in the samples was measured with a Packard scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Estimation of Particle Size by Settling Rates

According to Stokes' law, the sedimentation rate of emulsion particles is a function of their diameters. The particles were then centrifuged and the rate of formazan generation was computed as previously described (21). After 5 min of incubation at 37°C, 10 ml of ice-cold 0.15 M NaCl, 1 mM N-ethylmaleimide was added to the tubes which were then centrifuged at 1000 g for 10 min. The supernatant fluids were decanted, and formazan was extracted from the cell pellets with 4 ml of p-dioxane by heating at 85°C for 20 min. The optical density of the solutions was measured at 580 nm after clarification by centrifugation, and the rate of formazan generation was computed as previously described (21).

Assay of Ingestion by Measurement of Nitro Blue Tetrazolium Reduction

During ingestion, phagocytes reduce nitro blue tetrazolium to nitro blue tetrazolium formazan in the phagocytic vacuole (19), and the rate of reduction can be used as an indirect measurement of ingestion (20). The initial rate of nitro blue tetrazolium reduction was employed to determine the rate of uptake of fluorochemical liquid particles, because these particles cannot be separated from leukocytes by differential centrifugation. The particles, 0.2 ml, were added to 15-ml siliconized glass centrifuge tubes containing 0.8 ml of cells suspended in medium with nitro blue tetrazolium, 0.20 mg/ml, prepared and standardized as previously described (21). After 5 min of incubation at 37°C, 10 ml of ice-cold 0.15 M NaCl, 1 mM N-ethylmaleimide was added to the tubes which were then centrifuged at 1000 g for 10 min. The supernatant fluids were decanted, and formazan was extracted from the cell pellets with 4 ml of p-dioxane by heating at 85°C for 20 min. The optical density of the solutions was measured at 580 nm after clarification by centrifugation, and the rate of formazan generation was computed as previously described (21).

Fixation of[125I]C3 to Albumin Particles

Purified C3 was labeled with 125I (22). Purified C3, specific activity 6 X 10⁵ cpm/µg, was added to fresh human serum or heat-inactivated human serum (final radioactivity 5 X 10⁵ cpm/ml). Serum, 0.2 ml, containing the labeled C3 was added to 0.2 ml of albumin particles prepared with fluorochemical liquid to facilitate washing. The sera and particles were incubated at 37°C for 20 min. The particles were then washed five times with 10 ml of human serum albumin solution, 20 mg/ml in 0.15 M NaCl, pH 7.0. Radioactivity associated with the washed albumin-fluorochemical particles was measured with a Packard Auto-Gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Effects of Heat-Labile Opsonin: Human Leukocytes

The effect of fresh serum on the initial rate of ingestion of albumin particles by human leukocytes in the presence of 1 mM MgCl₂ and saturat-
ing particle concentrations is shown in Fig. 1. The leukocytes ingested unopsonized particles, but the ingestion rate was increased when the particles were preincubated with fresh human serum. A 1:1 ratio (vol/vol) of serum to particles had a maximal activating effect, and this proportion was utilized in subsequent experiments. Particles treated with fresh serum remained opsonized even after excess serum was removed by washing. Wright-stained smears of samples taken from the incubations revealed that band forms, granulocytes, and monocytes ingested unopsonized and opsonized particles. Engulfment was easily assessed by this technique, since ingested particles appeared as clear vacuoles in the cells, but precise quantitative assessment of the relative contribution of these cells could not be achieved. Band forms or monocytes did not constitute more than 10% of the total phagocytes. Table I shows that the opsonic effect of human serum was abolished by heat or zymosan. It was equivalent in normal, C2-deficient, and hereditary angioneurotic edema serum containing no detectable C2 or C4 (23).

However, serum from a patient with hereditary C3 deficiency (15) had no opsonic activity unless the serum was fortified with C3. As shown in Table II, [125I]C3 was fixed to albumin particles incubated with fresh serum but not with heated serum. In the absence of divalent cations, fresh serum did not opsonize albumin particles (Table III). Magnesium alone rendered the serum opsonically active, calcium alone did so to a lesser degree, and both ions together restored maximal opsonic activity to the serum.

Fig. 2 shows the effect of particle concentration on the initial ingestion rate of unopsonized albumin particles and of albumin particles treated with fresh serum. Although the opsonized particles

|TABLE I| Opsonic Activity of Normal, Abnormal, and Modified Human Serum for Albumin Particles
|-----------------|-----------------|-----------------|
|Serum            | Human leukocytes| Rabbit alveolar macrophages|
|                 | µg paraffin oil/10^6 phagocytes/min | µg paraffin oil/mg protein/min |
|None             | 16 ± 0.9*       | 28 ± 1.9        |
|Normal           | 42 ± 0.8        | 54 ± 2.2        |
|C2-deficient     | 41 ± 2.1        | 53 ± 3.5        |
|Hereditary angioneurotic edema | 39 ± 0.6 | 56 ± 2.9 |
|Heated (56°C, 30 min) | 16 ± 1.1 | 25 ± 2.3 |
|Zymosan-treated  | 14 ± 0.7        | 22 ± 2.4        |
|C3-deficient     | 16 ± 0.5        | 28 ± 1.7        |
|C3-deficient + C3| 35 ± 1.4        | —               |

* Mean ± SD for samples from three separate incubation flasks.

|TABLE II| Correlation of Opsonization and Binding of [125I]C3 to Albumin Particles
|-----------------|-----------------|-----------------|
|Particles incubated with: | Rate of uptake of the particles by human leukocytes | [125I]C3 bound to the particles |
|                 | µg formazan/10^6 phagocytes/min | % |
|Heated human serum | 0.25 ± 0.15* | 2 ± 0.5 |
|Fresh human serum  | 0.92 ± 0.11    | 15 ± 1 |

* Mean ± SD of triplicate assays.

Effects of Heat-Labile Opsonin: Alveolar Macrophages

Alveolar macrophages from rabbits also ingested unopsonized albumin particles and en-
TABLE III

Effect of Divalent Cations on the Opsonic Activity of Fresh Human Serum for Albumin Particles

| Serum                                  | Initial rate of ingestion by human leukocytes (µg paraffin oil/10⁷ phagocytes/min) |
|----------------------------------------|-----------------------------------------------------------------------------------|
| Dialyzed                               | 24                                                                                |
| + 1 mM MgCl₂                           | 48                                                                                |
| + 1 mM CaCl₂                           | 34                                                                                |
| + 1 mM CaCl₂ + 1 mM MgCl₂              | 58                                                                                |
| Undialyzed                             | 60                                                                                |
| Heated                                 | 21                                                                                |

Particles were opsonized with the indicated sera and then washed with albumin solution before being added to the cells as described in the text.

The rate of engulfment of either opsonized or unopsonized albumin particles by human leukocytes or alveolar macrophages was unaffected by the presence or absence of K⁺, by 10⁻⁴ M ouabain, or by substitution of choline⁺ for Na⁺.

**Effects of Monovalent Cations and Ouabain**

The rate of engulfment of either opsonized or unopsonized albumin particles by human leukocytes or alveolar macrophages was unaffected by the presence or absence of K⁺, by 10⁻⁴ M ouabain, or by substitution of choline⁺ for Na⁺.

**Effects of Ca²⁺ and Mg²⁺: Human Leukocytes**

Human leukocytes required divalent cations in the extracellular medium in order to ingest either unopsonized or opsonized albumin particles (Table II). Magnesium was more effective than calcium (Table IV). Magnesium increased the

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TABLE IV  
Effect of Divalent Cations on the Initial Rate of Ingestion of Unopsonized and of Opsonized Albumin Particles

| Cell type              | Albumin particles | No additions | 1.5 mM CaCl₂ | 1.5 mM MgCl₂ | 1 mM EDTA |
|-----------------------|-------------------|--------------|--------------|--------------|----------|
| Human leukocytes      | Unopsonized       | 0            | 5 ± 0.2      | 13 ± 1.0     | 0        |
|                       | Opsonized         | 0            | 45 ± 2.3     | 53 ± 0.9     | 0        |
| Rabbit alveolar macrophages | Unopsonized | 0            | 7 ± 0.5      | 12 ± 0.5     | 0        |
|                       | Opsonized         | 25 ± 1.4     | 51 ± 3.1     | 74 ± 2.6     | 14 ± 1.1 |

The cells were washed twice with 0.15 M NaCl and suspended in 0.13 NaCl containing 15 mM Tris-HCl, pH 7.4, and the indicated additions. The analysis of ingestion was performed as described in Materials and Methods. The values given are means ± SD of triplicate incubations.

**Figure 5** Effect of particle concentration on the initial rate of ingestion of unopsonized albumin particles by human leukocytes suspended in varying Mg²⁺ concentrations. The inset shows the same data expressed as a double-reciprocal plot.

$V_{max}$ of the engulfment rate of unopsonized albumin particles but did not alter the apparent $K_m$ (Fig. 5). Mg²⁺ also accelerated the $V_{max}$ of the ingestion of opsonized albumin particles without altering the apparent $K_m$. The effects of Mg²⁺ and Ca²⁺ on the ingestion rate in the presence of saturating particle concentrations differed depending on whether the albumin particles were opsonized or not. First, the concentrations of magnesium or calcium associated with the greatest ingestion rates were significantly lower for opsonized than for unopsonized albumin particles (Fig. 6). Secondly, concentrations of calcium or magnesium over 1 mM had small but reproducible inhibitory effects with opsonized but not with unopsonized particles. Thirdly, the shapes of the divalent cation activation curves for unopsonized particles were sigmoidal whereas the curves for opsonized particles were hyperbolic.

**Effects of Ca²⁺ and Mg²⁺: Alveolar Macrophages**

The effects of these divalent cations on the ingestion rate of unopsonized and opsonized albumin particles by alveolar macrophages were generally similar to those observed with human leukocytes. Mg²⁺ increased the $V_{max}$ of unopsonized or opsonized albumin particles without altering the apparent $K_m$. Added Mg²⁺ or Ca²⁺ were required for the ingestion of unopsonized albumin particles by alveolar macrophages, and Mg²⁺ was more effective than Ca²⁺ (Table IV). The most effective Mg²⁺ and Ca²⁺ concentrations were lower for opsonized than for unop-

**Figure 6** Effect of Mg²⁺ or Ca²⁺ concentration on the initial rate of ingestion of a saturating concentration of unopsonized (○) or opsonized (●) albumin particles by human peripheral blood leukocytes.
sonized albumin particles (Fig. 7). The Mg\(^{++}\) and Ca\(^{++}\) activation curves for opsonized particles were again hyperbolic while those for unopsonized particles were sigmoidal (Fig. 7). However, alveolar macrophages differed from human leukocytes in that they ingested opsonized albumin particles to some extent in the absence of added divalent cations and even in the presence of EDTA (Table IV). In addition, Mg\(^{++}\) or Ca\(^{++}\) at concentrations greater than 1 mM inhibited more strikingly the initial rate of ingestion of opsonized albumin particles by the macrophages (Fig. 7). The ingestion rate of unopsonized particles was inhibited only by very high concentrations of Ca\(^{++}\) or Mg\(^{++}\).

Effects of Mn\(^{++}\) and Co\(^{++}\)

Since Mg\(^{++}\) and Ca\(^{++}\) influenced the ingestion rate, the effects of other divalent cations at a concentration of 1 mM were tested, and the results are shown in Table V. The effects of Co\(^{++}\) and Mn\(^{++}\) on human leukocytes and rabbit alveolar macrophages ingesting albumin particles varied depending on whether the particles were opsonized or not. These agents increased the rate of ingestion of unopsonized particles (relative to the effect of Ca\(^{++}\) or Mg\(^{++}\)) but decreased the rate of engulfment of opsonized particles.

Binding of Calcium to Albumin Particles

When albumin particles were incubated with serum in the presence of \( ^{45}\text{Ca} \) and subsequently dialyzed against EDTA and NaCl, essentially no \( ^{45}\text{Ca} \) was associated with the particles, and there was no difference in radioactivity between particles treated with fresh or heated serum. As described above and shown in Table IV, however, the particles incubated with fresh serum and then dialyzed against EDTA and NaCl were ingested by alveolar macrophages in the absence of added divalent cations.

Effect of Heat-Labile Opsonin and Divalent Cations on Particle Size

As shown in Table VI, neither fresh serum nor divalent cations altered the sedimentation rate of albumin-fluorochemical particles, despite the fact that these agents markedly stimulated the rate of ingestion of these particles by human leukocytes, as determined by measuring the rate of nitro blue tetrazolium reduction.

| Table V | Effect of Divalent Cations on the Initial Rate of Ingestion of Unopsonized and Opsonized Albumin Particles |
|---------|--------------------------------------------------------------------------------------------------------|
| Cell type | Albumin particles | CaCl\(_2\) | MgCl\(_2\) | NaCl | CoCl\(_2\) |
| Human leukocytes | Unopsonized | 5 ± 0.1 | 10 ± 0.3 | 19 ± 1.9 | 17 ± 2.1 |
| | Opsonized | 69 ± 4.0 | 98 ± 4.4 | 34 ± 2.3 | 51 ± 2.9 |
| | | \(\mu g\) paraffin oil/10\(^7\) phagocytes/\(\mu l\) |
| Rabbit alveolar macrophages | Unopsonized | 9 ± 0.4 | 10 ± 0.3 | 30 ± 1.6 | 15 ± 1.2 |
| | Opsonized | 63 ± 2.2 | 71 ± 4.1 | 61 ± 2.9 | 47 ± 1.9 |

Human leukocytes or rabbit alveolar macrophages were washed twice with 0.15 M NaCl and suspended in 0.13 M NaCl containing 15 mM Tris-HCl, pH 7.4, and the indicated divalent cations at a final concentration of 1 mM. The values given are means ± SD for triplicate incubations.
TABLE VI

Effect of Fresh Serum and of Divalent Cations on Sedimentation Rates of Fluorochemical Particles and on the Rate of Ingestion of the Particles by Human Leukocytes

| Additions         | Settling time (min) | Initial rate of ingestion (µg formazan/10^7 phagocytes/min) |
|-------------------|---------------------|-------------------------------------------------------------|
| None              | 89 ± 5              | 0                                                           |
| 1 mM CaCl₂        | 87 ± 2              | 0.28 ± 0.06                                                 |
| 1 mM MgCl₂        | 86 ± 2              | 0.72 ± 0.11                                                 |
| Fresh serum       | 86 ± 2              | 0.72 ± 0.11                                                 |

Measurements of sedimentation and of ingestion were performed as described in Materials and Methods. Divalent cations, when present, were added to the settling cuvettes or to the centrifuge tubes containing the cells. Serum was incubated with the particles before adding them to the cuvettes or to cells. The settling times given are the times for the optical densities of the cuvettes to reach one-half of their initial value. Means ± SD of triplicate determinations are given.

DISCUSSION

Use of Albumin-Paraffin Oil Particles to Study Ingestion

Paraffin oil containing oil red 0 when emulsified with a variety of substances provides a substrate for assaysing the initial rate of ingestion by phagocytes from a number of species. Depending on the agent used to coat the oil droplets and on the choice of phagocytic cells, the avidity of ingestion and the response to opsonic factors varies. For example, guinea pig peritoneal exudate granulocytes ingest albumin particles avidly, and treatment of the particles with either fresh serum or antibody to albumin does not accelerate this ingestion rate (13). However, these same cells ingest *Escherichia coli* lipopolysaccharide-coated paraffin oil droplets efficiently only if the particles are first treated with fresh serum (17). Rabbit alveolar macrophages and suspensions of human blood leukocytes consisting primarily of mature granulocytes ingest albumin-coated paraffin oil particles at a reasonably rapid rate. Opsonization of these particles with fresh serum (or with antibody to albumin) stimulates the rate of engulfment (11, 24-26). Since the basal rate of ingestion is rapid enough to be easily measured, and because there is a significant increment in the rate of uptake of serum-treated particles, this particular combination of particles and phagocytes is suitable for examining the kinetics of activation of ingestion by divalent cations and heat-labile opsonin.

The paraffin oil particles are heterodisperse, and the ingestion assay detects the mass of paraffin oil taken up by the cells. Therefore, the dramatic effects of divalent cations and of serum could be the result of marked alterations in average particle size rather than of changes attributable to work by the phagocytes. However, particle sedimentation rates (as well as light microscopy) were employed to monitor average particle size, and no such changes were detectable.

Nature of the Heat-Labile Opsonin for Albumin Particles

Fresh serum opsonizes particles by fixing C3 to their surfaces. This fixation may occur by means of reactions involving antibody and the early components of the classical complement pathway (20, 27-29) or else by reactions involving the incompletely defined properdin (or C3 activator) system which does not require antibody (30, 31, 17). The opsonic activity of serum for albumin particles was destroyed by heat or zymosan. It was absent from C3-deficient serum but could be found in such serum after reconstitution with purified C3. Fixation of C3 to albumin particles in the presence of fresh but not of heated serum was demonstrated by means of radioactive C3. The opsonic effect on the particles was retained after washing, indicating that the opsonin was bound to them. All of these findings implicated a role for complement in the heat-labile opsonic activity of serum for the albumin particles. The observation that the opsonic power of sera missing C4 or C2, components of the classical complement pathway, was normal and the probability that no antibody to albumin existed in the sera used suggested that opsonization of the particles involved C3 fixation by means of the properdin system. The divalent cation requirements for opsonization were similar to those observed for the opsonization of *E. coli* lipopolysaccharide-coated particles, a process shown to depend on the properdin system (17). The manner in which the albumin particles activate the properdin mechanism remains to be determined. It is possible that albumin bound to the paraffin oil droplet surface is de-
Kinetic Approach to the Mechanism of Phagocytosis

Weisman and Korn recognized that the initial rate of ingestion of polystyrene or polystyrene particles by Acanthamoeba could be analyzed in a manner similar to that for rates of enzyme reactions (32). Phagocytes suspended with particles were considered as enzyme and substrate, respectively. Random contact between cells and particles was provided by agitation. The internalization of the particles constituted the catalytic reaction which resulted from cell-particle complex formation. The rate of product formation, i.e. the intracellular appearance of particles, at different substrate concentrations was a function of the effectiveness of this complex. As evidence to support the validity of this approach, these investigators and others (13, 33) found that the initial rate of particle ingestion by phagocytes was constant with time and that initial rates of uptake exhibited first order and saturation kinetics with respect to particle concentration. Weisman and Korn obtained $K_m$ measurements for uptake velocities of particles of varying size. They concluded from these values that particle mass, rather than diameter or number, determined the rate of uptake and subsequently provided direct morphologic evidence for this concept (34).

In this study the kinetic approach has been applied to mammalian phagocytes which can be activated by certain proteins bound to particles and by divalent cations. Magnesium, calcium, and C3 bound to albumin particles stimulated the rate of ingestion of the particles by human leukocytes and rabbit alveolar macrophages. The maximal velocity of uptake was accelerated by these agents without significant alteration of the apparent $K_m$ of the reaction, suggesting that the activation of engulfment involved stimulation of the work of internalization, i.e. pseudopod formation and cellular spreading, and not enhancement of cell-particle affinity. This conclusion concerning affinity is based on kinetic analysis of the overall reaction rates, and the interpretation will require confirmation by direct methods. Some (35–37) but not all (38) investigators have observed binding of C3-coated erythrocytes to various phagocytes. Such attachment could have physiologic importance aside from its effect on engulfment. However, current techniques for analyzing cell-particle binding directly are at best semiquantitative and improved methods will be required to assess the specificity of binding and its relevance to the rates of engulfment.

The interaction of the opsonin and divalent cations may have provided some clues to possible modes of action of these agents. First, heat-labile opsonin decreased the concentration of divalent cations required for a maximal rate of ingestion by human leukocytes. Secondly, the absolute requirement for divalent cations to promote ingestion of opsonized particles by alveolar macrophages was abolished when the cells were incubated with opsonized particles, although the cations did influence the ingestion rate. EDTA and divalent cations at concentrations under 1 mM inhibited and stimulated, respectively, the rate of ingestion. Thirdly, concentrations of divalent cations greater than 1 mM inhibited the rate of ingestion of opsonized particles by both cell types, effects seen only at very high concentrations of the cations when cells were incubated with unopsonized particles. Fourthly, with both human leukocytes and rabbit alveolar macrophages the divalent cation activation curves were sigmoidal for unopsonized particles, suggesting that the activation process was of a cooperative nature. This characteristic was not apparent for the ingestion of opsonized particles by either cell type. Finally, Mn$^{++}$ and Co$^{++}$ had different effects on ingestion, depending on whether unopsonized or opsonized particles were utilized.

These findings are all consistent with the explanation that the heat-labile opsonin facilitates the action of divalent cations at the site or sites which activate engulfment. The inhibitory effect of the divalent cations at higher concentrations, which may or may not have physiologic relevance, is also potentiated by the heat-labile opsonin. This effect is particularly dramatic in the case of alveolar macrophages where the opsonin may even permit utilization of endogenous divalent cation pools which are not chelated by EDTA in the extracellular medium. The fact that ingestion of opsonized particles occurred even in the presence of EDTA makes it unlikely that the serum-treated particles were merely contaminated with divalent cations or that the cations leaked into the medium from the cells.

Both calcium and magnesium must be present in serum in order for optimal opsonization to
occur. However, the failure to detect binding of calcium to the particles as a result of incubation with fresh serum suggests that opsonization with fresh serum does not merely serve to bring particle-bound divalent cations into contact with specific sites on the plasma membrane of the phagocyte. The particle-bound opsonin may function instead to enhance the transfer of divalent cations from on or within the plasma membrane to intracellular sites which activate engulfment. Although the molecular nature of these loci is unknown, contractile proteins with divalent cation-sensitive ATPase activities have been isolated from phagocytic leukocytes and are possible candidates (39–40).

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