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THE ENZYMATIC HYDROLYSIS OF *p*-NITROPHENYL ETHYL PHOSPHONATES BY MAMMALIAN PLASMAS

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Abstract—The enzyme in rabbit plasma primarily responsible for the hydrolysis of *p*-nitrophenyl ethyl alkyl and phenylalkylphosphonates has a pH optimum much more alkaline than the enzyme responsible for the hydrolysis of paraoxon. This enzyme is also distinctly less easily inhibited by EDTA and Ba^{2+} than is 'para-oxonase'. The ease of hydrolysis of these compounds by rabbit, guinea pig, and human plasma was greatly affected by the length of the carbon chain linked to the phosphorus. In addition to the effect of the length of the carbon chain the rates of hydrolysis were shown to depend on the activity of the leaving group of the phosphonates.

NUMEROUS investigators have studied the enzymes of blood and tissues which hydrolyze and thus inactivate organophosphorus esters (O'Brien,[1] Heath,[2] Mounter[3]). However, there has been no systematic investigation of the effect of changes of structure in a homologous series of organophosphorus esters on their activity as substrates for these enzymes. This paper presents the results of such a study using a series of *p*-nitrophenyl ethyl *n*-alkylphosphonates and *p*-nitrophenyl ethyl phenylalkylphosphonates as substrates for the hydrolytic enzymes present in rabbits, guinea pig, and human plasma.

MATERIALS AND METHODS

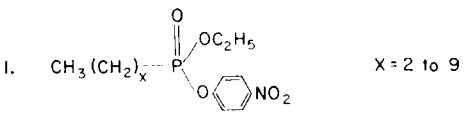
Plasma from individual rabbits and humans, and pools of plasma from three to four guinea pigs were used in each experiment as the source of the enzyme. The plasma was prepared from heparinized blood (7 units heparin/ml) drawn just before use.

The phosphonates were of the general formula seen in Fig. 1. In the series of *n*-alkylphosphonates the number of carbon atoms in the straight chain varied from three to ten; with the phenylalkylphosphonates, the number of carbons between the phenyl group and the phosphorus varied from zero to four. The synthesis* and chemical and biochemical properties of these compounds are given in Fukuto and Metcalf[4] and Becker *et al.*[5] In all experiments the phosphonates were weighed out just before use and dissolved in acetone to give a 2×10^{-2} M solution from which the 1×10^{-3} M stock solution in acetone was prepared.

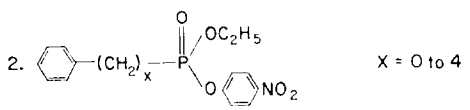
Except when otherwise stated, the rates of hydrolysis were determined at pH 8·45, 37°, and $3{\cdot}33 \times 10^{-5}$ M substrate. The buffer used to maintain this pH was 0·02 M

* The di-*p*-nitrophenyl phenylethylphosphonate was synthesized by D. Canham, B Boone, and E. Boger, the *p*-nitrophenyl ethyl 4-chlorobutylphosphonate and the *p*-nitrophenyl ethyl 6-chlorohexylphosphonate were synthesized by B. Boone. The details of synthesis, etc., will be published by them later.

Tris, 0·15 M NaCl with a pH of 8·52; this gave a pH for the entire mixture of 8·45. The rates of enzymatic hydrolysis were determined by measuring the rate of liberation of *p*-nitrophenol in a Beckman DU spectrophotometer at 410 mμ. The chamber for the cuvets was kept at 37°. Cuvets to which the plasma was to be added, containing 2·35 ml of buffer, 0·1 ml of the stock solution of phosphonate, and 0·05 ml of acetone,



ethyl *p*-nitrophenyl *n*-alkylphosphonate



ethyl *p*-nitrophenyl *n*-phenylalkylphosphonate

FIG. 1. The structure of the *p*-nitrophenyl ethyl *n*-alkyl and phenylalkylphosphonate esters.

and a cuvet to serve as a blank containing 0·15 ml acetone, 2·35 ml buffer, and 0·5 ml plasma, were allowed to equilibrate in the cuvet chamber for 5 min. At the end of that time 0·5 ml of plasma was added and the increase in optical density measured at various intervals depending on the rate of the reaction. The optical density readings were plotted against time, and the slope of the initial linear portion of the curve was used as the measure of the rate of the reaction. When necessary, the slopes (optical density increase/5 min) were transformed by means of a standard curve into micromoles of *p*-nitrophenol per liter liberated per 5 min. A standard curve was prepared for each pH at which the reaction was run. Where required, the rates of hydrolysis were corrected for nonenzymatic hydrolysis; under our conditions, this was necessary only above pH 10·2. In all experiments where the pH was varied, the pH of the reaction mixture was determined after the reaction was completed with a Cambridge research model pH meter.

The variation in enzyme activity from one plasma to another within a species was not great (See Table 1). Nevertheless, in order to reduce this source of variation as

TABLE 1. THE COMPARISON OF THE HYDROLYSIS RATE OF $3{\cdot}33 \times 10^{-5}$ M *p*-NITROPHENYL ETHYL *n*-PENTYLPHOSPHONATE AT pH 8·45 BY RABBIT, GUINEA PIG, AND HUMAN PLASMA

Species	Number of individual plasmas or pools	Mean (mean hydrolysis rate ± S.E.*)
Rabbit†	8	3·75 ± 0·029
Guinea pig‡	8	0·67 ± 0·0039
Human†	5	2·00 ± 0·011

* Micromoles of nitrophenol liberated/liter/5 min.
† Plasma obtained from individual animals.
‡ Plasma pools used.

much as possible, the rates of hydrolysis were usually determined relative to the rate of hydrolysis of the pentylphosphonate,† which was run as a standard in all experiments.

EXPERIMENTS AND RESULTS

The nature of the enzyme in rabbit plasma hydrolyzing phosphonate esters

As expected for an enzymatic reaction, the rate of enzymatic hydrolysis of the phosphonates was found to depend on the concentration of the substrate. As seen in Fig. 2, there is the usual linear relationship between the reciprocal of the velocity of the reaction, $1/v$, and the reciprocal of the substrate, $1/s$. The maximal velocity, V_{max} of the pentylphosphonate is 23 μmoles nitrophenol liberated/liter per 5 min, and the Michaelis constant, K_m, is $2{\cdot}0 \times 10^{-3}$ M for the reaction of pH 8·45.

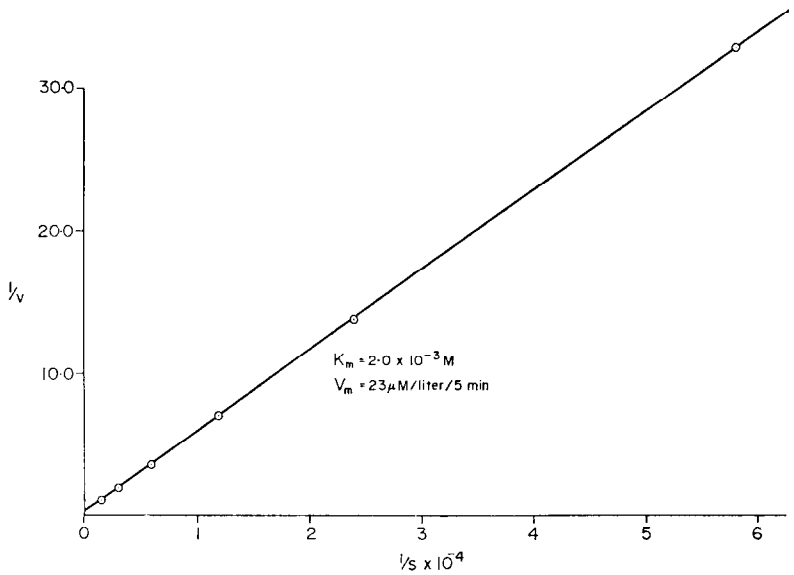


FIG 2. The linear relationship between the reciprocal of the velocity of hydrolysis ($1/v$) by rabbit plasma and the reciprocal of the substrate concentration ($1/s$). The substrate is *p*-nitrophenyl ethyl pentylphosphonate.

Because of the difference in pH one cannot really compare the K_m for the hydrolysis of paraoxon by rabbit plasma as found by Aldridge[6] with the K_m of pentylphosphonate found here. Aldridge has reported that the pH optimum for the hydrolysis of paraoxon (*p*-nitrophenyl diethyl phosphate) by rabbit serum was pH 7·3 and Main[7] found the 'para-oxonase' from sheep serum had the same pH optimum. Preliminary experiments made it evident that the pH optimum for the hydrolysis of the pentylphosphonate ester by rabbit plasma was above pH 8. Since our reaction conditions were distinctly different from those of Aldridge and of Main, the pH activity curves of paraoxon and the pentylphosphonate were compared in the same experiments under the same conditions. In these experiments, 0·02 M Tris buffers were used throughout. As can be seen in Fig. 3 the pH optimum for the hydrolysis of paraoxon was pH 7·49,

* For convenience in what follows, the compounds will be named purely by the alkyl or phenylalkyl group attached to the phosphorus with the presence of *p*-nitrophenyl ethyl groups being understood.

essentially the same as that found by Aldridge and by Main, whereas the pH optimum for the hydrolysis of the pentylphosphonate was above pH 9·0.

In further experiments using no Tris, but Sorenson glycine buffers,[8] the pH optimum was around pH 10·6 (Fig. 4). Much less extensive studies with human and guinea

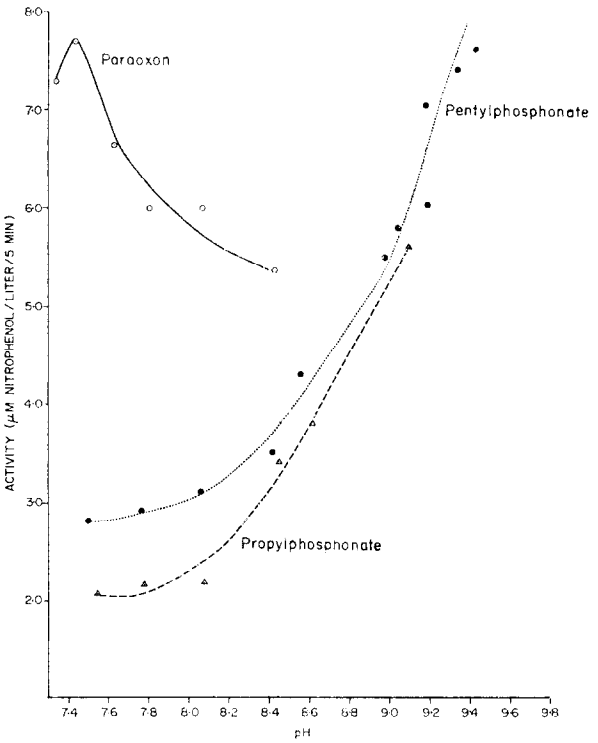


FIG. 3. The comparison of the effect of pH on the activity of rabbit plasma in hydrolyzing paraoxon (——), *p*-nitrophenyl ethyl *n*-pentylphosphonate (· · ·), and *p*-nitrophenyl ethyl *n*-propylphosphonate (- - -).

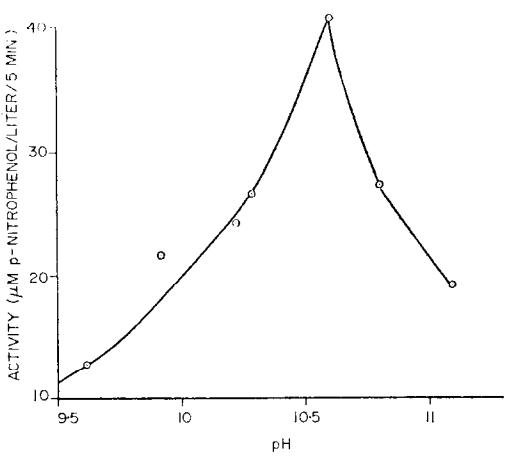


FIG. 4. The effect of pH on the activity of rabbit plasma in hydrolyzing *p*-nitrophenyl ethyl *n*-pentylphosphonate.

pig plasma made it clear that the pH optimum for the hydrolysis of pentylphosphonate with plasma from these species was also above pH 9·0.

The very wide separation in pH optima for the hydrolysis of paraoxon and the pentylphosphonate ester indicated that different enzymes were primarily responsible for the hydrolysis of the two substrates. It then became of interest to see whether it was the length of the carbon chain in the phosphonate or some other feature that caused it to be primarily hydrolyzed by an enzyme other than the 'para-oxonase.' The effect of pH on the ability of rabbit plasma to hydrolyze the propylphosphonate ester was therefore tested. As Fig. 3 shows, the pH activity curve for the hydrolysis of the propylphosphonate parallels that of the pentylphosphonate.

Although it was recognized as desirable to work at the optimum pH for the hydrolysis of the phosphonate esters, we also wished to be as near as possible to physiologic pH. This led to the compromise choice of pH 8·45 as the pH employed in most of the work to be described.

Main[7] showed that ethylenediamine tetraacetic acid (EDTA) and Ba^{2+} inhibit the hydrolysis of paraoxon by the enzyme he isolated from sheep plasma. We therefore compared the effect of these inhibitors on the hydroysis by rabbit plasma of the pentylphosphonate and of paraoxon. In this experiment the effects of EDTA (final concentration 0·005 M) and $BaCl_2$ (0·005 M) on the hydrolysis of paraoxon and the pentylphosphonate were studied at both pH 7·6 and 8·45 with the results seen in Table 2.

TABLE 2. THE INHIBITION BY EDTA AND Ba^{2+} OF THE HYDROLYSIS BY RABBIT PLASMA OF PARAOXON AND *p*-NITROPHENYL ETHYL *n*-PENTYLPHOSPHONATE

		pH 7·6		pH 8·5	
Substrate $3·3 \times 10^{-5}$ M	Inhibitor 0·005 M	Activity (μmoles/liter per 5 min)	Inhibition (%)	Activity (μmoles/liter per 5 min)	Inhibition (%)
Pentylphosphonate	None	3·2		3·9	
	$BaCl_2$	1·7	48	3·3	15
	EDTA	0·6	83	2·5	37
p-Oxon	None	5·4		3·8	
	$BaCl_2$	2·0	63	1·5	60
	EDTA	0·00	100	0·00	100

EDTA inhibited the breakdown of paraoxon completely at both pH's, and gave 83% inhibition of the hydrolysis of pentylphosphonate at pH 7·6 but only 37% inhibition at 8·5 $BaCl_2$ gave 60% inhibition of paraoxon hydrolysis at both pH's, but 48% inhibition of pentylphosphonate breakdown at pH 7·6 and only 15% at pH 8·5. Thus the inhibition of the hydrolysis of paraoxon by the two substances was apparently independent of pH. On the other hand, the activity of the two inhibitors on the hydrolysis of the pentylphosphonate was not only distinctly less than on the hydrolysis of paraoxon, but the degree of inhibition decreased with increase of pH.

The influence of the structure of the phosphonates on their ease of enzymatic hydrolysis by rabbit, guinea pig, and human plasma

The comparisons in the activity of the various phosphonates were all made at only one substrate concentration ($3·33 \times 10^{-5}$ M) and at pH 8·45. It was realized

that the relationships obtained might be somewhat different if the K_m for each substrate at various pH's had been determined. This was not done because of the work involved, and also because the fundamental purpose of the study was to provide information that could be correlated with the toxicity of the phosphonates.

The relative rates of hydrolysis of the alkylphosphonates by rabbit, guinea pig, and human plasma are seen in Fig. 5. The butylphosphonate exhibits a minimum in

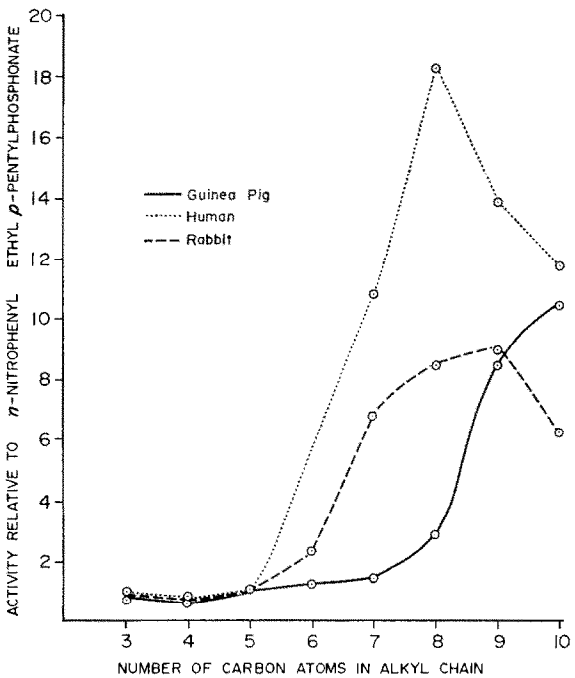


FIG. 5. The relative rates of hydrolysis of *p*-nitrophenyl ethyl *n*-alkyl phosphonates as a function of the number of carbon atoms in the alkylphosphonate side chain by rabbit plasma (- - -), human plasma (· · ·), and guinea pig plasma (———). Ethyl *p*-nitrophenyl *n*-pentylphosphonate equals 1.

activity with the plasma from all three species. The maximal activity is obtained with the octylphosphonate in human plasma and with the nonylphosphonate in rabbit plasma. With guinea pig plasma the decyl is distinctly more active than the nonylphosphonate. The comparisons of the activity of the octyl, nonyl, and decylphosphonates were made on the same guinea pig, human, and rabbit plasmas at the same time.

The relative rates of hydrolysis of the phenylalkylphosphonates by plasma from the same three species are pictured in Fig. 6. In all cases the benzylphosphonate is hydrolyzed least rapidly. There is an increase in activity with the phenylphosphonate, but this is relatively greater with the human plasma than with rabbit plasma, and relatively much greater with guinea pig plasma. There is a distinct maximum in activity at the phenylpropylphosphonate with rabbit and human plasma, but this maximum is shifted to the phenylethylphosphonate with guinea pig plasma.

The rates of hydrolysis by rabbit and guinea pig plasma of four other phosphonates were determined in addition to those already considered. The compounds and their rates of hydrolysis are seen in Table 3. The di-*p*-nitrophenyl phenylethylphosphonate was so insoluble that the highest concentration at which it could be studied was $1{\cdot}0 \times 10^{-5}$ M. Because of this low concentration, and the necessity to test the *n*-pentylphosphonate at the same concentration, we experienced much greater variation

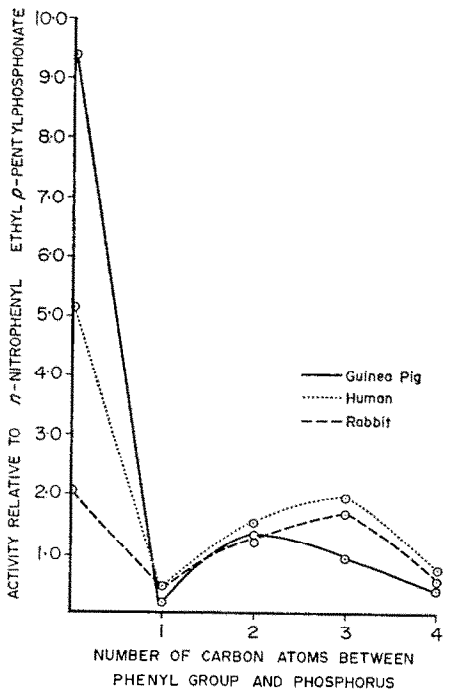


FIG. 6. The relative rates of hydrolysis of *p*-nitrophenyl ethyl phenylalkylphosphonates as a function of the number of carbon atoms in the alkyl portion of phenylalkylphosphonate by rabbit plasma (- - -), human plasma (· · ·), and guinea pig plasma (———). Ethyl *p*-nitrophenyl *n*-pentylphosphonate equals 1.

from one experiment to another than was the case with the other compounds. Table 3 shows the great differences obtained in three independent determinations of the hydrolysis rates of the di-*p*-nitrophenyl phenylethylphosphonate. Nevertheless, it is evident that the di-*p*-nitrophenyl phenylethylphosphonate is split very rapidly.

DISCUSSION

Augustinsson and Heimburger,[9] on the basis of summation experiments, concluded that two A esterases existed in rabbit serum capable of hydrolyzing organophosphorus esters. One of them, 'A esterase I', (Ref. 2, p. 167), hydrolyzes di-isopropylphosphorofluoridate (DFP), and ethyl N,N-dimethyl phosphoramidocyanate (Tabun), but not paraoxon or tetraethylpyrophosphate (TEPP), whereas the other 'A esterase II' (Ref. 2, p. 167), hydrolyzes paraoxon and probably TEPP, but not DFP or Tabun. Aldridge[6] and Mounter[10] showed that A esterase I of rabbit serum hydrolyzed both DFP and *p*-nitrophenyl acetate. Mounter *et al.*[11] showed that the pH

optimum for the hydrolysis of *p*-nitrophenyl acetate is pH 7·8; the pH optimum was not determined with DFP as the substrate.

Our work indicates that the enzyme in rabbit plasma primarily responsible for the hydrolysis of the phosphonate esters has a pH optimum much more alkaline than A esterase II (paraoxonase), and is also distinctly less easily inhibited by EDTA and Ba^{2+}. Accepting that the pH optimum of A esterase I is, in fact, at pH 7·8, one has to accept that the enzyme splitting phosphonate esters differs from A esterase I. Thus our work suggests that in rabbit plasma there exist at least three A esterases: A esterase I, A esterase II, and the enzyme described here, A esterase III. In addition, the limited work we have done on the effect of pH on the activity of guinea pig and human plasma in splitting phosphonate esters suggests that an enzyme similar to A esterase III also exists in these species.

TABLE 3. THE RATES OF ENZYMATIC HYDROLYSIS, RELATIVE TO *p*-NITROPHENYL ETHYL *n*-PENTYLPHOSPHONATE, OF FOUR PHOSPHONATE ESTERS

	Rates of hydrolysis	
	Rabbit	Guinea pig
p-Nitrophenyl ethyl 4-cholrobutylphosphonate	1·10	0·65
p-Nitrophenyl ethyl 6-chlorohexylphosphonate	2·25	0·85
p-Nitrophenyl ethyl 2-phenylethylenephosphonate*	2·80	7·6
Di *p* nitrophenyl phenylethylphosphonate†	7·1, 10·6, 17·8	27·5

* Also called *p* nitrophenyl ethyl styrylphosphonate.

† Tested at $1·0 \times 10^{-5}$ M instead of $3·3 \times 10^{-5}$ M as with all other compounds.

Our data do not allow the conclusions that A esterase II is completely incapable of splitting phosphonate esters, nor that A esterase III cannot hydrolyze paraoxon. It is possible, for example, that the decrease in effectiveness of Ba^{2+} and EDTA on the hydrolysis of pentylphosphonate (Table 2) as the pH is raised is an expression of the ability of A esterase II to act on the phosphonate. Other explanations, however, are at least as plausible.

Even though the difference in specificity between the two enzymes may not be absolute, it is at the very least considerable. The only difference between *p*-nitrophenyl diethyl phosphate (*p*-oxon) and *p*-nitrophenyl ethyl propylphosphonate is the substitution of a carbon atom in the phosphonate for an oxygen in one of the ethoxy groups in paraoxon; nevertheless, the two enzymes clearly distinguish between them. The basis for such differentiation is not the length of the groups; there is very little difference between the propyl or ethoxy group in this regard. It presumably is in the possession of a P–O bond in one case and a P—C bond in the other. This would then be another example of the effect on biochemical reactivity of substituting a P—O for a P—C bond.[12–14]

Much work on the relationship of the structure of phosphorus esters to their ability to inhibit susceptible esterases (B esterases) has shown that the inhibitory ability depends on the activity of the 'leaving group'* as well as on the structure of

* The leaving group in the case of the phosphonate esters studied here is the *p*-nitrophenyl group.

the remainder of the molecule.[1, 2, 15] The same factors evidently affect the activity of the phosphonate esters as substrates for the A esterase, strengthening the conclusion that the mechanism of the inhibition of B esterases by phosphorus esters and the substrate activity of phosphorus esters on A esterases is fundamentally the same, at least for the phosphorylation part of the reaction.[1, 2, 15]

The activity of the leaving group is measured by the ease with which the ester is hydrolyzed nonenzymatically.* The much higher substrate activity of di-*p*-nitrophenyl phenylethylphosphonate and the nitrophenyl ethyl 2-phenylethylenephosphonate compared to the phenylethylphosphonate could be because of the higher activity of the leaving groups of the first two compounds ($K_1 = 190 \times 10^{-4}$ and $26{\cdot}9 \times 10^{-4}$ min^{-1} respectively) compared to the last ($6{\cdot}6 \times 10^{-4}$ min^{-1}). The increase in substrate activity of the phenylphosphonate, compared to the benzylphosphonate, also can be correlated with the increased leaving tendency of the *p*-nitrophenyl in the phenylphosphonate ($K_1 = 26{\cdot}4 \times 10^{-4}$ min^{-1}) compared to the benzylphosphonate ($K_1 = 7{\cdot}05 \times 10^{-4}$ min^{-1}).

The differences in substrate activity among the rest of the phosphonates studied cannot, however, be explained by differences in the leaving tendency of the *p*-nitrophenyl group. From the propyl to the heptylphosphonate there is no real change in the rate of nonenzymatic hydrolysis, with a decrease occurring with the octyl, and a further decrease with the decyl.[4, 5] The substrate activity curves in Fig. 6 are completely different. Similarly, from the benzyl to the phenylbutylphosphonate there is no variation in the nonenzymatic rates of hydrolysis even though the rates of enzymatic breakdown change distinctly. Thus the changes in substrate activity of these compounds depend on the structure of the alkyl and phenylalkyl groups and presumably reflect the 'goodness of fit' of these groups to the enzyme. The exact interpretation to be put on this 'goodness of fit' in terms of the mechanism of action of the A esterase on these phosphonates must await further work on the extensively purified enzyme.

* The nonenzymatic, first-order hydrolysis constants, K_1, were measured at pH 8·3, 0·067 M PO_4, 37°.[4, 5]

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