A COMPARISON OF THE SPECIFICITY OF INHIBITION BY PHOSPHONATE ESTERS OF THE FIRST COMPONENT OF COMPLEMENT AND THE ANTIGEN-INDUCED RELEASE OF HISTAMINE FROM GUINEA PIG LUNG

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A large and heterogeneous group of esterases are inhibited by organophosphorus inhibitors such as diisopropyl fluorophosphate (DFP), and the phosphonate esters (1–3). For some of these enzymes there is direct evidence, and for others good presumptive evidence, that the inhibition is due to the phosphorylation of the hydroxyl group of a serine present in the active center[1] of the enzyme. This phosphorylation is deemed to be essentially the same as the process by which the substrate of these enzymes acylates the active center (4, 5). The difference between substrate and inhibitor is that the acylated enzyme is very rapidly deacylated, restoring its activity, whereas the phosphorylated enzyme is dephosphorylated very slowly or not at all. To stress the substrate-like activity of organophosphorus inhibitors, they have been termed "hemisubstrates" (6). In accord with the substrate nature of these inhibitors, distinct differences in reactivity with different enzymes are observed (7). This allows these enzymes to be characterized, not only in the usual manner by their reactivity with complete substrates, but also by the pattern of their reactivity with hemisubstrates such as phosphonate esters.

The first component of guinea pig complement is an esterase (8, 9) inhibited by organophosphorus compounds such as DFP (10, 11) and phosphonate esters (3). The antigen-induced release of histamine from perfused, sliced guinea pig lung is also inhibited by DFP suggesting that an esterase is involved (12). The additional finding that aromatic amino acid esters were potent inhibitors of the antigen-induced histamine release led to the hypothesis that the DFP-inhibited esterase has a chymotrypsin-like substrate specificity (12). It was realized however that there is no rigorous, direct

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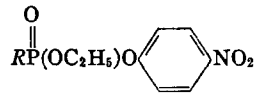
[1] "Active center" is used here in the same sense as by Vallee as referring "... to all those features of primary, secondary and tertiary structures ... which are required for substrate binding, specificity or hydrolysis of the substrate (29)."

evidence bearing on this point (reference 13, p. 44). The striking similarities and several differences between the hemolytic action of complement, and *in vitro* antigen-induced release of histamine summarized previously (12) have not provided a basis for a definite conclusion as to the role of complement in this latter reaction.

In this study, a number of *p*-nitrophenylethyl phosphonates of the structure



where *R* is phenyl alkyl, alkyl, ω-chloroalkyl, and ω-aminoalkyl have been tested for their ability to inhibit the activated first component (C'1a) of guinea pig complement, and the antigen-induced release of histamine from sliced guinea pig lung. In this manner, the antigen-antibody–activated esterases, respectively involved in the two reactions were further characterized and simultaneously compared.

Materials and Methods

Phosphonate Inhibitors.—The *p*-nitrophenylethyl alkyl, phenyl alkyl, and the ω-chloroalkyl phosphonates have been described previously (7, 14, 15). The synthesis and properties of *p*-nitrophenylethyl ω-aminoalkyl phosphonates will be described subsequently by Mr. Robert Statz, Mr. Dennis Werber, and Mr. Gim Tijoe of American University. These esters are unstable. They break down to yield the inactive phosphonic acid and free *p*-nitrophenol. In all cases, the concentration of phosphonate ester was measured in terms of the actual bound *p*-nitrophenol as assayed on the day of experimentation.

Buffers.—Three buffers were used in the study of the inhibition of C'1a: triethanolamine-buffered saline (TBS) with added magnesium, calcium, and gelatin (16); "low ionic strength (0.065 μ) buffer," pH 7.4 with mannitol, as described in reference 17 except that triethanolamine was substituted for veronal; and "low ionic strength (0.065 μ) buffer," pH 8.0.

The reaction of inhibitor with EAC'1a, 4 was performed in the buffer of low ionic strength, pH 8.0. The low ionic strength prevented dissociation of C'1a from the cell during the reaction (17), and pH 8.0 was used in order for the results to be directly comparable with previous work on other enzymes (7, 15).

Tyrode's buffer was employed for the study of histamine release.

Guinea Pig Serum.—Fresh frozen guinea pig serum absorbed three times with sheep erythrocytes at 0°C (reference 18, p. 151) was the source of the complement used in the preparation of EAC'1, 4, 2 and other cell intermediates. Commercial fresh frozen or lyophilized complement reconstituted with the diluent provided was employed in the study of the inhibition of whole complement action by phosphonates.

Red Cells.—Sheep red cells were collected in sterile Alsever's and just before use, washed, and suspended in TBS buffer at a concentration of 2.0×10^9/ml. They were sensitized with an equal volume of five times the optimal concentration of commercial hemolysin. The preparation of C'2, EAC'4, EAC'1, 4, and C'EDTA has been described previously (reference 18, p. 133). EAC'1a, 4, 2 were prepared as described (reference 18, p. 133) except that the EA were cooled to exactly 0°C prior to adding 2.0 ml of fresh guinea pig serum per 100 ml of EA; the guinea pig serum and EA were left in contact at 0°C for 10 minutes before centrifugation and washing.

Inhibition of C'1a.—Ten ml of EAC'1a, 4 (1.33×10^8/ml, 50 to 100 effective sites per cell)

in low ionic strength buffer, pH 8.0, was added to 10 ml of a 1:100 dilution of a stock solution of phosphonate in acetone. Ten ml of a 1:50 dilution of the stock inhibitor in 50 per cent acetone was used in the case of the aminoalkyls. All solutions were brought to 25.0°C before mixing, and were maintained at 25.0°C in a water bath during the reaction. One ml aliquots were removed at 3 minutes and 10 minutes after mixing, and after that at 10-minute intervals for 60 minutes. The samples were immediately added to 5 ml of ice-cold, low ionic strength buffer, pH 7.4, and the cells immediately centrifuged, washed in the latter buffer, resuspended

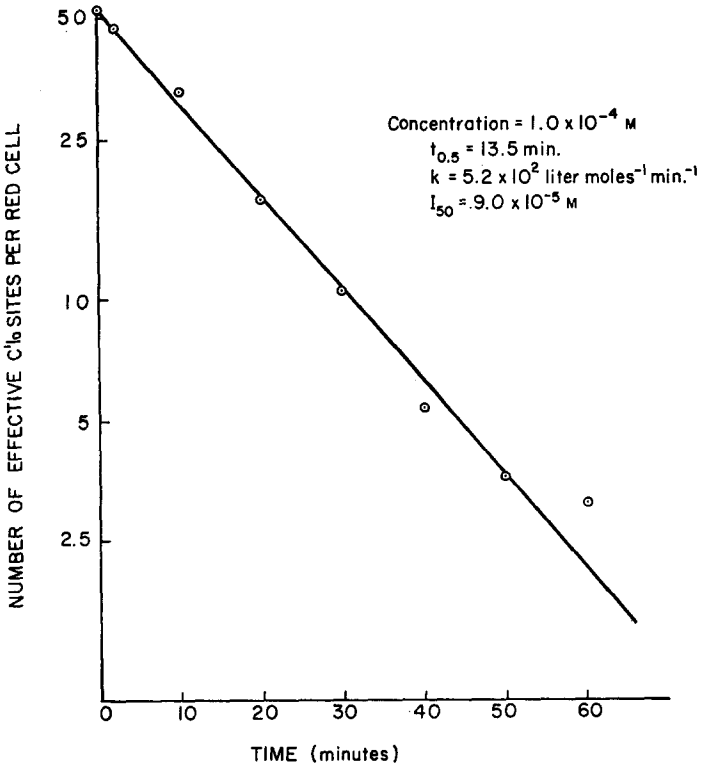


FIG. 1. The progressive, irreversible inhibition of C'1a by *p*-nitrophenylethyl phenyl phosphonate.

in 1 ml TBS and stored at 1°C until the sampling was complete. At this time, a 1:75 dilution of cells was prepared from the contents of each tube. Five-tenths ml of this was added to 0.5 ml of EAC'4 and assayed for the number of effective EAC'1a, 4 sites remaining, essentially as described in reference 19. If necessary the number of effective sites remaining was corrected for any non-specific loss exhibited in the control.

In the case where inhibitory activity was low because the compounds were too insoluble to be run at a sufficiently high concentration the sampling was done at 20-minute intervals for 2 hours.

A control tube containing 10 ml of EAC'1a, 4, to which 10 ml of 1 per cent acetone in low ionic strength buffer, pH 8.0, was added, was sampled at zero time, 30 minutes, and 1 hour.

If the 2 hour time was employed the control tubes were sampled also at 90 and 120 minutes.

The reaction between organophosphorus inhibitors and susceptible enzymes is a progressive, bimolecular one; with sufficient excess of inhibitor over enzyme the rate is first order, independent of the enzyme concentration, and directly proportional to inhibitor concentration (reference 1, p. 120). This also holds for the reaction of phosphonate esters with C'1a, as is demonstrated in Fig. 1. From the slope of the straight line obtained by plotting the logarithm of the effective sites (s) remaining against the time (t) at a given inhibitor concentration (I), the bimolecular rate constant (k) was calculated by means of Equation 1:

$$\log_{10} s = \frac{-kI}{2.303t} \tag{1}$$

For ease of comparison with previous work, and also for convenience of presentation, the so called I_{50}, the molar concentration of inhibitor giving 50 per cent inhibition when inhibitor and cells were allowed to stand 15 minutes at 25°C was calculated by means of Equation 2:

$$I_{50} = \frac{0.0463}{k} \tag{2}$$

From the I_{50}, the negative logarithm of the I_{50}, termed pI_{50} was obtained.

Inhibition of Whole Complement by Phosphonate Inhibitor.—The inhibition of whole guinea pig hemolytic complement by phosphonate inhibitors at 37°C was studied as described in reference 3. The activity remaining was expressed in terms of the ratio of the volume of a given dilution of complement without inhibitor giving 50 per cent hemolysis to the volume of the same dilution of complement with inhibitor also giving 50 per cent hemolysis (3).

Histamine Release.—Guinea pigs were sensitized with 10 per cent egg albumin as described (12) except that one to two intraperitoneal booster injections of 1 mg egg albumin in alum were given 2 to 6 weeks prior to the day of experimentation. Perfused, sliced, washed, lung tissue was prepared and divided into replicate samples as described (12). The replicate samples were washed twice and suspended in 3.0 ml warm Tyrode's solution with or without inhibitor. The solution of inhibitor was prepared by adding 0.1 ml of stock inhibitor in acetone to 59.9 ml of Tyrode's solution; control solution without inhibitor contained the same concentration of acetone (0.17 per cent). This concentration of acetone was found not to interfere with histamine release by antigen. Ten seconds after the addition of inhibitor to tissue 1.0 mg of egg albumin per ml of reaction mixture was added. After 15 minutes at 37°C the supernate was removed and assayed for histamine (12).

The comparison of the activity of a given series of phosphonate inhibitors, or of one inhibitor with another, was made within the same experiment using triplicate tissue samples for each concentration of each inhibitor.

Enzymes exist in blood and tissues which are capable of hydrolyzing and thus inactivating organophosphorus esters (20). In order to be certain that hydrolysis by the lung tissue was not responsible for any differences in inhibitory activity, the ability of perfused, sliced lung tissue to hydrolyze each of the phosphonate esters used was tested by measuring the increase of free p-nitrophenol after a 15 minute reaction period at 37°C. None of the phosphonates described here were hydrolyzed to an extent greater than 2 per cent of the initial concentration of phosphonate ester, showing that the differences observed could not be due to an accelerated breakdown of a particular phosphonate.

RESULTS

Inhibition of Complement Activity.—

Inhibition of C'1a: Fig. 2 illustrates the effect of structural alterations within the series of phenyl alkyl (Fig. 2, *A*), alkyl (Fig. 2, *B*), and ω-chloroalkyl phos-

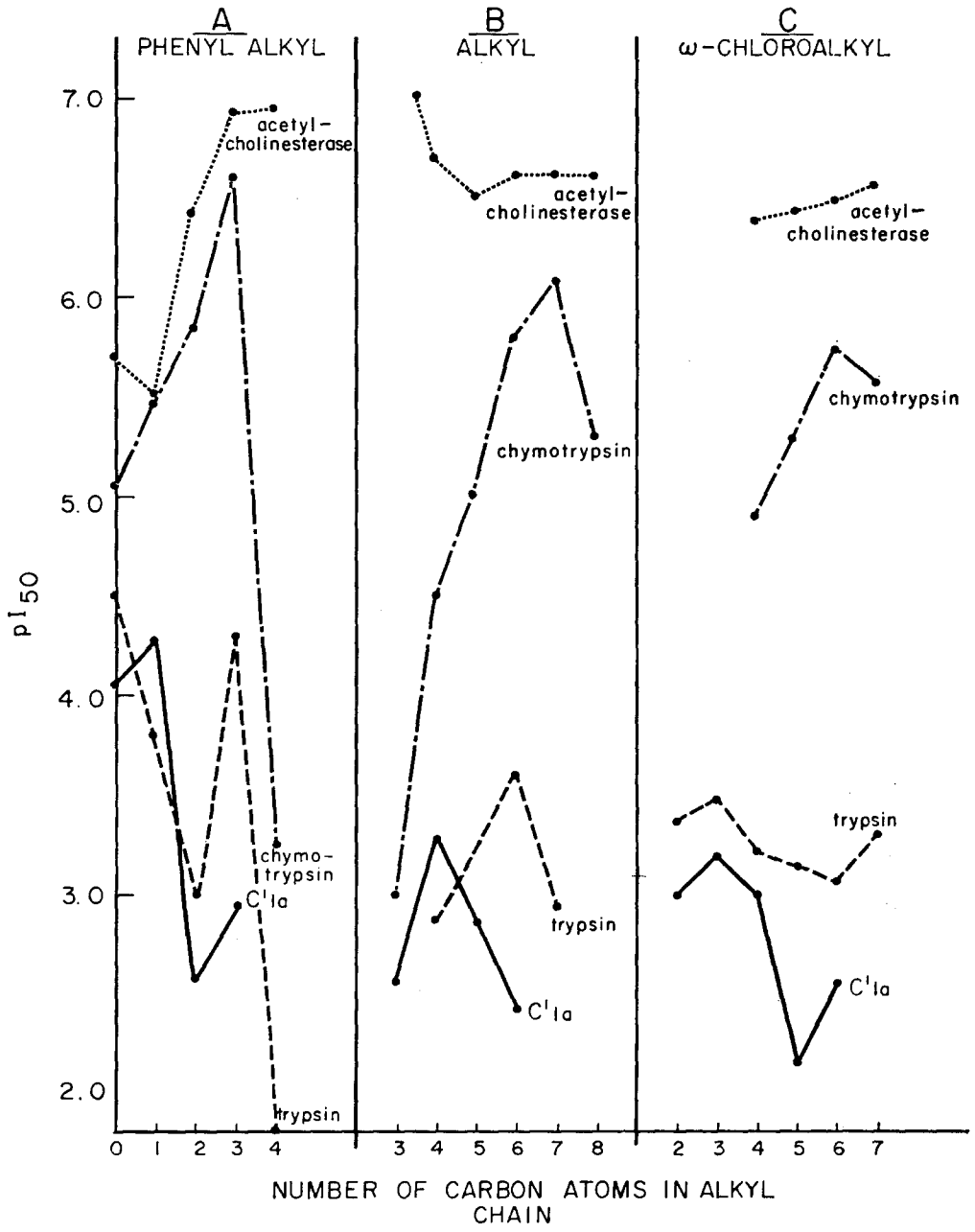


FIG. 2. The relationship between the structure of the *p*-nitrophenylethyl phenyl alkyl (*A*), alkyl (*B*), and ω-chloroalkyl-phosphonates, (*C*), and the inhibition of acetylcholinesterase, ... ; chymotrypsin, —·—·—· ; trypsin, — — —; and C'1a, ————.

phonates (Fig. 2, *C*) on the inhibition of C'1a. For comparison, the inhibitory activity of these same compounds against "true" acetylcholinesterase, trypsin, and chymotrypsin reported previously (7, 15) is also plotted. The inhibition pattern for acetylcholinesterase differs completely from any of the other enzymes.

In the phenyl alkyl series, C'1a shows a distinct peak in susceptibility to inhibition with the benzyl, and a minimum with the phenyl ethyl phosphonate. Trypsin shows one maximum at the phenyl phosphonate and another at the phenyl propyl phosphonate, but like C'1a has a minimum sensitivity to phenyl ethyl phosphonate. Chymotrypsin demonstrates a single peak in sensitivity at the phenyl propyl phosphonate.

In the alkyl phosphonate series, the optimum activity against C'1a, trypsin, and chymotrypsin occurs respectively with butyl phosphonate, hexyl phosphonate, and heptyl phosphonate.

TABLE I

Effect of a Terminal Amino Group on Inhibition of C'1a

	I_{50}
5-Aminopentyl phosphonate	1.5×10^{-5}
6-Aminohexyl phosphonate	3.5×10^{-5}
Pentyl phosphonate	1.4×10^{-3}
Hexyl phosphonate	3.7×10^{-3}

With the ω-chloroalkyl phosphonates, chymotrypsin gives a single peak at the 6-chlorohexyl phosphonate, whereas both C'1a and trypsin show peak activity at the 3-chloropropyl phosphonate. However, C'1a has a sharp minimum in susceptibility to inhibition with the 5-chloropentyl phosphonate, whereas with trypsin, a much more shallow, but distinct minimum occurs at the 6-chlorohexyl phosphonate.

Table I demonstrates that placing an amino group on the terminal carbon of the pentyl and hexyl phosphonate increases their inhibitory potency against C'1a approximately 100-fold.

Inhibition of whole complement: The inhibitory activity of the phenyl alkyl phosphonates against the hemolytic activity of commercial fresh frozen complement is depicted in Fig. 3, *A* and *B*. Fig. 3, *A* demonstrates that a straight line relationship is obtained when the logarithm of the per cent activity remaining is plotted against the concentration of *p*-nitrophenylethyl phenylphosphonate. This result is expected from Equation 1, when the time, t, is held constant, and the inhibitor concentration is varied. The time, t, that the inhibitor has to act is presumably the interval between the activation of C'1p to C'1a and the action of C'1a on C'4 and C'2. This time, of course, is unknown,

but from the results it is apparently constant. Thus, the concentration giving 50 per cent inhibition of whole complement action can by symbolized by I_{50*}, the * signifying that the time is not known. The value for I_{50*} is obtained directly from the plot of the per cent activity remaining *versus* concentration of the inhibitor. The negative logarithm of I_{50*} then becomes pI_{50*}. In Fig. 3, *B*,

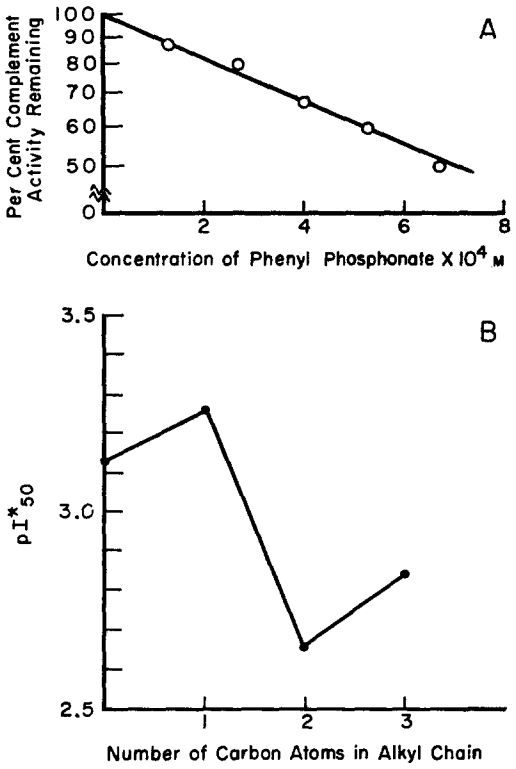


FIG. 3. (*A*), The inhibition of whole complement activity (C′) by increasing concentrations of *p*-nitrophenylethyl phenyl phosphonate. (*B*), The relationship between the structure of the *p*-nitrophenylethyl phenyl alkyl phosphonates and the degree of inhibition of whole complement.

pI_{50*} is plotted against the number of carbon atoms in the alkyl chain of the phenyl alkyl phosphonate series. It is evident that as with C′1a (Fig. 2, *A*) the benzyl phosphonate gives maximum and the phenyl ethyl phosphonate gives minimum inhibition.

Fresh frozen guinea serum obtained from guinea pigs in our own laboratory or commercial lyophilized complement was used to test the inhibitory activity of the alkyl phosphonates. When the logarithm of the per cent activity remaining was plotted against the concentration of the propyl, butyl, or pentyl phos-

phonate, a straight line extrapolating to 100 per cent activity at zero concentration was not obtained with the fresh frozen serum. However, with the commercial lyophilized complement a straight line extrapolating to 100 per cent at zero concentration was observed and a peak in the inhibitory activity was obtained with the butyl phosphonate.

Inhibition of Antigen-Induced Histamine Release.—The inhibition given by varying concentrations of the phenyl, 3-chloropropyl, benzyl, and butyl phos-

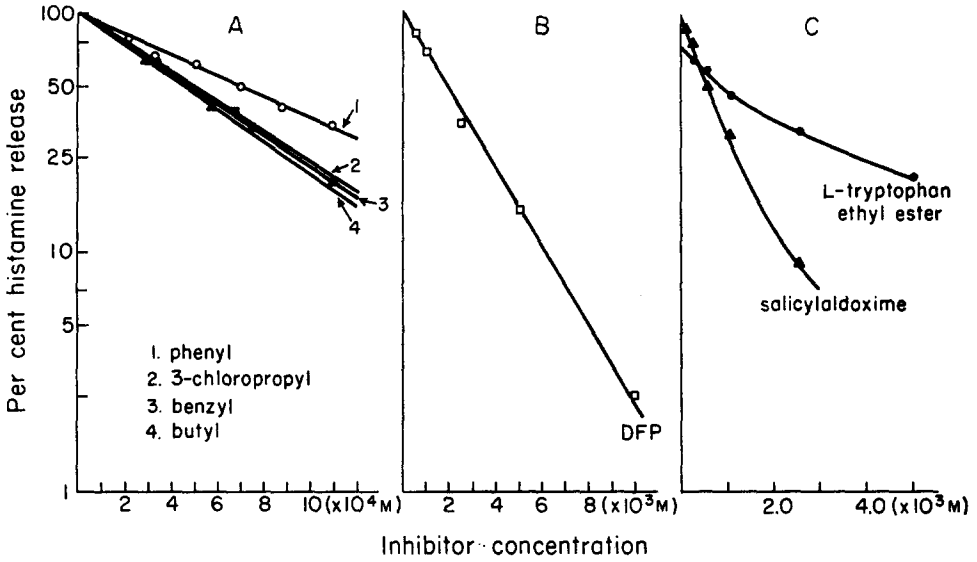


FIG. 4. The relationship between the logarithm of the per cent of histamine release remaining and the concentration of (*A*) phosphonate inhibitors, (*B*) diisopropyl phosphofluoridate (data from reference 12) and (*C*) L-tryptophan ethyl ester and salicylaldoxime (data from references 12 and 21 respectively). Antigen-induced histamine release in buffer without inhibitor was arbitrarily assigned a value of 100 per cent.

phonates are seen in Fig. 4, *A*. All of the inhibitors gave a straight line when the logarithm of the per cent histamine release remaining was plotted against the inhibitor concentration. Replotting of the data (Fig. 4, *B*) for inhibition of histamine release by diisopropyl phosphofluoridate reported previously (12) demonstrated the same relationship, even to 97.5 per cent inhibition. The previously reported data for inhibition of histamine release by salicylaldoxime (21) and L-tryptophan ethyl ester (12) failed to give a straight line extrapolating to 100 per cent at zero inhibitor concentration (Fig. 4, *C*).

The concentration giving 50 per cent inhibition, I_{50*} was obtained for each phosphonate by plotting the logarithm of the per cent histamine release remaining against inhibitor concentration. From this the pI_{50*} was calculated. The *,

as with whole complement inhibition, signifies that the time interval over which the inhibitor is acting is unknown.

In Fig. 5, the pattern of inhibition (pI_{50*}) of antigen-induced histamine release given by the phenyl alkyl, alkyl, and chloroalkyl phosphonates is com-

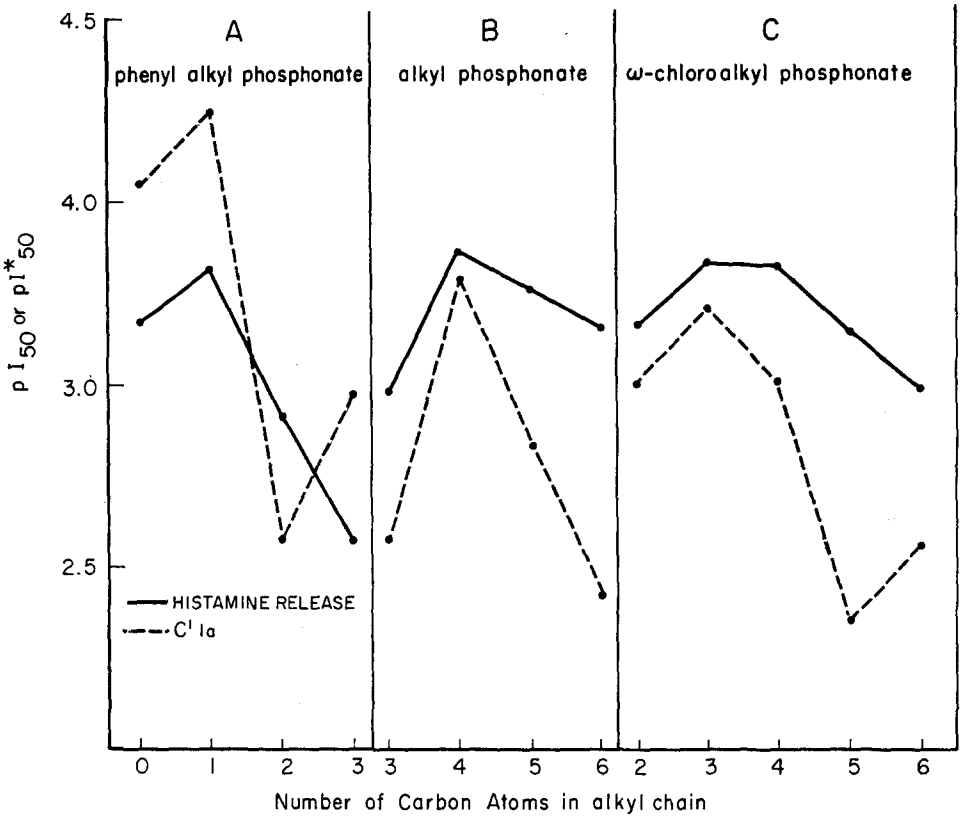


FIG. 5. The relationship between the structure of the *p*-nitrophenylethyl phenyl alkyl (*A*), alkyl (*B*), and ω-chloroalkyl phosphonates, (*C*), and the inhibition of antigen-induced histamine release from guinea pig lung and of activated first component of guinea pig complement, (C'1a).

pared to the inhibition of C'1a given by these same compounds. In the phenyl alkyl series (Fig. 5, *A*) benzyl phosphonate gives peak inhibition of both histamine release and C'1a but there is no minimum in inhibition of histamine release with the phenylethyl phosphonate. In the alkyl series (Fig. 5 *B*), butyl phosphonate shows peak activity against both histamine release and C'1a. In the chloroalkyl series (Fig. 5, *C*) 4-chlorobutyl phosphonate is as active an inhibitor of histamine release as the 3-chloro compound whereas the 3-chlorophos-

phonate is distinctly more active as an inhibitor of C'1a. Moreover the 5-chloropentyl phosphonate is a stronger inhibitor of histamine release than the 6-chlorohexyl phosphonate, although the reverse is true of the inhibition of C'1a.

The inhibition of histamine release by 5-aminopentyl and 6-aminohexyl phosphonate was compared to that given by pentyl and hexyl phosphonate (Table II). The addition of an amino group to the terminal carbon on the pentyl and hexyl phosphonates considerably depresses the ability of the phosphonates to prevent histamine release; this contrasts markedly with the increased inhibitory activity against C'1a produced by the same substitution (Table I).

As is also seen in Table II, the presumed reversible inhibitor TAMe (tosyl L-arginine methyl ester) gave no inhibition of histamine release whether it

TABLE II

Effect of Basic Terminal Group on the Inhibition of Anaphylactic Histamine Release

	Concentration $\times 10^4$	Inhibition	I_{50}* $\times 10^4$
	M	*per cent*	*M*
5-Aminopentyl phosphonate	5.0	19	16
Pentyl phosphonate	5.0	47	5.5
6-Aminohexyl phosphonate	5.0	9.4	44
Hexyl phosphonate	3.3	26	7.4
TAMe‡	100	0	
TAMe preincubated 5 minutes	100	0	

‡ Tosyl L-arginine methyl ester.

was added to the tissues in the standard fashion, 10 seconds before adding the antigen, or allowed to preincubate with the tissue 5 minutes before addition.

DISCUSSION

It is evident that the pattern of inhibition of these phosphonate esters against C'1a is as unique as that observed previously for acetyl-cholinesterase, chymotrypsin, and trypsin (7, 14). The pattern of inhibition of acetylcholinesterase is completely different from that of chymotrypsin, trypsin, and C'1a (Fig. 2). Chymotrypsin has only a few points of likeness to the latter two enzymes. C'1a and trypsin, however, although obviously different have an over-all similarity in the specificity of their reactions with the various phosphonates: in the phenyl alkyl series both enzymes give a minimum in activity with phenylethyl phosphonate; in the chloroalkyl series, 3-chloropropyl phosphonate gives maximum inhibition of both enzymes and with the two enzymes an increase in chain length leads to compounds showing a distinct mimimum in activity; moreover, placing an amino group on the terminal carbon of the alkyl phosphonate clearly facilitates the reaction with both enzymes. C'1a and trypsin might, therefore,

be called "parazymes"[2]; *i.e.*, enzymes which have a similar although not identical specificity. Here, the relation is defined in terms of the specificity of reaction with the hemisubstrates, not the complete substrates.

The pattern of reactivities of the phosphonate esters given by the various enzymes is a reflection of the structure of the enzyme, and more particularly of the active center of the enzyme. Thus, the inhibition by phosphonates can be used to draw inferences as to the nature of the active center in the same manner as have the reactions of enzymes with complete substrates.

The presence of an anionic site in trypsin at a definite distance from the esteratic site is suggested by the fact that positively charged amino acids such as lysine and arginine are required in the preferred substrates for trypsin. The minimum in inhibition of trypsin given by the 6-chloro-hexyl phosphonate might be due to the repulsion of the electron-rich chloro group by the putative anionic site (15). By the same reasoning, the minimum in the inhibition of C'1 given by the 5-chloropentyl phosphonate could be due to the possession of a similar anionic group. The difference between trypsin and C'1a, or at least one of the differences, might then be in the distances between the anionic and esteratic sites in the two enzymes; the distance in C'1a possibly being less than in trypsin. The above reasoning is in accord with both the fact that C'1a is active against TAMe, and not against lysine ethyl ester.

With certain organophosphorus inhibitors and certain enzymes it has been demonstrated that the elements of structure which are of importance for the activity of a complete substrate with an enzyme are the same as the elements of structure which are of importance for the reaction of the hemi-substrate with the same enzyme (7, 22, 23). This can be expected to be true only if the acylation reaction is the rate-limiting step in the reaction of the enzyme with the complete substrate (7, 24) because this is the reaction which corresponds to phosphorylation by the organophosphorus inhibitor.

Acylation is apparently the rate-limiting reaction of fatty acid esters with trypsin (25), and the alkyl phosphonate giving optimum inhibition of trypsin corresponds in chain length to the fatty acid ester showing maximum substrate activity with the same enzyme (7). The marked increase of inhibitory activity of the aminoalkyl phosphonates with both trypsin (26) and C'1a (Table I) compared to the corresponding alkyl phosphonates suggests that acylation is rate-limiting in the reaction of these enzymes with esters of the basic amino acids. The addition of an amino group to the pentyl phosphonate increases its inhibitory activity against trypsin 4000-fold (26) but only 100-fold against C'1a. This is in accord with the fact that lysine methyl ester is a good substrate for trypsin, but is not a substrate of C'1a, (9). This quantitative correspondence between the structural requirements for a good substrate and a good inhibitor

[2] We wish to thank Dr. George Fostoropoulous for suggesting the prefix for this term.

can be taken as additional evidence that the acylation reaction is rate-limiting with these substrates.

When the deacylation reaction is rate-limiting there is no longer any necessary structural relationship between substrate and inhibitor. Deacylation is the rate-limiting step in the reaction of chymotrypsin with substrates containing aromatic amino acids (4, 5). This may explain the finding that phenylethyl phosphonate, corresponding in length of alkyl chain to phenylalanine is not the best of the phenyl alkyl phosphonate inhibitors of chymotrypsin (Fig. 2, *A*) (7). C'1a is capable of hydrolyzing substrates containing aromatic amino acids (9, 27), nevertheless, the best phenyl alkyl phosphonate inhibitor of C'1a is the benzyl and not phenylethyl phosphonate. This lack of correspondence between the structural requirements for inhibitory and substrate activity suggests that with C'1a, the deacylation reaction is rate-limiting.

The pattern of inhibition of histamine release by the alkyl, phenyl alkyl, and chloroalkyl phosphonates is similar to the inhibition of C'1a by these same compounds (Fig. 5 *A*, *B*, and *C*). This is additional evidence for the previous suggestion (12) that the activation of an organophosphorus-inhibitable esterase is necessary for the antigen-induced histamine release of guinea pig lung. The linear dose-response curve of inhibition of histamine release by organophosphorus inhibitors (Fig. 4, *A* and *B*) is further support for such an hypothesis. This is underlined by the difference in dose-response curve given by L-tryptophan ethyl ester and salicylaldoxime which are presumably reversible competitive inhibitors. An additional implication of the straight line relationship between the logarithm of the per cent histamine release remaining and the concentration of organophosphorus inhibitor is that the amount of histamine released is directly proportional to the concentration of activated esterase.

Although there is a great similarity in the patterns of inhibition of C'1a and of histamine release given by the phosphonate esters there are also distinct and definite differences. In contrast to C'1a, there is no minimum in the inhibition of histamine release by the phenyl ethyl phosphonate; there is no well defined peak in inhibition of histamine release by the 3-chloropropyl phosphonate; no minimum in inhibition by the 5-chloropentyl phosphonate; and the aminoalkyl phosphonates are distinctly less active inhibitors of histamine release than the corresponding alkyl phosphonates (Table II).

In determining whether these differences signify that different enzymes are operating in the two systems there are several factors to be considered. The inhibition of C'1a, involves the inactivation of a single enzyme already existing in the activated state, whereas in the inhibition of histamine release, we are forced to assume that it is the inactivation of only one enzymatic step which is being studied. Moreover, in this latter system the esterase exists in an inactive form, and only after activation is it susceptible to inhibition by the phosphonate (12). The fact that a linear relation exists between the logarithm of the per-

centage histamine release remaining and the concentration of inhibitor (Fig. 5, *A* and *B*) not only indicates that the necessity for activation does not cause any disturbance in the reaction of organo-phosphorus inhibitor with enzyme, but also that a single step is being inhibited. This suggestion is further strengthened by the finding with whole complement where there is also a requirement for the activation of an organophosphorus-inhibitable esterase. In this instance, the linear relationship in the inhibition of whole complement by the phosphonates is obtained (Fig. 3, *A*), and there is a parallelism between the pattern of inhibition given by the phenyl alkyl phosphonates acting on C'1a and on whole complement (Fig. 3, *B*).

The phosphonates inhibit C'1a on the surface of the red cell, and there is no question of there being differences in the access of the various inhibitors to the enzyme. In the case of the inhibition of histamine release from sliced guinea pig lung this question can not be so easily dismissed. The target cell, presumably the mast cell, is imbedded in the tissue and there very well may be barriers limiting access of the inhibitor to the target cell either on the basis of the size of the inhibitor, lipid solubility, or charge.

These presumed barriers do not prevent egg albumin, the antigen, with a molecular weight over 100 times that of the inhibitor from initiating histamine release within 15 seconds of its addition to the finely chopped sensitized tissue (12). On the other hand, the inhibitors are distinctly more lipid-soluble than egg albumin raising the possibility that the pattern of inhibition reflects the lipid solubility of the inhibitors. This is not likely, since in no case is the maximum of inhibition given by either the most water-soluble or most lipid-soluble compound of any of the homologous series studied. The poor inhibition of histamine release exhibited by the aminoalkyl phosphonates, or by TAMe (Table II) could be due to the binding of the positively charged inhibitors by some element of the tissue. There is no present evidence for or against this possibility. Our tentative conclusion, however, is that the organophosphorus-inhibitable enzymes in the two guinea pig systems, serum complement, and antigen-induced histamine release from lung are similar but different; *i.e.* they are parazymes.

This conclusion immediately raises the question of whether the enzyme in quinea pig lung is in fact "chymotrypsin-like" as has been suggested (12). In the phenyl alkyl series, there is a difference of two carbon atoms between the benzyl phosphonate giving peak inhibition of histamine release, and phenyl propyl phosphonate giving peak inhibition with chymotrypsin. Similarly, the peak is shifted by 3 carbon atoms in the alkyl series and between 2 and 3 carbon atoms in the chloroalkyl series. However, the aminoalkyl compounds are not only less active as inhibitors of histamine release, but as inhibitors of chymotrypsin as well (26). The differences in the patterns of inhibition of histamine release and of chymotrypsin inactivation by the phosphonates are more evident

than the similarities. From this latter point of view, the guinea pig lung enzyme can not be considered chymotrypsin-like. However, if chymotrypsin-like implies the capability of splitting characteristic chymotrypsin substrates such as aromatic amino acid esters, the answer must be more equivocal. It has already been pointed out that no necessary connection between the structure of complete substrates and hemisubstrate need exist unless the acylation of the enzyme by the complete substrate is rate-limiting. This certainly is not true of the reaction of chymotrypsin, nor probably of C'1a with aromatic amino acid–containing substrates and very well might not be true of the guinea pig lung enzyme. Therefore, the dissimilarity in the patterns of reactivity of chymotrypsin and guinea pig lung enzyme with phenyl alkyl phosphonates can not be used as an argument that the guinea pig lung enzyme is incapable of reacting with aromatic amino acid–containing substrates.

The suggestion that the guinea pig lung enzyme is chymotrypsin-like was based on wholly indirect reasoning relating the presence of an organophosphorus-inhibitable step to the ability of histamine release to be depressed by aromatic amino acid–containing derivatives. Here, the ability of the enzyme to bind these aromatic amino acid derivatives and the ability of the enzyme to be phosphorylated by the corresponding phenyl alkyl phosphonates involving as they both do the initial part of the reaction of the enzyme would be expected to be more comparable. On this basis, the difference in inhibitory pattern of the phenyl alkyl phosphonates for histamine release and chymotrypsin is probably significant. It therefore seems probable that the aromatic amino acid derivatives would not be particularly good inhibitors of the guinea pig lung esterase and thus, the inhibition observed is due to their acting at another step in the sequence.

This last conclusion is in accord with the recent finding that the primary site of inhibition of complement by aromatic amino acid derivatives is not on the C'1, organophosphorus-inhibitable step, but on one of the later C'3 steps (28). If the conclusion in regard to the site of inhibition of histamine release by aromatic amino acid derivatives is correct this would be another general similarity with the complement system. It is felt that the differences and similarities between the histamine release reaction and complement reaction which have been detailed here and elsewhere (12) can best be reconciled by the hypothesis that they are two parazymous systems; *i.e.*, systems made up of components which are similar but not identical in their reactivity.

SUMMARY

The ability of a number *p*-nitrophenylethyl alkyl, phenyl alkyl, chloroalkyl, and aminoalkyl phosphonates to inhibit the activated first component (C'1a) of guinea pig complement, and the antigen-induced release of histamine from sliced, perfused guinea pig lung has been compared. C'1a in its reactivity with

these phosphonates is distinctly more similar to trypsin than to any of the other enzymes studied previously. It is suggested that both trypsin and C'1a possess an anionic group in the active center of the respective enzyme, but the distance between the anionic and esteratic site in C'1a might be less than in trypsin.

The pattern of inhibition of histamine relase by the alkyl, phenyl alkyl, and chloroalkyl phosphonates is similar to the inhibition of C'1a by these compounds, although distinct differences are apparent. The aminoalkyl phosphonates are distinctly less active inhibitors of histamine release than the corresponding alkyl phosphonates, whereas the reverse is true of the inhibition of C'1a. On the basis of these differences, it is tentatively concluded that the organophosphorus-inhibitable enzymes in the guinea pig systems studied here are similar but not identical.

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