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Base catalysed phosphate diester hydrolysis

Nicholas H. Williams* and Paul Wyman

Department of Chemistry, Sheffield University, Sheffield, UK S3 7HF.
E-mail: N.H.Williams@Sheffield.ac.uk; Fax: 0114 2738673; Tel: 0114 2229469

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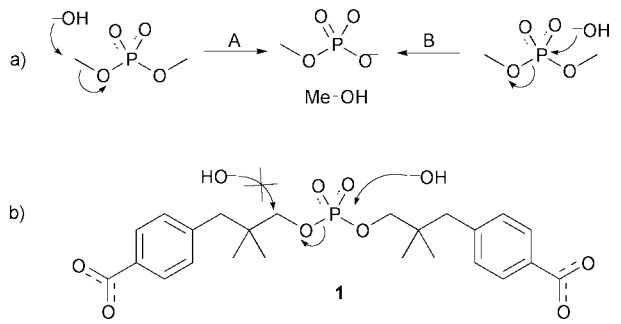
The rate of attack of hydroxide on dialkyl phosphate diesters is far slower than previously estimated, allowing us to estimate the stability of the diester link in DNA and showing that ethylene phosphate is 10^{11} fold more reactive towards attack by hydroxide than an acyclic diester (1000 fold more than previously estimated).

Nature has selected phosphate diesters to hold together the genetic code. This linkage needs to be very stable to keep the sequence of bases intact, but is also the site at which DNA is hydrolysed in the course of its repair and destruction by nucleases.[1] To be able to understand the efficiency of the enzymes (and ribozymes) which catalyse hydrolysis, it is important to quantify the background reactivity. This information will also define the catalytic efficiency which must be achieved by artificial catalysts before they can be used as useful substitutes for natural nucleases. As this is not the most reactive site of DNA,[2] direct measurement of the hydrolysis reaction at phosphorus is not possible and so the hydrolysis of simpler phosphate diesters has to be studied. Here we report the rate of hydroxide catalysed hydrolysis of phosphate diesters with poor leaving groups, revealing that the phosphate diesters in DNA are far more resistant to hydrolysis than has been previously estimated.

The hydrolysis of phosphate diesters with alkoxy leaving groups is extremely slow in the absence of efficient catalysts. Westheimer[3] reported the rate of hydroxide attack on dimethyl phosphate at high temperatures, but Bunton *et al.*[4] used isotopically labeled solvent to show that the majority of the reaction occurs by C–O cleavage (pathway A, Scheme 1a), giving the same products but showing that the attack at phosphorus (pathway B, Scheme 1a) must be slower still. This was confirmed by Westheimer and Haake,[5] and modifying the original rate data to allow for competing attack at carbon gives 6.8×10^{-12} M^{-1} s^{-1} as the best estimate for DNA reactivity with hydroxide at 25 °C.[6] Recently, Wolfenden and Radzicka re-examined the hydrolysis of dimethyl phosphate, and by extrapolating kinetic data from high temperatures arrived at an estimate of 130 000 years as the half life for DNA at pH 6.8 and 25 °C.[7] More detailed study revealed that only pathway A was occurring, and so only an upper limit for the biologically relevant hydrolysis reaction can be derived from these data.[8] These later data also showed that hydrolysis of monomethyl phosphate proceeds only by P–O cleavage and is rapid enough to occur to a similar extent as solvent incorporation under the conditions of the original labeling studies. This could account for the reported isotope labeling results, without involving any P–O cleavage in the initial reaction of dimethyl phosphate. We decided to try and establish the reactivity of dialkyl phosphodiesters to hydrolysis by studying **1** (Scheme 1b) which has a leaving group pK_a comparable to that in DNA.



Scheme 1 (*a*) Pathways for hydrolysis of dimethyl phosphate. (*b*) Compound **1** has pathway A selectively hindered.

Compound **1**† uses the neopentyl effect to prevent attack at carbon without hindering attack at the phosphorus. The steric effect of an alkyl group can significantly retard substitution rates when it is branched α or β to a reaction centre, but the impact of branching at the γ position is minimal.[9] Indeed, the hydrolysis of alkyl 4-nitrophenyl phosphate is only slowed ~2 fold when the alkyl group is changed from methyl to neopentyl.[10] To facilitate analysis by HPLC, we appended aromatic rings, which required the addition of carboxylate groups for solubility in aqueous solution. These anionic groups are distant to the reacting diester, so in a polar, protic solvent such as water, we expect no electrostatic impact on its reactivity; initial studies (using ^{1}H NMR) confirmed that dineopentyl phosphate had the same reactivity as **1**. To study the hydrolysis of **1**, we sealed 0.5 ml aliquots of a 10 mM solution of the diester (with 4-methylbenzoic acid as an internal reference) in 1 M KOH in stainless steel reaction vessels with a PTFE liner and graphite gasket. These were kept at constant temperature by being immersed in a circulating oil bath and removed for analysis at various times.

We investigated the site of reaction by using 94% ^{18}O labeled water. Stopping the reaction after ~50% reaction at 240 °C and analysing the solution by LC-MS revealed that the carboxylate oxygens of **1** and the product alcohol were isotopically labeled at solvent levels, but that the alcohol OH was still at natural abundance—and so the biologically relevant hydrolysis of a phosphate diester mimicking the DNA link is being observed.

Over the range 160–260 °C, we could observe the disappearance of the diester and corresponding appearance of the alcohol product by HPLC analysis of aliquots removed at various time intervals (Fig. 1).‡ Under these conditions, the monoester intermediate does not accumulate to any significant extent. Maintaining the ionic strength at 1 M with KCl, we also measured the rates at 0.33 M KOH, and observed a 3 fold decrease in rate, showing that in 1 M base, the reaction is catalysed by hydroxide.§

In Fig. 2, we plot this data as the log of the second order rate constant for the hydroxide attack against 1/*T* which shows the expected linear relationship. The activation enthalpy is 129 ± 4 kJ mol^{-1} and the activation entropy is −102 ± 9 J mol^{-1} K^{-1}, consistent with a bimolecular reaction and very similar to the entropy of activation (−117 ± 7 J mol^{-1} K^{-1}) measured for the hydroxide catalysed hydrolysis of methyl 4-nitrophenyl phosphate.[11] Extrapolating this data to 25 °C predicts a second order rate constant for attack of hydroxide at the phosphate diester of $10^{-15 \pm 0.5}$ M^{-1} s^{-1}. (*i.e.* half life at pH 14 is ~20 million years!).

If base catalysis is the dominant reaction at pH 7,[12] then the rate of hydrolysis of dialkyl diesters at neutral pH and ambient temperature would be ~10^{-22} s^{-1}. This means that *staphyloccocal nuclease*, which has a turnover number of 95 s^{-1},[13] is

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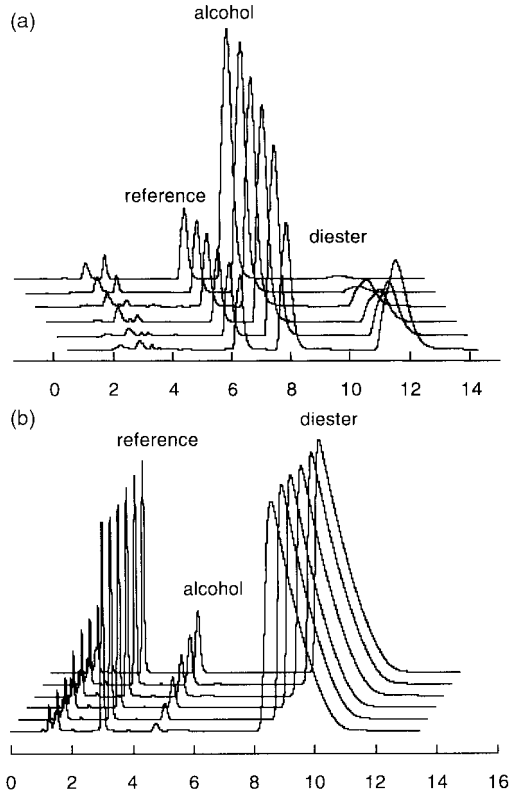

Fig. 1 HPLC traces for the hydrolysis of **1** in 1 M KOH. Upper traces at 240 °C (250 mm column); lower traces at 180 °C (150 mm column). Detection is at 236 nm and retention times are in minutes.

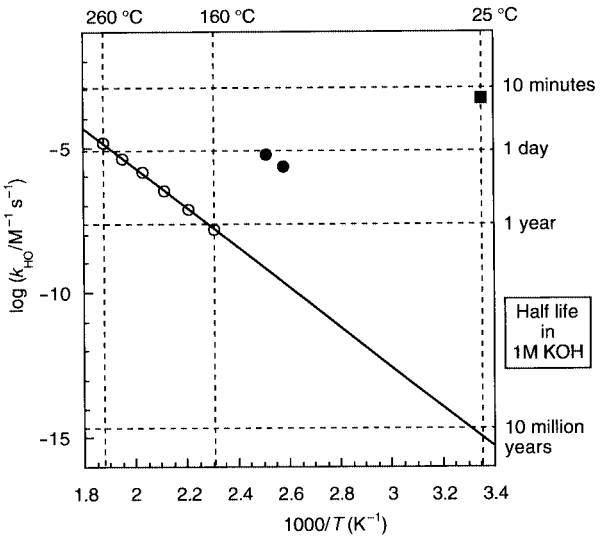

Fig. 2 Arrhenius plot of the second order rate constant for the hydrolysis of **1** (open circles) by hydroxide. The filled circles are the second order rate constant for hydroxide attack on dimethyl phosphate at 125 and 115 °C, and the filled squares for attack on ethylene phosphate at 25 °C.[5]

accelerating the rate of reaction (of the bound diester) by some 23 orders of magnitude. This data also means that the enhanced reactivity of ethylene phosphate is significantly greater than previously estimated. Constraining the ester groups in a 5 membered ring raises the rate constant for attack by hydroxide to 5×10^{-4} M^{-1} s^{-1} at 25 °C.[3] Our data shows that this represents about 10^{11} fold rate enhancement compared to the acyclic compound, 1000 fold more than previously estimated[5] suggesting that rationalisations of this observation will require further study.

Finally, the previously reported rates of reaction of dimethyl phosphate with hydroxide[3] are about 10 000 fold faster than predicted by extrapolating our data (Fig. 2), and so are unlikely to have any significant contribution from pathway B. Approximating the steric effect of the 5′ position of DNA to an isobutyl substituent (which retards S_N2 attack at carbon by an oxyanion in a protic solvent by about 100 fold compared to a methyl substituent[9a]) means that DNA hydrolysis through hydroxide attack at this carbon may be faster than attack at phosphorus. Apparently, biological catalysts have evolved to accelerate attack at phosphorus, rather than the lower energy reaction in solution which would be more energetically efficient.

In conclusion, we have measured the rate of hydroxide attack at the phosphorus of a dialkyl phosphate diester, and found it to be far slower than previously estimated. Consequently, nucleases are considerably more proficient than previously appreciated and the target for making useful artificial nucleases is even more challenging than has previously been appreciated. To be able to artificially manipulate DNA in the same way, catalysts will have to be developed which provide a rate acceleration of about 18 orders of magnitude to reduce the half life to useful levels.

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Notes and references

† Diester **1**: δ_H (250 MHz, D_2O) 0.85 (12H, s, CH_3), 2.60 (4H, s, CH_2Ar), 3.45 (4H, d, *J* 4.1, CH_2O), 7.25 (4H, d, *J* 8.2, ArH), 7.75 (4H, d, *J* 8.2, ArH); δ_P (101 MHz, D_2O) 1.69; MS(ES$^-$), 477 (M − H$^+$); **1**·0.5H_2O Anal. Calcd. for $C_{24}H_{32}O_{8.5}P$: C, 59.13; H, 6.62. Found: C, 59.26; H, 6.80. Alcohol: δ_H (250 MHz, $CDCl_3$) 0.80 (6H, s, CH_3), 2.60 (2H, s, CH_2Ar), 3.30 (2H, s, CH_2O), 7.25 (2H, d, *J* 9.4, ArH), 7.80 (2H, d, *J* 9.4, ArH); MS(EI$^+$) Calcd. for $C_{12}H_{16}O_3$: 208.109945. Found: 208.110790.

‡ HPLC analysis: 10 μl of each aliquot was injected onto a Luna RP C-18 5 μm column (either 4.6 × 150 mm or 4.6 × 250 mm) and eluted isocratically with 60% 20 mM sodium phosphate buffer (pH 7), 40% methanol, and monitored at 236 nm. The integrated peaks were normalised against the internal reference and observed rate constants obtained either from a first order fit or by initial rate analysis.

§ The observed pseudo-first order rate constants are: in 1 M KOH, 260 °C, 1.5×10^{-5} s^{-1}; 240 °C, 4.0×10^{-6} s^{-1}; 220 °C, 1.3×10^{-6} s^{-1}; 200 °C, 3.6×10^{-7} s^{-1}; 180 °C, 7.2×10^{-8} s^{-1}; 160 °C, 1.8×10^{-8} s^{-1}. In 0.33 M KOH, 260 °C, 3.9×10^{-6} s^{-1}; 240 °C, 1.2×10^{-6} s^{-1}; 200 °C, 8.4×10^{-8} s^{-1}. These data were averaged to obtain the second order rate constants plotted in Fig. 2.

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