THE REQUIREMENT OF SERINE ESTERASE FUNCTION IN COMPLEMENT-DEPENDENT ERYTHROPHAGOCYTOSIS

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A heterogeneous group of cell-bound serine esterases (esterases with a reactive serine residue in the active center of the enzyme molecule) have been identified as necessary participants in a number of cellular processes including the antigen-induced release of histamine from sensitized slices of guinea pig lung (1) and from rat peritoneal mast cells sensitized with rat homocytotropic antibody (2) or rabbit anti-rat gamma globulin (3), and in the chemotactic responses of rabbit macrophages (4) and polymorphonuclear leukocytes (PMNs) (5). The participation of serine esterases in these processes was primarily revealed by the use of phosphonate esters, organophosphorus compounds which act as selective inhibitors of this class of enzymes. These organophosphorus inhibitors have proved to be invaluable tools for obtaining information on at least one of the biochemical steps involved in some of the complex functions of living cells.

The inhibitory activity of organophosphorus compounds such as diisopropyl fluorophosphate (DFP) and the phosphonate esters is based on their behavior as quasi-substrates for serine esterases. Unlike the reaction between the enzyme and its natural substrate, however, in which a serine residue in the active center of the enzyme is acylated and the activity of the enzyme then restored by a subsequent deacylation step, the interaction between enzyme and inhibitor results in the more or less irreversible phosphorylation of the reactive serine residue and, thus, inactivation of the enzyme (6). Because of the behavior of organophosphorus inhibitors as quasi-substrates for serine enzymes, the activity of various inhibitors differs according to the substrate reactivity of each susceptible esterase, and the pattern of the relative inhibitory effects of various compounds on a given enzyme forms a distinctive reaction profile, the "inhibition profile", which can be used to characterize that enzyme. Using several series of p-nitrophenylethyl phosphonate ester inhibitors, for example, specific inhibition profiles have been recorded for trypsin, chymotrypsin, acetylcholinesterase, (7, 8) and the first component of complement, $\overline{C1}^1$ (1). The serine enzymes implicated in the cellular processes mentioned above have also been characterized in this manner (1, 5). Additional information on the substrate reactivity of an inhibitable enzyme can also be obtained by determining the type of natural substrate which, when mixed with an

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[1] 1968. Nomenclature of Complement. Bull. World Health Organ. **39**:935.

organophosphorus inhibitor, is able to compete with the inhibitor for the active center of the enzyme and, in so doing, protect the enzyme from inactivation by the inhibitor.

Using p-nitrophenylethyl phosphonate ester inhibitors, Ward and Becker previously implicated two serine esterases in the complement-dependent chemotaxis of rabbit PMN leukocytes based on two different profiles of enzyme inhibition (5). One enzyme, a so-called "activatable esterase", appears to exist in or on the leukocyte in a precursor state until it is activated for its chemotactic function by the chemotactic factor (C567). This esterase was detected by exposing leukocytes to phosphonate inhibitors during the time when chemotactic factor-induced cell migration was occurring. Inhibition of chemotaxis produced in this manner was referred to as "chemotactic factor-dependent inhibition". The second enzyme, an already activated esterase was detected by exposing leukocytes to phosphonate inhibitors for a limited perior prior to their contact with chemotactic factor and before the onset of chemotaxis. Inhibition of the so-called "activated esterase", achieved by this preincubation procedure, was termed "cell-dependent inhibition". The activated enzyme was further characterized as a possible acetylase or acetylesterase, according to the ability of simple acetate esters to protect against cell-dependent inhibition of chemotaxis (15).

We were interested in whether similar serine esterases play a common role not only in chemotaxis, but in phagocytosis as well, and have studied the effect of p-nitrophenylethyl phosphonate ester inhibitors on complement-dependent erythrophagocytosis. We found that phagocytosis can be inhibited by phosphonate esters in a manner indicative of the necessary participation of an activated esterase in the phagocytic process. Suggestive evidence for the function of a second, activatable esterase in phagocytosis was also obtained. The profile of inhibition of the activated esterase of phagocytosis closely resembled that of the activated esterase implicated in chemotaxis, suggesting, therefore, that the same enzyme may be involved in both processes. Attempts to further ascertain the relationship between these enzymes, however, were unsuccessful.

Materials and Methods

Animals.—Hartley strain guinea pigs of both sexes and various weights were used as donors of phagocytic cells. The "Principles of Laboratory Animal Care" as outlined by the National Society for Medical Research were followed throughout these studies.

Buffers.—EDTA-TBS (ethylenediaminetetraacetate–triethanolamine buffered saline), a triethanolamine buffer, pH 7.4, containing 0.01 M EDTA (9), was used for washing and storage of sheep red blood cells (E) and sensitization of E with rabbit anti-sheep red cell hemolysin (A). GVB, (gelatin veronal buffer) a low ionic strength veronal buffer, pH 7.4, containing calcium and magnesium ions and glucose (10) was employed mainly in sensitizing EA with C components. A modified Hanks' buffer was used as the basic medium for phagocytosis. Hanks' balanced salt solution was obtained as a 10 × concentrate without sodium bicarbonate (Microbiological Associates, Inc., Bethesda, Md.). This was diluted tenfold with distilled water and then further with 5% glucose so that the final ionic strength of the medium was 0.12. 1 M Tris (hydroxymethyl) aminomethane buffer (Fisher Scientific Co., Fair Lawn, N. J.), pH 7.4, was added to a final molarity of 0.02.

Phagocytes.—Polymorphonuclear leukocytes (PMNs) were obtained from casein-induced peritoneal exudates in guinea pigs. Each of a group of three to eight animals was injected intraperitoneally with 10 ml of 4% casein (Nutritional Biochemical Corp., Cleveland, Ohio) adjusted to pH 7.4 in sterile normal saline. 16 hr later, each animal was injected intraperitoneally with 30 ml sterile normal saline buffered to pH 7.4 with 0.005 M sodium phosphate containing heparin (Riker Laboratories, Inc., Northridge, Calif.), 10 USP units per ml, and the peritoneal exudate collected by gravity through a 15 gauge hypodermic needle with gentle massage of the animal's abdomen. Cells from various animals were ordinarily pooled, washed at least twice in large volumes of pH 7.4, 0.005 M phosphate-buffered saline without heparin, and suspended to the desired concentration in modified Hanks' buffered salt solution. At least 95% and ordinarily over 99% of cells collected and treated in this manner were viable, based on their ability to exclude trypan blue vital dye. An average of 90% of the leukocytes present in these preparations were PMN cells. PMN cells were responsible for ingesting approximately 95% of all the red cells phagocytosed, even in inhibited preparations, as shown by microscopic examination.

Sheep Red Blood Cells Sensitized with Antibody and Complement (EAC1423).—Sheep red blood cells were obtained in Alsever's solution, weekly, from the Walter Reed Army Animal Farm, Ft. Meade, Md. Cells were washed thoroughly in cold EDTA-TBS buffer, suspended to a concentration of 2×10^9 per ml in EDTA-TBS and stored at 0°C for up to 1 wk. Cells were always rewashed just prior to use.

Rabbit anti-sheep red blood cell hemolysin was obtained in 50% glycerin (Markham Laboratories, Inc., Chicago, Ill.) and kept refrigerated at 4°C until used.

Fresh guinea pig serum was used as a source of C components. A functionally pure fraction of C2 was prepared as described in reference 11, and frozen at −70°C until used. A partially purified C3 fraction was obtained using the procedure as described for "series XXVI, column 2" in reference 12.

Sheep red cells were sensitized with antibody as described in reference 13, using the highest concentration of antibody which did not cause hemagglutination. EAC14 were reacted with C2 and C3 at 30°C using amounts of these latter components calibrated to give minimal hemolysis but promote maximal phagocytosis. EAC1423 rarely gave more than 4% lysis when incubated in GVB or modified Hanks' buffer for 30 min at 37°C, but could be lysed by the addition of C-EDTA. EAC1423 also were active in immune adherence.

Organophosphorus Inhibitors.—A series of *p*-nitrophenylethyl phosphonate esters of the following general structure were used:

$$\mathrm{RP} \overset{\displaystyle \overset{O}{\|}}{\underset{O}{\diagdown}} \overset{OC_2H_5}{\diagup} \langle\!=\!\rangle NO_2$$
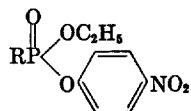
The relative activity of these phosphonate esters in inhibiting various esterases depends largely on specific structural variations in the R group of the compound. The R represents either an alkyl, phenylalkyl, *w*-aminoalkyl, or *w*-chloroalkyl chain of varying number of carbon atoms. These compounds will be referred to in these experiments according to the specific nature of the R group present, and have been described previously (7, 8). Phosphonates were dissolved in acetone, and diluted 1:100 in modified Hanks' buffer just prior to use. Unless specified otherwise, phosphonate esters were used in concentrations known to be completely soluble in aqueous solution. Diisopropyl fluorophosphate (DFP) was obtained from Dr. Bernard Jandorf, Edgewood, Md. Cyclohexyl butylphosphonofluoridate was synthesized in the Department of Immunochemistry, Walter Reed Army Institute of Research, Washington, D. C.

Phagocytosis.—Phagocytosis was performed and measured essentially as described by Gigli and Nelson (14). Briefly, leukocytes and sensitized sheep red cells were mixed together in approximately equal numbers in a medium consisting of four parts modified Hanks' buffer and one part GVB, then shaken constantly in a 37°C water bath. A 30 min incubation period was used based on preliminary experiments which showed that phagocytosis was at least 90%
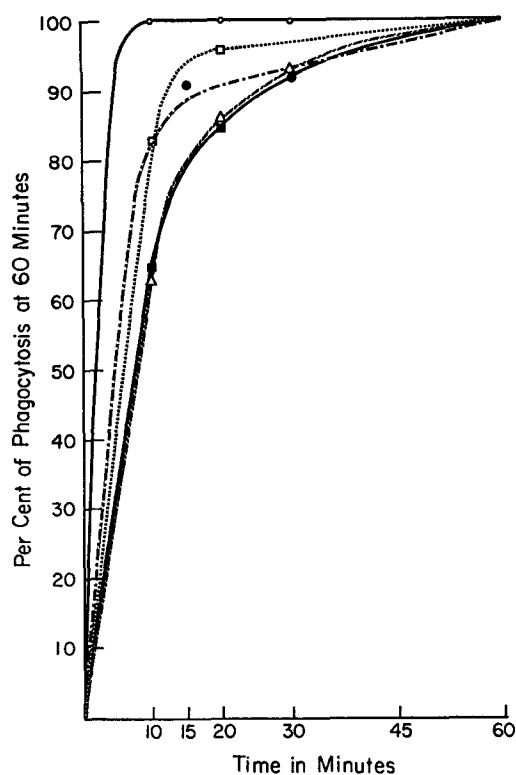

FIG. 1. Time course of phagocytosis. Five experiments performed on separate days are plotted. The amount of phagocytosis which occurred at 60 min was given a value of 100%. Phagocytosis can be seen to be over 60% completed in the first 10 min and at least 90% completed by 30 min.

completed in this interval (Fig. 1). The extent of phagocytosis was calculated by osmotically lysing red cells which were uningested by the end of the incubation period and measuring the difference between the amount of hemoglobin liberated from these remaining cells and the total amount of hemoglobin initially present in the form of red blood cells (14). In most experiments, phagocytosis was performed in 25 or 50 ml siliconized Erlenmeyer flasks containing 4×10^7 leukocytes and 5×10^7 EAC1423 in a total volume of 5 ml. In later experiments, siliconized 15×90 mm flat bottom test tubes (Walter Reed Army Institute of Research Glass Working Department) containing 4×10^6 leukocytes and 5×10^6 EAC1423 in a total volume of 0.2 ml were used. The conditions of the experiments were otherwise identical to earlier experiments. All tests were performed in duplicate or triplicate. Phagocyto-

sis was ordinarily confirmed in each experiment by microscopic examination of the prepara-tions. In most experiments, 65–75% of the sensitized sheep red cells present were phagocytosed in preparations lacking phosphonate inhibitors.

Inhibition of Phagocytosis by Phosphonate Esters.—The effect of phosphonate esters on phagocytosis was tested by two procedures. In the first procedure, a phosphonate ester in modified Hanks' buffer at twice the desired final concentration was added to an equal volume of leukocytes previously suspended to 2×10^7 cells per ml in the same buffer, and incubated at 37°C for 30 min with constant shaking in a water bath. At the end of this period, the mixture was diluted five-fold with modified Hanks' buffer and centrifuged at room tempera-ture at 70 g for 10 min. The cells were washed in 10 times their original volume and resuspended to the desired cell concentration in modified Hanks' buffer. EAC1423 were then added, and the procedure for performing and measuring phagocytosis followed as outlined above. In-hibition of phagocytosis which resulted from this procedure, namely, allowing phosphonate esters to interact with phagocytes and washing away the unreacted phosphonate prior to the initiation of phagocytosis, will be termed "cell-dependent inhibition", in conformity with the term used for inhibition of chemotaxis produced in this manner (5). Cell-dependent inhibition is thought to be due to the reaction of phosphonates with an esterase which exists in PMN leukocytes in an activated state directly susceptible to inhibition by phosphonate esters.

The second procedure, the so-called "phagocytosis-dependent inhibition" procedure, was designed to detect serine esterases activated during the course of phagocytosis which function in the phagocytic process. In this procedure, the effect on phagocytosis of exposing leukocytes to phosphonate inhibitors while phagocytosis was taking place was examined. Since it was essential to define the extent to which any inhibition achieved in this manner could be attrib-uted specifically to an effect on esterases activated during phagocytosis, rather than to an effect on esterases already activated prior to the onset of phagocytosis, the extent of cell-dependent inhibition which occurred was also measured in each instance. Inhibition due to an effect on activatable esterases activated during the course of and as part of the phagocytic process was considered to have been demonstrated only when exposure of leukocytes to phos-phonate inhibitors during the course of phagocytosis resulted in significantly greater inhibition than that which could be produced by exposing the same batch of leukocytes to the same concentration of inhibitor for a comparable period prior to the initiation of phagocytosis. The second procedure was performed as described above in the procedure for cell-dependent in-hibition and then washed. Those leukocytes which had not been preincubated with phos-phonates were resuspended either in buffer alone or in buffer containing a given phosphonate ester. The EAC1423 was immediately added, and the procedure for determining phagocytosis followed as outlined. In order to compensate for any contribution to the optical density (OD) of the lysate made by breakdown products of phosphonates, the OD of the lysate of leuko-cytes to which phosphonates, but not EAC1423, had been added was also measured.

In all experiments, the final concentration of acetone in each sample, with or without phosphonate, was adjusted to 0.5%. In preliminary experiments, this concentration of acetone was shown to be without any appreciable effect on phagocytosis.

In order to establish that inhibition of phagocytosis induced by phosphonate esters occurred in a manner compatible with the known enzyme-inactivating properties of these compounds, the effects on the inhibition of phagocytosis of the duration of exposure of leukocytes to phosphonate inhibitors, the concentration of inhibitor used, and variations in the temperature and pH of the reaction were examined. The general cell-damaging effects of inhibitory concen-trations of phosphonate esters as measured by trypan blue exclusion was also determined.

Chemotaxis.—The micropore filter method (18) was the standard assay procedure utilizing peritoneal exudate cells obtained in guinea pigs in the manner described above. Values for chemotaxis were expressed as numbers of migrating cells counted in 5 random microscopic

fields at low power. The lower compartments contained as chemotactic factor 10% guinea pig serum activated by preincubation with an antigen-antibody precipitate (19) made up from rabbit IgG rich in antibody to bovine serum albumin.

Trypan Blue Studies.—The cytotoxic effects of the phosphonate esters used were estimated as a function of the inability of leukocytes to exclude the vital dye, trypan blue (Matheson Scientific, Inc., Cincinnati, Ohio). Dye exclusion tests were performed as described (5).

RESULTS

Kinetics of Cell-Dependent Inhibition of Phagocytosis.—The time course of cell-dependent inhibition of phagocytosis was determined by employing rep-
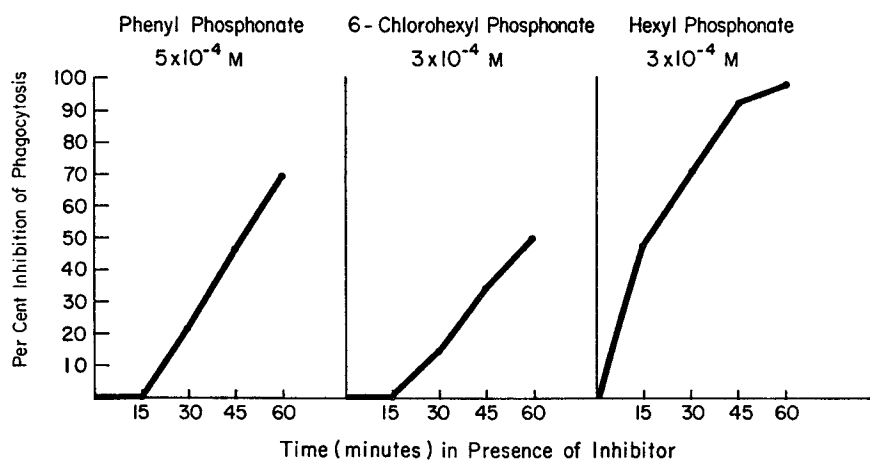

Fig. 2. Time course of cell-dependent inhibition of phagocytosis. Results using a representative of each of three series of phosphonate esters are plotted. Inhibition can be seen to be progressive with time in each instance.

resentatives of three of the four homologous series of phosphonate esters utilized during the course of these experiments. The results are illustrated in Fig. 2. As can be seen, there is a progressive increase in inhibition of phagocytosis as the duration of exposure between leukocytes and inhibitor increases. When phenyl phosphonate and 6-chlorohexyl phosphonate were used at the designated concentration, inhibition was not observed until the period of exposure exceeded 15 min. This apparent lag in inhibition was not seen when higher concentrations of phenyl phosphonate $(1 \times 10^{-3}\text{M})$ were used and did not always occur when 6-chlorohexyl phosphonate was used at $3 \times 10^{-4}\text{M}$.

Concentration Dependence of Cell-Dependent Inhibition of Phagocytosis.—As expected, cell-dependent inhibition of phagocytosis caused by phosphonate esters was a direct function of the concentration of the ester used (Fig. 3). The same phosphonates employed in the previous experiments were tested, keeping the time of incubation constant (30 min) while varying the concentration of each

inhibitor. As illustrated in Fig. 3, above a certain threshold concentration of each inhibitor, the inhibitory effect of each compound was proportional to the concentration of phosphonate used.

Influence of pH and Temperature on Cell-Dependent Inhibition of Phagocytosis.—The effect of pH on phosphonate-induced inhibition of phagocytosis was tested in five experiments. The pH of the medium in which leukocytes were exposed to phosphonate esters varied in these experiments over a range of 6.8–8.1. Each mixture maintained its pH during the period of incubation within 0.1 pH units. In four experiments, pentyl phosphonate $(4 \times 10^{-4}\text{M})$ was em-
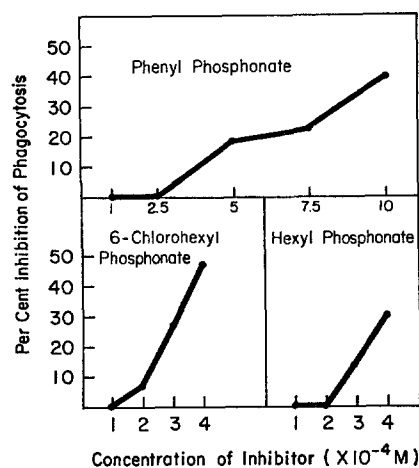

FIG. 3. Concentration dependence of cell-dependent inhibition of phagocytosis. Results using a representative of each of three series of phosphonate esters are pictured. Inhibition can be seen to be proportional to the concentration of inhibitor used.

ployed as the inhibitor; hexyl phosphonate $(2 \times 10^{-4}\text{M})$ was used in one experiment. As Fig. 4 shows, some variation in results was encountered from one experiment to the next. However, in four of the five experiments, inhibition was significantly greater at or near pH 7.4, than at a lower pH and, in all experiments, a maximum inhibitory effect occurred at the most alkaline part of the pH range tested.

The effect of temperature on the inhibitory activity of the phosphonate esters was tested in three separate experiments. In each experiment, pentyl phosphonate $(4 \times 10^{-4}\text{M})$ was incubated with leukocytes for 30 min at a temperature of 37°C, and at room temperature $(23 \pm 1°\text{C})$, and the inhibitory activity of this compound at the different temperatures of reaction compared. As Table I indicates, in each instance the inhibitory activity of pentyl phosphonate proved to be significantly greater at the higher temperature of reaction, as would be expected for an enzymatic reaction.

Profiles of Cell-Dependent Inhibition of Phagocytosis by Phosphonate Esters.—
In each of the four series of phosphonate esters used to test inhibition (alkyl, phenylalkyl, w-chloroalkyl, and w-aminoalkyl phosphonates), the effect of varying the length of the alkyl chain on the relative inhibitory capacity of the compound was determined. The concentration of all members of a homologous
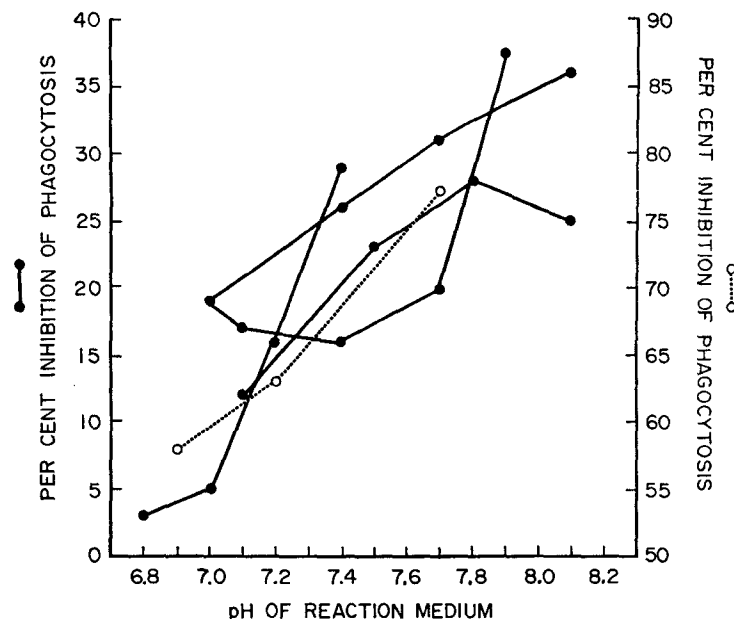

FIG. 4. The influence of pH on the cell-dependent inhibition of phagocytosis. Leukocytes were exposed to pentyl phosphonate (●——●) or hexyl phosphonate (O——O) at 37°C for 30 min prior to phagocytosis. The pH of the reaction medium was adjusted using 0.02 M Tris buffer. Phagocytosis was performed at pH 7.4.

series of phosphonate esters were kept the same within each experiment, and the relative inhibitory activity of each member of a series of phosphonate esters, one to another, was defined using a single batch of cells. Fig. 5 shows the results obtained when the four series of inhibitors were preincubated with guinea pig polymorphonuclear leukocytes. In the phenylalkyl series, the phenyl and benzyl phosphonates were inactive at 3 × 10⁻⁴M. Used at a concentration of 1 × 10⁻³M, however, these compounds did inhibit phagocytosis, though the activity of benzyl phosphonate was extremely small. Profound inhibition occurred when phenylethyl phosphonate was used at 3 × 10⁻⁴M and even greater inhibition occurred at this concentration when phenylpropyl phosphonate was used. Thus, in this series of inhibitors, placement of one carbon atom between the phenyl ring and the phosphorus atom resulted in a significant loss of po-

tency, whereas further additions of one and two carbon atoms caused a marked and progressive increase in the inhibitory capacity of the compound. In both the alkyl and chloroalkyl series, the capacity of each series of compounds increased as the alkyl chain was lengthened from 3 to 6 carbon atoms (see both

TABLE I

Effect of Temperature on the Inhibitory Activity of Pentyl Phosphonate

Experiment	Per cent inhibition of phagocytosis with cells incubated at:	
	37° C	RT (23 ± 1°C)
A	23	11
B	27	0
C	22	6

Leukocytes were exposed to pentyl phosphonate at a concentration of 4×10^{-4} M for 30 min and washed free of unreacted phosphonate before phagocytosis was allowed to proceed. Phagocytosis was performed at 37° C.
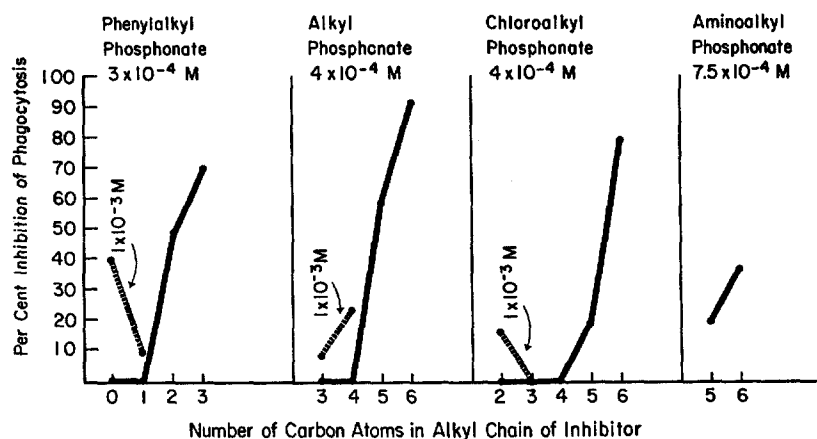

Fig. 5. Profile of cell-dependent inhibition of phagocytosis. The relative inhibitory activity of each series of compounds is plotted against the length of the alkyl chain in each compound.

Fig. 5 and Table IV). In the chloroalkyl series, there was a decrease in inhibitory activity in going from the 2-chloroethyl to the 3-chloropropyl phosphonate as shown by the experiment performed at 1×10^{-3}M phosphonate (Fig. 5). In the aminoalkyl series, neither of the two compounds tested, 5-aminopentyl nor 6-aminohexyl phosphonate, was particularly active in causing cell-dependent inhibition of phagocytosis. Some inhibitory activity was demonstrable, however, and a noticeable increase in activity occurred when the number of carbon atoms in the aminoalkyl chain was increased from 5 to 6.

Uptake of Trypan Blue by Cells Treated with Phosphonate Esters.—An estimate

of the extent of cell damage which resulted from the exposure of polymorpho-
nuclear leukocytes to phosphonate inhibitors was obtained based on the in-
ability of damaged cells to exclude the vital dye, trypan blue. Table II is a
representative summary of experiments in which trypan blue exclusion was
measured in cell preparations exposed to various phosphonate esters. Such
exposure did lead to significant cell damage in a number of instances. However,
as can be seen in Table II, with the phenylpropyl and pentyl phosphonates at
3×10^{-4}M and 2×10^{-4}M, respectively, cell-dependent inhibition of phagocy-

TABLE II

Uptake of Trypan Blue by Cells Incubated with Various Phosphonates

Phosphonate used	Concentration	Cells taking up dye	Inhibition of phagocytosis
	M	%	%
Phenylpropyl	3×10^{-4}	0	74
Pentyl	2×10^{-4}	0	27
Chlorobutyl	3×10^{-4}	8	0
Butyl	3×10^{-4}	11	0
Phenyl	1×10^{-3}	12	45
Benzyl	1×10^{-3}	13	5
Propyl	1×10^{-3}	3	20
Butyl	1×10^{-3}	24	32

Leukocytes were examined for their ability to exclude trypan blue after incubation with
designated phosphonates and prior to the initiation of phagocytosis. The data are grouped
according to experiments performed at three different times.

tosis occurred in the absence of appreciable cell damage, and significant cell
damage occurred with (phenyl, benzyl, propyl, and butyl phosphonates at
1×10^{-4}M) and without (chlorobutyl and butyl phosphonates at 3×10^{-4}M)
inhibition of phagocytosis. Thus, the cell damaging effects of phosphonate esters
as defined by dye exclusion was not a necessary accompaniment of significant
inhibition of phagocytosis.

As indicated in Materials and Methods, some assessment of phagocytosis was
also made in each of the experiments reported here by examining each prepara-
tion microscopically. In cell preparations in which appreciable cell-dependent
inhibition of phagocytosis had occurred, most of the uningested red cells were
frequently noted to be adherent to the surface of phagocytes. This suggested
that inhibition was not directed at the adherence phenomenon per se, but rather
at a step subsequent to this event. This was further supported by the observa-
tion that increasing the concentration of red cells and, thus, the likelihood of
contact between red cells and white cells did not change the per cent inhibition

by phosphonates as might be expected if some limitation of contact between EAC1423 and phagocytes was responsible for the decrease in phagocytosis induced by phosphonate esters. In addition, the time course of phagocytosis was found to be the same in both phosphonate inhibited and uninhibited cell preparations.

The Ability of Various Esters to Protect against Phosphonate Inhibition of Phagocytosis.—As will be discussed later, the profile of cell-dependent inhibition of phagocytosis using guinea pig PMNs closely resembles the profile of cell-dependent inhibition of the chemotactic activity of rabbit PMNs, suggesting the possibility that the same enzyme functions in both cell processes. The observation that simple acetate esters (ethyl acetate, glyceryl triacetate, butyl acetate, acetoxyacetic acid, and acetyl phosphate) protect rabbit PMNs against cell-dependent inhibition of chemotaxis (15) suggested experiments to examine the ability of various esters to protect guinea pig PMNs against phosphonate inhibition of phagocytosis. The entire series of esters described in reference 15 was employed in these experiments.

Each ester was preincubated with leukocytes in the presence of an inhibitory concentration of pentyl phosphonate (4 $\times$ 10^{-4}M), using various concentration of esters, ranging in ratio of ester to inhibitor from 10:1 to 120:1. Even at the highest concentration of esters used (5 $\times$ 10^{-2}M), none of the acetate esters reported to be protective against phosphonate inhibition of chemotaxis, or any other ester tested, was found to protect against inhibition of phagocytosis with the possible exception of acetyl phosphate. Acetyl phosphate (1 $\times$ 10^{-2}M), adenosine diphosphate (ADP) (3 $\times$ 10^{-3}M), and adenosine monophosphate (AMP) (1 $\times$ 10^{-2}M), (the latter two compounds not tested in chemotaxis) could be shown to exert a moderate (10–40%) protective effect against pentyl phosphonate inhibition in some, but not all experiments. In addition to this inconsistency, each of these phosphate esters exerted a limited (10–20%) but definite irreversible inhibitory effect when preincubated with leukocytes, further complicating the interpretation of results in the protection experiments. Adenosine triphosphate (ATP), tosyl-L-arginine methyl ester (TAMe), glycine ethyl ester, and acetyl-L-tyrosine ethyl ester also irreversibly inhibited phagocytosis when preincubated with leukocytes, raising the possibility that any protection afforded by these compounds may have been masked by the inhibitory effects of of the compounds themselves on phagocytosis.

Because of technical difficulties encountered with the use of rabbit PMNs, phagocytosis was studied in the experiments reported here, using guinea pig PMNs. In order to investigate the possibility that the difference in the protective effect of acetate esters on phosphonate inhibition of phagocytosis by guinea pig PMNs and chemotaxis of rabbit PMNs might be related to species differences in the enzyme systems involved, parallel experiments were performed to compare the effects of certain esters on inhibition of chemotaxis and phagocy-

tosis, using PMN leukocytes from the same species, the guinea pig.[2] A single batch of guinea pig PMNs was employed in each experiment, and all cells were treated identically before being assayed for chemotactic and phagocytic activity.

Table III lists the results of an experiment comparing the ability of five esters to protect against phosphonate inhibition of the chemotactic and phag-

TABLE III

Protection against Cell-Dependent Inhibition of Phagocytosis and Chemotaxis Using Guinea Pig Polymorphonuclear Leukocytes

PMNs Preincubated with pentyl phosphonate plus:	Inhibition of:	
	Phagocytosis	Chemotaxis
	%	%
———	39	90
Glyceryl triacetate	37	92
Ethyl acetate	39	47
Ethyl propionate	43	50
Ethyl butyrate	57	83
Butyl acetate	61	92

PMNs were incubated with pentyl phosphonate (4×10^{-4} M) in the presence or absence of one of the esters designated (at 1.6×10^{-2} M ester). At the end of 30 min incubation at room temperature, each mixture was diluted with buffer, the PMNs were washed, and chemotactic and phagocytic function was assayed.

It should be noted that ehtyl acetate and ethyl propionate, but not glyceryl triacetate or butyl acetate, are protective when guinea pig PMNs are used, whereas ethyl acetate, glyceryl triacetate, and butyl acetate, but not ethyl propionate were reported to be protective when rabbit PMNs are used, suggesting that the esterases involved in the two species may possess similar, but not identical, substrate reactivities.

ocytic activities of guinea pig PMNs. As can be seen, ethyl acetate and ethyl propionate reduced the inhibitory effect of pentyl phosphonate on chemotaxis by almost 50%, but did not interfere appreciably with phosphonate inhibition of phagocytosis. This disparity in the ability of ethyl acetate and ethyl propionate to protect against phosphonate inhibition of chemotaxis, but not phagocytosis, was reproducible. Ethyl butyrate and butyl acetate, without effect on chemotaxis, appeared to increase the inhibitory effect of pentyl phosphonate on phagocytosis. The reason for this is not readily apparent, since neither ester interfered with phagocytosis when used alone at 1.6×10^{-2}M. It is possible, however, that ethyl butyrate and butyl acetate may well have exerted some

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[2] Preliminary experiments using alkyl and chloroalkyl phosphonate esters had revealed a similarity in inhibition profiles for the guinea pig and rabbit activated esterases involved in chemotaxis.

effect on PMNs, which by itself was below the level of detection in the phago-
cytic assay system used.

It was noted also that phosphonate esters repeatedly exerted a profoundly
greater effect on chemotaxis than on phagocytosis. Whether this finding reflects
a qualitative or quantitative difference in the requirements of the enzymes
involved in the two processes, or is related in some way to differences in "sensi-
tivity" of the systems used for assaying these two processes could not be deter-
mined.

Phagocytosis-Dependent Inhibition of Phagocytosis.—In order to investigate
the possibility that other ("activatable") serine esterases activated during the
course of phagocytosis also perform important functions in the phagocytic
process, the effect of exposing phagocytes to various phosphonate esters during
the course of phagocytosis was examined. Phenylalkyl, alkyl, and w-chloroalkyl
phosphonates exposed in high concentration to leukocytes during the course of
phagocytosis were, for the most part, without effect on phagocytosis (Table
IV). When inhibition did occur, as in the case of 5-chloropentyl and 6-chloro-
hexyl phosphonate, less phagocytosis-dependent inhibition occurred than cell-
dependent inhibition. Consequently, the inhibitory effect exerted by 5-chloro-
pentyl and 6-chlorohexyl phosphonate during the course of phagocytosis could
not be distinguished from an effect which could be ascribed to inhibition of the
activated esterase demonstrated in the same experiment. Similar results were
obtained when 5-aminopentyl phosphonate was used for inhibition. In con-
tradistinction to these findings, however, 6-aminohexyl phosphonate caused
slightly but consistently greater phagocytosis-dependent than cell-dependent
inhibition. In an experiment not listed in Table IV, when the period of expo-
sure between 6-aminohexyl phosphonate (6 $\times$ 10^{-4}M) and leukocytes was
reduced to 10 min, an effect on phagocytosis was still observed when exposure
took place during the phagocytic process (14% inhibition), but did not occur
if exposure took place prior to phagocytosis. Exposure of leukocytes to 4-chloro-
butyl, 5-chloropentyl, and phenyl phosphonate for 10 min resulted only in
cell-dependent inhibition.

Because of the results obtained with 6-aminohexyl phosphonate, additional
organophosphorus inhibitors of serine esterases, namely, DFP and a newly
synthesized compound, cyclohexyl butylphosphonofluoridate, were used in
order to see if further evidence could be obtained for the existence of an acti-
vatable esterase operative in phagocytosis. Some inhibition of phagocytosis
was demonstrable at all four concentrations of DFP used, whether leukocytes
were exposed to DFP prior to or during phagocytosis (see Table IV). Below
5 $\times$ 10^{-3}M, this effect was decidedly more pronounced if the inhibitor was
exposed to leukocytes before the onset of phagocytosis. In the experiment
listed in Table IV, DFP, used at a concentration of 5 $\times$ 10^{-3}M, appeared to be
more effective if exposed to leukocytes during, rather than prior to phagocytosis.

However, a reverse effect was seen at this concentration in other experiments. On the other hand, cyclohexyl butylphosphonofluoridate, a phosphonate inhibitor of trypsin, chymotrypsin, and acetylcholinesterase (E. L. Becker,

TABLE IV

Inhibition of Phagocytosis Resulting from Exposure of Phosphonate Esters to PMN Leukocytes before or during Phagocytosis

Inhibitor	Concentration of inhibitor	Inhibition of phagocytosis	
		Inhibitor present during phagocytosis	Inhibitor present prior to phagocytosis
	M	%	%
Phenyl phosphonate	1.0×10^{-3}	0	22
Benzyl phosphonate	1.0×10^{-3}	0	16
Propyl phosphonate	1.0×10^{-3}	0	16
Butyl phosphonate	1.0×10^{-3}	0	29
2-Chloroethyl phosphonate	1.0×10^{-3}	0	27
3-Chloropropyl phosphonate	1.0×10^{-3}	0	12
4-Chlorobutyl phosphonate	1.0×10^{-3}	3	43
5-Chloropentyl phosphonate	$1.0 \times 10^{-3}*$	49	65
6-Chlorohexyl phosphonate	$1.0 \times 10^{-3}*$	56	97
5-Aminopentyl phosphonate	7.5×10^{-4}	9	22
5-Aminopentyl phosphonate	6.0×10^{-4}	0	0
DFP‡	5.0×10^{-3}	69	45
DFP	1.6×10^{-3}	13	31
DFP	8.0×10^{-4}	9	29
6-Aminohexyl phosphonate	7.5×10^{-4}	50	38
6-Aminohexyl phosphonate	6.0×10^{-4}	14	6
6-Aminohexyl phosphonate	5.0×10^{-4}	18	7
Cyclohexyl butylphosphonofluoridate	1.0×10^{-3}	54	18
Cyclohexyl butylphosphonofluoridate	6.7×10^{-4}	46	0
Cyclohexyl butylphosphonofluoridate	3.3×10^{-4}	0	0

* These compounds are not completely soluble at the concentrations listed so that the actual effective concentration was somewhat lower. The experiments reported using aminoalkyl phosphonates were performed on separate days, using these phosphonates at only one concentration on a given day.

‡ Diisopropyl fluorophosphate.

unpublished experiments) repeatedly demonstrated marked phagocytosis-dependent inhibition, but produced either relatively little or no cell-dependent inhibition of phagocytosis whatever. Typical results are listed on Table IV.

The possibility was considered that phagocytosis-dependent inhibition produced by cyclohexyl butylphosphonofluoridate might have been due to the

action of the phosphonate on EAC1423, rendering these cells unpalatable to the PMNs. This possibility was tested by incubating EAC1423 with 8×10^{-4} M cyclohexyl butylphosphonofluoridate for 30 min at 37°C, then washing the red cells free of the phosphonate, and comparing their ability to be phagocytosed with control EAC1423 which had been incubated with 0.5% acetone. EAC1423 treated with the phosphonate gave 61% phagocytosis, whereas control EAC1423 gave 56% phagocytosis, thus indicating that phagocytosis-dependent inhibition by cyclohexyl butylphosphonofluoridate was not the result of some action of the phosphonate on EAC1423.[3]

DISCUSSION

The p-nitrophenylethyl phosphonate esters, known inhibitors of serine enzymes, have been shown in these studies to produce "cell-dependent inhibition" of complement-dependent erythrophagocytosis in a manner indicative of the function of an activated esterase in the phagocytic process, as follows: (a) The phosphonate inhibition of phagocytosis was "irreversible"; that is, the inhibitory effect could be demonstrated after phagocytes were washed free of unreacted inhibitor. (b) The amount of inhibition produced was directly proportional to the concentration of inhibitor used. (c) The amount of inhibition produced was a direct function of duration of exposure between phagocytes and inhibitor. (d) Inhibition was influenced by the temperature of the reaction medium: greater inhibition resulted when phagocytes reacted with inhibitor for 30 min at 37°C than at 23 ± 1°C for the same period. (e) Inhibition was pH dependent. Over the pH range tested (6.8–8.1), the activity of a given concentration of inhibitor increased as the pH of the medium became more alkaline, with maximum activity occurring at pH 7.7 or higher.[4] (This is in line with the known pH optimum of about 8.0 for inactivation by DFP of a number of serine esterases (16). DFP is thought to act in a manner analogous to the action of phosphonate inhibitors utilized here, and was also shown in this report, to produce cell-dependent inhibition of phagocytosis.) (f) The inhibitory effects of the compounds used here were unrelated to any general damaging effects on phagocytic cells, at least to the extent measured by uptake of trypan blue, or to interference with steps preliminary to phagocytosis, namely, contact between phagocytes and EAC1423.

Whether the "activated enzyme", which participates in phagocytosis ordinarily exists in an activated state, or was activated in the process responsible for the accumulation of cells in the casein-induced peritoneal exudate, or by the conditions of incubation or handling of cells was not determined.

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[3] We are indebted to Dr. Richard Wistar, Department of Immunochemistry, Walter Reed Army Institute of Research, for this information.

[4] The fact that any change in the pH of the extracellular medium influenced the reaction between inhibitor and a cellular enzyme is remarkable in itself, and would tend to suggest that the inhibitable enzyme is located at or near the surface of the cell.

Four homologous series of p-nitrophenylethyl phosphonate esters were used to study cell-dependent inhibition of phagocytosis. An "inhibition profile" of the susceptible enzyme was obtained from the patterns of reactivity of this enzyme with the various phosphonate esters as reflected in the relative ability of these compounds to inhibit phagocytosis. Comparisons of the profile of inhibition characteristic of the activated esterase of phagocytosis with inhibition profiles of other serine esterases obtained with the phosphonate esters permits some suggestions as to the identity of the phagocytic enzyme.

On the one hand, the inhibition profiles of CĪ (1, 3), trypsin (7, 8), chymotrypsin (7, 8, 17) and acetylcholinesterase (7, 8, 17) each reveal patterns of reactivity which are distinctly different from that which characterizes the activated esterase implicated in phagocytosis. This is also true of the profiles of inhibition of the antigen-induced release of histamine from sensitized slices of guinea pig lung (1) and from rat peritoneal mast cells sensitized with rat homocytotropic antibody (2) or rabbit anti-rat and globulin (3). In addition, the activatable esterase of rabbit PMNs (5) and the activated esterase of rabbit macrophages (4), both involved in the chemotactic responses of these cells, possess inhibition profiles which are significantly different from that of the activated phagocytic esterase. These esterases, therefore, appear to be different from the activated esterase which functions in phagocytosis.

On the other hand, comparison of the profiles of cell-dependent inhibition of chemotaxis of rabbit PMNs (5) and cell-dependent inhibition of phagocytosis reveals remarkable similarities for each of the four series of phosphonate inhibitors used. In fact, except for two members of the alkyl series, the profiles of cell-dependent inhibition appear to be essentially identical for the two processes. In the alkyl series, butyl phosphonate appears to be slightly more active than propyl phosphonate in inhibiting phagocytosis in guinea pig PMNs whereas the reverse was reported to be true for inhibition of chemotaxis of rabbit PMNs (5). However, in unpublished experiments in which we examined the effect of phosphonate esters on chemotaxis of guinea pig PMNs, propyl phosphonate was found to be slightly more active of the two compounds in producing cell-dependent inhibition in fewer than half the experiments performed, and it exhibited less inhibitory activity in some experiments and equivalent activity in others. Thus, even the small difference in the inhibitory effects of propyl and butyl phosphonate on the two processes is reduced in significance when leukocytes of the same species are compared.

The fact that the cell-dependent inhibition profiles of chemotaxis of rabbit PMNs and of phagocytosis using guinea pig PMNs appear to be identical suggests that the same enzyme may be involved in both processes. However, this conclusion was made distinctly uncertain by the fact that certain esters shown to protect against phosphonate inhibition of chemotaxis did not protect against cell-dependent inhibition of phagocytosis. It will be recalled that simple acetate

esters were found to protect against phosphonate inhibition of chemotaxis of rabbit PMN leukocytes (15). Repeated attempts to demonstrate a similar protective effect of simple acetate esters (or any other ester tested) on phosphonate inhibition of phagocytosis, using guinea pig PMNs, were for the most part unsuccessful. Although acetyl phosphate, an ester shown to be protective in chemotaxis was found in some experiments to protect in the phagocytic system, this effect was inconstant and complicated by the fact that it exerted some inhibitory activity of its own on phagocytosis. Experiments performed to compare the protective effect of various compounds on phosphonate inhibition of chemotactic and phagocytic functions of cells from the same species of animal, the guinea pig, showed that certain of these esters could prevent cell-dependent inhibition of chemotaxis, but in the same experiment, a sample of the same cells were not protected against inhibition of phagocytosis.

An additional source of uncertainty as to the identity of the activated esterase of phagocytosis and chemotaxis was introduced by the finding in the same experiments that exposure of a given population of leukocytes to phosphonate inhibitors invariably resulted in a profoundly greater inhibitory effect on chemotaxis than on phagocytosis. Whether this quantitative difference in the inhibitory effects of phosphonates on chemotaxis and phagocytosis relates in some way to the disparity in the protective effects of certain acetate esters against phosphonate inhibition of chemotaxis and phagocytosis (such as different compartmentalization of the enzymes), in spite of involvement of the same enzyme in both processes, or whether these enzymes actually have different substrate specificities in the face of practically identical patterns of reactivity with various phosphonate esters cannot be determined at present.

As already mentioned, the ability of EAC 1423 to adhere to leukocytes which showed distinct cell-dependent inhibition is seemingly unimpaired. This indicates that in phagocytosis by macrophages (20) the engulfment phase is separable from the stage of adherence. The phosphonates apparently act on the engulfment step, but show little, if any, ability to interfere with the preliminary adherence step.

Using the phagocytosis-dependent procedure of inhibition, a search was made for the participation of an activatable esterase in phagocytosis. By this procedure, only suggestive evidence was obtained for the function of such an enzyme in phagocytosis. Exposure of leukocytes to most of the phosphonate esters or to DFP during the course of phagocytosis either had no appreciable effect on phagocytosis or resulted in distinctly less inhibition than that which could be accounted for by cell-dependent inhibition. One of the phosphonates used, however, 6-aminohexyl phosphonate, and cyclohexyl butylphosphonofluoridate, a compound with significant structural differences from the p-nitrophenyl phosphonate esters, consistently demonstrated more phagocytosis-dependent than cell-dependent inhibitory activity. The phosphonofluoridate was particu-

larly effective in this regard, giving almost 50% phagocytosis-dependent inhibition at a concentration (6.7 $\times$ 10^{-4}M) which produced no detectable cell-dependent inhibition. This same compound did not act on EAC1423 to prevent these cells from being phagocytosed. The ability of the cyclohexyl butylphosphonofluoridate to inhibit phagocytosis when PMNs and EAC1423 are together and its lack of effect on either cell by itself strongly suggests that in phagocytosis-dependent inhibition, the cyclohexyl butylphosphonofluoridate is inhibiting an esterase which is activated during the course of phagocytosis, that is, an activatable esterase. Further work is obviously required before this suggestion can be accepted and this work is now in progress. The potency of cyclohexyl butylphosphonofluoridate in producing phagocytosis-dependent inhibition, while causing relatively little cell-dependent inhibition of phagocytosis would imply that structurally similar compounds should be useful in further substantiating the function of an activatable serine esterase in phagocytosis and might also permit some characterization of the enzyme, based on the pattern of inhibitory activity of a series of such compounds. Such compounds are not yet available but are currently being synthesized with these objectives in mind.

SUMMARY

The *p*-nitrophenyl ethyl phosphonate esters have been shown to inhibit complement-dependent erythrophagocytosis when exposed to guinea pig polymorphonuclear leukocytes prior to the initiation of phagocytosis. Inhibition of phagocytosis occurred in a manner characteristic of the well-defined capacity of phosphonate esters to inactivate serine esterases: inhibition was irreversible, dependent upon the temperature of reaction and pH of the reaction medium, and proportional to the concentration of inhibitor used and the duration of exposure between leukocytes and inhibitor. Phosphonate inhibition was further shown to be independent of any general cell damaging effects of the compounds used. The phagocytic enzyme inhibited by phosphonate esters apparently exists in or on leukocytes in an already activated state prior to the initiation of the phagocytic process.

The inhibitory profile of the activated phagocytic esterase was found to be essentially identical to the profile of inhibition previously obtained for the activated chemotactic esterase of rabbit polymorphonuclear leukocytes, suggesting that the same enzyme may function in both chemotaxis and phagocytosis. Various substrates including acetate esters reported to protect the activated chemotactic esterase from inhibition by phosphonate esters did not exhibit a clear protective effect in the phagocytic system and attempts to define the relationship between the two enzymes were unsuccessful.

Suggestive evicence was also obtained for the requirement of the function of a second, activatable esterase in the phagocytic process.

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