Intramolecular General Acid Catalysis of Intramolecular Nucleophilic Catalysis of the Hydrolysis of a Phosphate Diester

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The dianion (8) of bis-2-carboxyphenyl phosphate (half-life 10.2 min at 39 °C) is hydrolysed *ca.* 10^{10} times faster than diphenyl phosphate. The reaction is accounted for in terms of intramolecular general acid catalysis by the *ortho*-CO_2H of one salicyl group of the breakdown of the penta-covalent intermediate formed by the addition to phosphorus of the carboxylate group of the other. The general acid catalysis part of the process is unexpectedly inefficient.

In a series of papers in the early seventies we showed that the neighbouring carboxylate group is a powerful nucleophilic catalyst for the hydrolysis of phosphate esters.[1-3] The effective molarity (EM) [4] of CO_2^- in the reaction of phenyl salicyl phosphate (1), for example, is 6×10^8M. This reaction is thought [1] to involve a penta-covalent intermediate (2), for which pseudorotation [5] is inhibited by the presence of the two negatively charged substituents in the equatorial plane.

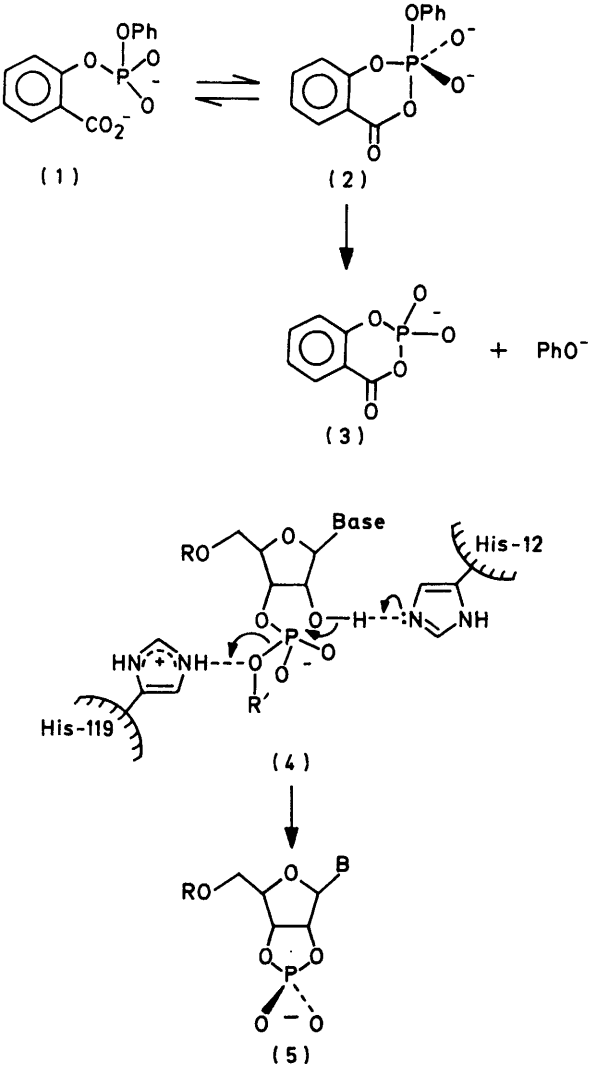
Consequently [5] only the two apical substituents, the carboxylate and phenolate groups of (2), can be lost when it breaks down. Of the three good potential leaving groups on phosphorus these are the best and worst, respectively,[3] so that the rate-determining step for the formation of the cyclic acyl phosphate (3) must be the breakdown of (2).[1]

Intramolecular nucleophilic catalysis of phosphate diester hydrolysis is also involved in the hydrolysis of ribonucleic acid catalysed by ribonuclease A.[6,7] The enzyme reaction involves the formation of the cyclic phosphate ester (5).

Here the neighbouring 2′-hydroxy-group of the ribose residue is the nucleophile, assisted by histidine-12 of the enzyme acting as a general base (4). This displacement also presumably involves a penta-covalent intermediate (not shown), although the evidence demands only that displacement should be in-line, with inversion at phosphorus.[8] Since the reaction is close to symmetrical (both leaving groups are ribose oxygens) catalysis only of the addition step would leave the breakdown of the intermediate rate-determining, however, this step is thought to be subject to catalysis also, by protonated histidine-119 acting as a general acid.

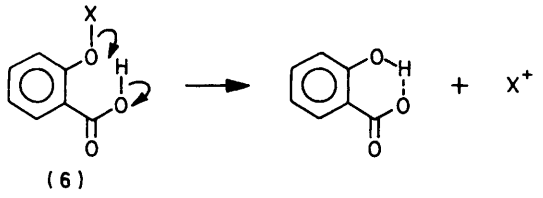
No detailed model of this process is available. Diesters containing the 2-hydroxyethyl group cyclise readily in specific acid- and base- catalysed reactions, but there is only scanty evidence for general species catalysis of the formation of the five-membered cyclic phosphates.[9] In no instance has general acid–base catalysis been properly characterised for even one of the two stages of the reaction. A major problem is the relative inefficiency of intramolecular general acid–base catalysis.[4] There seems no prospect at present of devising an efficient intramolecular model for the ribonuclease reaction, because the effective molarities of general acids and general bases in aliphatic systems are generally no more than 1—10M. Efficient intramolecular general acid catalysis is observed only in derivatives of salicylic acid and related systems,[4] where EMs as high as 10^5M have been observed in reactions (6) involving the loss of the salicylate phenol oxygen from acetals,[10] sulphates,[11] and phosphate monoesters.[12]

Efficient intramolecular general acid catalysis of the breakdown of the penta-covalent intermediate in the hydrolysis of a phosphate diester would therefore be expected if the leaving group were salicylate, as long as the breakdown step were rate determining. Since this is known to be the case for the hydrolysis of the aryl salicyl phosphates (1) described above,[1] we prepared the phosphate diester (7) from salicylic acid, which should therefore be hydrolysed with both intramolecular nucleophilic catalysis by one, ionised, carboxy-group and intramolecular general acid catalysis by the second.



Experimental

Inorganic salts were of analytical-reagent grade. Distilled water was distilled twice more from all-glass apparatus.



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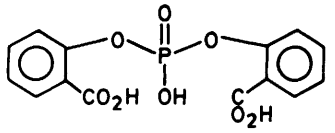


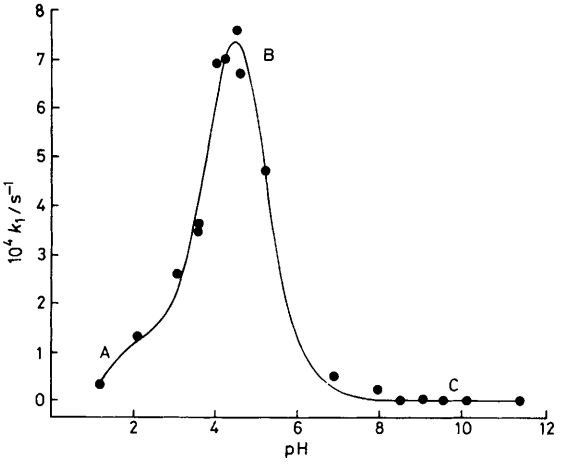
(7)

Bis-(2-carboxyphenyl) Hydrogen Phosphate (7).—This compound was prepared only with considerable difficulty, because it is rapidly decomposed in solution, no doubt by reactions involving the neighbouring carboxy-groups,[1-3] initially to an equimolar mixture of salicyl phosphate and salicylic acid. Protecting groups were therefore essential, but most methods of deprotection, particularly the hydrogenolysis of benzyl groups, proved too slow even in aprotic solvents. We therefore devised a two-stage method of deprotection, using trimethylsilyl iodide to remove esterifying groups, thus generating the stable tris(trimethylsilyl) ester. The trimethylsilyl groups were then removed rapidly under mild conditions to generate (7) in a separate operation.

Bis-(2-benzyloxycarbonylphenyl) Methyl Phosphate.—A solution of benzyl salicylate (979 mg, 4.3 mmol) in dry tetrahydrofuran (2 ml) was added to a suspension of NaH (200 mg, 4.2 mmol) in the same solvent (5 ml) under nitrogen. Injection of methyl phosphorodichloridate (2 mmol) produced a fine precipitate of NaCl. The reaction was stirred for a further 30 min at room temperature, then the solvent removed under reduced pressure. The residue was taken up in dry diethyl ether, and this solution washed with dilute NaOH. The aqueous phase was extracted twice with diethyl ether, and the combined organic layers dried and evaporated to leave a buff-coloured gum. Preparative t.l.c. on silica [eluant light petroleum (b.p. 40—60 °C) –diethyl ether 20 : 80] yielded the *triester* as an oil (891 mg, 82%) (Found: M^+, 532.1273. $C_{29}H_{25}O_8P$ requires M, 532.1287); m/z 532 (2%), 425(2), 318(6), and 303(100); δ($CDCl_3$) 7.95—6.65 (18 H, m, aromatic H); 5.2 (4 H, s, benzyl CH_2), and 3.78 (3 H, d, J 11 Hz, $POCH_3$).

Bis-(2-trimethylsilyloxycarbonylphenyl) Trimethylsilyl Phosphate.—Mixed triester (50 mg) was dissolved in CCl_4 (1.5 ml) in an n.m.r. tube flushed with N_2 and sealed with a rubber septum cap. The addition of 250 μl (6 equiv.) of trimethylsilyl iodide produced an immediate yellow precipitate, which re-dissolved on mixing. Ester exchange was followed by the changes in the ^1H n.m.r. spectrum. Demethylation was almost instantaneous, but debenzylation was complete only after 3 days. During this time a dark red viscous oil separated out at the bottom of the tube. (The oil also formed when the reaction was carried out on a larger scale with stirring.) Finally, the solvent was removed *in vacuo* to leave the tris(trimethylsilyl) ester of (7) as a dark red oil, which was not characterised, but used as required to generate (7).

Bis-(2-carboxyphenyl) Hydrogen Phosphate (7).—The tris(trimethylsilyl) ester (26 mg, 4.7×10^{-8} mol) was dissolved in acetonitrile (2 ml) containing 5% of water. The reddish solution was allowed to stand at room temperature for 20 min, then the solvent removed under reduced pressure to leave (7) as a glass, which was used without purification. The structure (7) follows from the method of preparation; from the absence of signals, other than those of the salicylic acid ring protons in the ^1H n.m.r.; from the pH–rate profile (Figure) for hydrolysis, which clearly depends on two free carboxy-groups; and from the course of the hydrolysis reaction, which can be monitored in the u.v. This shows the release of 2 equiv. of salicylate in two consecutive steps: the u.v. spectrum after the first stage is complete, can be measured at high pH, and was shown to be that expected for an equimolar mixture of salicyl phosphate and salicylate; the rate of the second stage is identical to that measured previously, under the same conditions, for the hydrolysis of salicyl phosphate.[12]



The pH–rate profile for the hydrolysis of (7), at 39 °C and ionic strength 1.0M (KCl). The rate constants are derived as described in the text, and represent the first stage of hydrolysis, to salicylic acid and salicyl phosphate. The points are experimental, and the curve calculated, using the rate and dissociation constants given in the Table

Kinetic Methods and Results

The hydrolysis of (7) was followed by measuring the rate of release of salicylic acid at 298.5 nm, which is the isosbestic point for salicylic acid and its anion, at 39.0 ± 0.1 °C and ionic strength 1.0M (KCl), in the thermostatted cell compartment of a Zeiss PMQ II spectrophotometer. Reactions were carried out under pseudo-first-order conditions, but the first-order plots showed two phases at most pHs, an initial rapid reaction being followed by a second, slower change. The rate of the second stage could be measured accurately where the first stage was more than about three times faster, and was shown to be equal to the known rate of hydrolysis of salicyl phosphate under these conditions.[12] The results were therefore fitted by the method of least-squares to a model for consecu-

$$A \xrightarrow{k_1} B + C; \qquad B \xrightarrow{k_2} C \qquad (1)$$

tive first-order reactions (1) where $[C] = 2[A_o] - [A_o]\{(2k_2 - k_1)e^{-k_1 t} - k_1 e^{-k_2 t}\}/(k_2 - k_1)$ and k_2 is the known [12] rate constant for the hydrolysis of salicyl phosphate under the conditions used. The values obtained for k_1 are compared with those for k_2 in the Table.

The rate constant (k_1) for the hydrolysis of (7) to salicylic acid and salicyl phosphate is itself a composite constant,

Rate constants for the hydrolysis of bis-(2-carboxyphenyl) phosphate (7), at 39 °C and ionic strength 1.0M (KCl)

Conditions [a]	pH	k_1/s^{-1}	k_2/s^{-1} [b]
0.1M HCl	1.18	3.27×10^{-5}	7.50×10^{-6}
0.01M HCl	2.11	1.28×10^{-4}	1.33×10^{-5}
Formate buffer	3.06	2.61×10^{-4}	4.00×10^{-5}
Formate buffer	3.56	3.48×10^{-4}	6.55×10^{-5}
Formate, 0.5M	3.60	3.64×10^{-4}	6.71×10^{-5}
Acetate buffer	4.03	6.92×10^{-4}	9.38×10^{-5}
Acetate buffer	4.26	7.01×10^{-4}	1.09×10^{-4}
Acetate buffer	4.54	7.60×10^{-4}	1.36×10^{-4}
Acetate buffer	4.63	6.72×10^{-4}	1.42×10^{-4}
Acetate buffer	5.24	4.74×10^{-4}	1.39×10^{-4}
Phosphate buffer	6.87	4.92×10^{-5}	2.42×10^{-5}
Tris buffer	7.94	2.14×10^{-5}	1.67×10^{-6}
Tris buffer [c]	8.52	2.62×10^{-6}	
Carbonate buffer [c]	9.04	1.67×10^{-6}	
Carbonate buffer [c]	9.53	5.27×10^{-7}	
Carbonate buffer [c]	10.11	6.02×10^{-7}	
0.01M NaOH [c]	11.39	1.05×10^{-6}	
Derived constants	k_1'	$1.31 \pm 0.85 \times 10^{-4}$	
	k_2'	$1.13 \pm 0.17 \times 10^{-3}$	
	k_3'	$1.18 \pm 0.71 \times 10^{-6}$	
	pK_1	1.21 (K_1 $6.2 \pm 5.8 \times 10^{-2}$)	
	pK_2	4.00 (K_2 $9.94 \pm 5.2 \times 10^{-4}$)	
	pK_3	5.08 (K_3 $8.4 \pm 3.0 \times 10^{-5}$)	

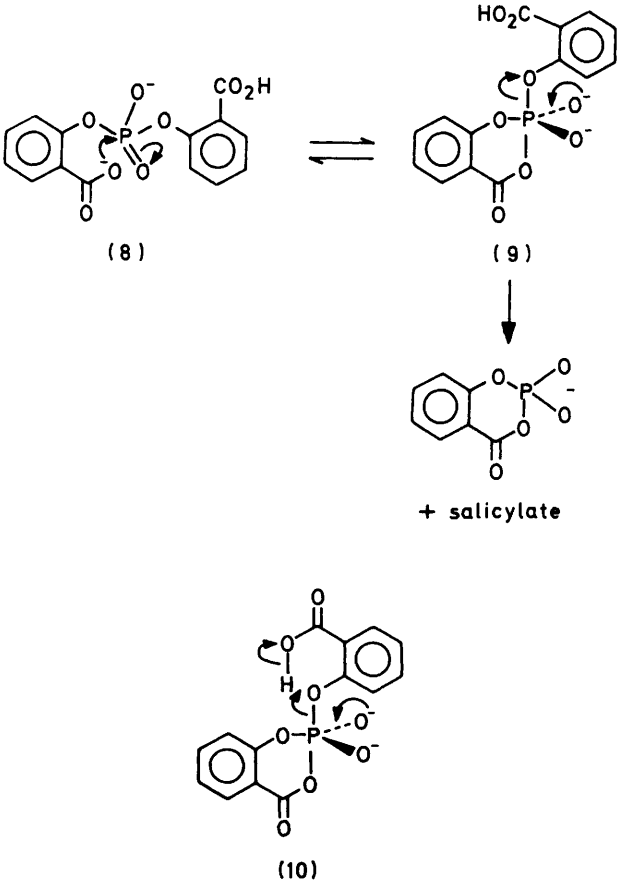
[a] Buffers were 0.05M unless otherwise stated. [b] Data from ref. 12 and R. H. Bromilow, Thesis, University of Cambridge, 1971. [c] Rate constants under these conditions were measured by the initial-rate method. See text.

made up of contributions from various ionic forms of the substrate. Approximate values for k_1', k_2', and k_3', the first-order rate constants for the hydrolysis of the mono-, di-, and tri-anion, were obtained from the three separate regions, A, B, and C of the pH–rate profile for k_1 (Figure); the rate of hydrolysis of the tri-acid appears to be negligible. This allowed the calculation of approximate (apparent) pK_as and thence, by iteration, the best set of apparent pK_as and rate constants for the three ionic forms of (7). The curve shown in the Figure is calculated, using pK_1 1.21, pK_2 4.00, and pK_3 5.08. The rate constants are listed in the Table. Slow reactions, at pH > 8.5, were measured by the initial-rate method; infinity values were measured by adding portions to 50% formate buffer, pH 3.56, where hydrolysis is rapid. After 10 half-lives under these conditions the sample was diluted 210 times with the original high-pH buffer and the end-point read.

Discussion

The pH–rate profile for the hydrolysis of (7) (Figure) is dominated by the rapid hydrolysis near pH 4.5 in the region where the concentration of the dianion (8) is expected to be a maximum. The rate constant (k_2') for the reaction of the dianion is 1.13×10^{-3} s^{-1}, corresponding to a half-life of 10.2 min at 39 °C. This may be compared with a predicted half-life of 180 years for diphenyl phosphate in water at 100 °C in the absence of catalysis.[13] The rate enhancement associated with the introduction of one carboxy and one carboxylate group is thus of the order of 10^{10}.

A rate enhancement of this magnitude is *prima facie* evidence for a mechanism involving nucleophilic catalysis.[4] The reaction of the dianion is *ca.* 1 000 times faster than that of the trianion, which is itself 3.4 times slower than that of the dianion of 2-carboxyphenyl phenyl phosphate. These latter reactions may be presumed to go by the same intramolecular nucleophilic catalysis mechanism:[1] the seven-fold reduction in the intrinsic reactivity at phosphorus of the trianion of (7) being a not unexpected electrostatic effect of the *ortho*-carboxylate group. This is absent in the reaction of the dianion (8), which can be written:



The effect of the *ortho*-CO_2H on the leaving group could be catalytic, or simply electronic. In the hydrolysis of diaryl 2-carboxyphenyl phosphates, where there appears to be no barrier to endocyclic displacement, the acyl phosphorane leaving group has an effective pK_a of 8.52.[3] This may be taken as a lower limit for the effective pK_a of the (conjugate acid) of the salicylic acid leaving group of (8) or (9), allowing a reliable estimate of 2.76×10^{-4} s^{-1} for the maximum expected rate of hydrolysis of (8) if the *ortho*-CO_2H acts simply by electron withdrawal.

The observed rate of hydrolysis of the dianion (8) is four times greater than this estimated maximum. This is a small factor, but large enough to leave little doubt that the *ortho*-CO_2H group is acting catalytically; and we conclude that the mechanism of breakdown of the penta-covalent intermediate (9) does indeed involve intramolecular general acid catalysis by the carboxy-group, (10).

The question then arises, why is this catalysis so inefficient? The rate enhancement observed may be greater than four-fold, but it is not likely to amount to much more than one order of magnitude. This is in sharp contrast to the efficient general acid catalysis observed in other cases,[10-12] discussed above, where the salicylate monoanion is a leaving group. It is not likely that the rate-determining step reverts to the formation of the penta-covalent intermediate: breakdown still appears to be rate-determining for 2-carboxyphenyl 4-nitrophenyl phosphate, which is hydrolysed nine times faster than the

dianion (8). So it appears that intramolecular general acid catalysis of the breakdown of (10) is genuinely relatively inefficient.

A likely explanation is that the efficiency of catalysis by the *ortho*-CO_2H group depends on the timing of P–O cleavage. High EMs are observed for the hydrolysis of compounds, such as salicyl phosphate and 2-methoxymethoxybenzoic acid, which have very late transition states (P–O, C–O cleavage well advanced), because the departure of the salicylate monoanion leaves behind a high-energy species (metaphosphate, oxocarbenium ion). The penta-covalent intermediate (9) is itself a high-energy species, breaking down to two relatively stable fragments, salicylate and the cyclic acyl phosphate. For this process the transition state will be relatively early and it may be that insufficient negative charge has developed on the leaving group oxygen in the transition state to trigger the proton transfer step (described in refs. 12 and 10) in its most efficient mode.

Acknowledgements

We are grateful to Dr. J. Robertshaw and Mr. J. D. Mersh for help with the equations for consecutive first-order reactions and curve fitting, respectively, and to S.E.R.C. for support (K. W. Y. A.).

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Received 7th December 1982; *Paper* 2/2047