Phosphodiester linkages, including those that join the nucleotides of DNA, are highly resistant to spontaneous hydrolysis. The rate of water attack at the phosphorus atom of phosphodiester is known only as an upper limit, based on the hydrolysis of the dimethyl phosphate anion. That reaction was found to proceed at least 99% by C–O cleavage, at a rate suggesting an upper limit of 10⁻¹⁵ s⁻¹ for P–O cleavage of phosphodiester anions at 25°C. To evaluate the rate enhancement produced by P–O cleaving phosphodiesterases such as staphylococcal nuclease, we decided to establish the actual value of the rate constant for P–O cleavage of a simple phosphodiester anion. In dineopentyl phosphate, C–O cleavage is sterically precluded so that hydrolysis occurs only by P–O cleavage. Measurements at elevated temperatures indicate that the dineopentyl phosphate anion undergoes hydrolysis in water with a half-life of 30,000,000 years at 25°C, furnishing an indication of the resistance of the internucleotide linkages of DNA to water attack at phosphorus. These results imply that staphylococcal nuclease (kcat = 95 s⁻¹) enhances the rate of phosphodiester hydrolysis by a factor of 10¹⁷. In alkaline solution, thymidylyl-3'-5'-thymidine (TpT) has been reported to decompose 10²-fold more rapidly than dineopentyl phosphate. We find however that TpT and thymidine decompose at similar rates and with similar activation parameters, to a similar set of products, at pH 7 and in 1 M KOH. We infer that the decomposition of TpT is initiated by the breakdown of thymidine, not by phosphodiester hydrolysis.

DNA hydrolysis | DNA stability | nucleic acid | rate enhancement | phosphate ester

Phosphoric acid diesters are, in general, exceedingly unreactive in water (1–3), so that the phosphodiester linkages that join the nucleotides of DNA are highly resistant to spontaneous hydrolysis. By extrapolation of earlier model experiments at elevated temperatures, the uncatalyzed hydrolysis of dimethyl phosphate in neutral solution was found to proceed with an estimated rate constant of ~2 × 10⁻¹³ s⁻¹ at 25°C, corresponding to a half-time of 140,000 years. That reaction was found to proceed at least 99% by C–O cleavage, suggesting an upper limit of ~1 × 10⁻¹⁵ s⁻¹ at 25°C on the rate constant for spontaneous P–O cleavage of a phosphodiester anion, the reaction that is catalyzed by many phosphodiesterases (4).

More recently, a rate constant of 6 × 10⁻⁷ s⁻¹ has been reported for the decomposition of thymidylyl-3'-5'-thymidine (TpT) at 80°C in 1 M KOH (5). Extrapolation of the results obtained earlier for dimethyl phosphate hydrolysis in neutral solution (4), to 80°C, would indicate a rate ~10³-fold slower. That discrepancy might indicate a major role for catalysis by hydroxide, but the hydrolysis of another dialkyl phosphodiester, bis-3-(4-carboxyphenyl)neopentyl phosphate (Np³P), in which γ-branching of the leaving alcohol prevents C–O cleavage (Fig. 1), also proceeds ~10²-fold more slowly in 1 M KOH (6).

In an effort to resolve that discrepancy, and to evaluate the approximate rate enhancement produced by phosphodiesterases such as staphylococcal nuclease (7), we sought to establish the value of the rate constant for P–O cleavage of a simple phosphodiester anion. In the present work, we determined the rate of spontaneous hydrolysis of dineopentyl phosphate (Np²P), in which P–O cleavage occurs, but C–O cleavage is precluded by steric effects (Fig. 1) and cannot occur through elimination. We also measured the reactivity of a methyl triester analogue and reinvestigated the decomposition of TpT in 1 M KOH at 80°C.

**Results**

Rate constants were obtained for Np²P (0.01 M) hydrolysis at 25°C in anion-forming buffers (0.1 M potassium formate, acetate, phosphate, borate, and carbonate) whose pH had been determined at 25°C and also in solutions containing HCl (0.1–1.0 M) and KOH (0.1–1.0 M). For Np³P, C–O cleavage by nucleophilic substitution is sterically precluded and cannot occur through elimination. Hence, only P–O cleavage occurs, as was demonstrated by mass spectrometric analysis of the products of hydrolysis in H₂¹⁸O. Very similar rate constants were obtained for hydrolysis over the range from pH 6.5 to 13 (Fig. 2). Fig. 3 shows an Arrhenius plot of typical results obtained in 0.1 M potassium phosphate buffer (pH 6.8). Extrapolation of those results indicates that, at 25°C, the apparent first-order rate constant is 7 × 10⁻¹⁶ s⁻¹ for hydrolysis of the Np²P anion. A more complete Arrhenius plot, based on 68 data points gathered over the range between pH 6.5 and 13, indicates that ΔH‡ = 29.5 ± 0.7 kcal/mol for this reaction, and TΔS‡ = –8.5 ± 1.0 kcal/mol. Data for the hydrolysis of Np³P at 250°C agree closely with these data (Fig. 2).

In strongly alkaline solution, hydroxide ion catalysis became apparent at KOH concentrations >0.1 M. The results of an Arrhenius plot of rate constants obtained in 1 M KOH were extrapolated to give k₂₅ = 1.4 × 10⁻¹⁵ s⁻¹ for this reaction, with ΔH‡ = 29.5 kcal/mol and TΔS‡ = –8.0 kcal/mol, very similar to those observed for the water reaction (data not shown). This rate is closely comparable with the rate of hydrolysis in 1 M KOH of Np³P in which the leaving alcohol similarly prevents C–O cleavage, but P–O cleavage is expected to be unimpeded (6). The rate of Np²P hydrolysis also increased at pH values ≤ 6 (Fig. 2), consistent with water attack on uncharged Np²P. Solubility limitations precluded determination of rate constants for Np³P hydrolysis at low pH values where the ester is mostly protonated, except at high temperatures near 250°C. For that reason, the thermodynamics of activation could not be determined for the reaction of the neutral species.

To estimate the difference in reactivity between the neutral and anionic diesters, we measured the rate of hydrolysis of the triester methyl Np³P at 25°C in 1 M NaOH, using the methyl group as a surrogate for protonation of the phosphate. The phosphate diester products were methyl Np³P and Np³P in a ratio of 1:4. Partitioning the observed rate constant (5 × 10⁻⁶ s⁻¹) between Np³P and methanol displacement gives a rate constant of 1 × 10⁻⁸ s⁻¹ for Np³P expulsion. The loss of methanol

**Conflict of interest statement:** No conflicts declared.

**Abbreviations:** TpT, thymidylyl-3'-5'-thymidine; Np³P, dineopentyl phosphate; Np²P, bis-3-(4-carboxyphenyl)neopentyl phosphate.

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from Np\textsubscript{2}P might in principle proceed by either C–O or P–O bond cleavage; that distinction was not investigated.

As noted earlier, a surprisingly rapid rate of internucleotide hydrolysis ($k_{1000}/H_{11005}$ $6/11003$ $7$ s$^{-1}1$) has been reported for TpT (5). In 1 M KOH at 80°C, TpT was found to decompose $\approx5$ orders of magnitude more rapidly than would be expected from the present findings for Np2P as well as the earlier findings for Np\textsuperscript{*}2P (6). To investigate the source of that discrepancy, we examined the reaction of TpT in 1 M KOH at 80°C and also in potassium phosphate buffer (0.1 M, pH 6.8).

We were able to duplicate the findings of Takeda et al. (5), observing decomposition of TpT at 80°C in 1 M KOH. We also observed decomposition, proceeding at a similar rate, in potassium phosphate buffer (0.1 M, pH 6.8) at 80°C. We found, however, that under both sets of conditions, thymidine itself is unstable, and that both thymidine and TpT decompose at comparable rates in such a way as to obscure the rate of cleavage (if any) of the phosphodiester bond of TpT. Moreover, at pH 14, both thymidine and TpT decompose at 80°C, with similar pseudo-first-order rate constants and activation parameters, to roughly the same set of products, as indicated by proton NMR analysis (see Fig. 5, which is published as supporting information on the PNAS web site). UV analysis indicated that opening of the pyrimidine ring proceeds with the same rate constant.

At pH 6.8 (0.1 M potassium phosphate buffer), thymidine and TpT decomposed with rate constants and activation parameters that were identical within experimental error. However, at pH 6.8, decomposition leads to a different set of products from those observed in 1 M KOH. Under these conditions, proton NMR and UV spectroscopic analysis showed that both substrates are converted quantitatively to thymine, with no significant degradation of the pyrimidine ring. The hydrolysis of thymidine to thymine, as indicated by UV analysis, was observed earlier in neutral and acid solution (8).

**Discussion**

Table 1 summarizes the rate constants, enthalpies, and entropies of activation for diester hydrolysis observed in this study and earlier work. The similarity between the extrapolated value of $k_{25} = 7 \times 10^{-16}$ s$^{-1}$ for Np2P and the approximate upper limit on the rate constant for P–O cleavage of dimethyl phosphate ($\approx1 \times 10^{-15}$ s$^{-1}$ at 25°C) that was indicated by earlier experiments on dimethyl phosphate (1) suggests that P–O cleavage is not sterically impeded in Np2P. It seems reasonable to infer that the extrapolated rate constant of $k_{25} = 7 \times 10^{-16}$ s$^{-1}$, equivalent to a half-time of 31,000,000 years at 25°C, can be considered typical of apparent water attack on the phosphorus atom of simple dialkyl phosphate anions. Because the activation parameters for the hydroxide-catalyzed and spontaneous reactions are very similar, the form of the pH rate profile at high pH will not change at lower temperature. Hydroxide attack at the diester anion appears to become a significant contributor to hydrolysis only at very high pH.

As to the actual mechanism by which Np2P is hydrolyzed, it is of interest that the rate constant for Np2P hydrolysis, extrapolated to 100°C from the present results, is larger by 3 orders of magnitude than would be expected by extrapolation to $pK_a$ 15.5 of a Brønsted plot based on the rates of hydrolysis of anions of diaryl phosphate esters of alcohols with $pK_a$ values ranging from 4 to 8.5 (3). In further contrast to our observations (Fig. 2), these data also show that hydroxide-catalyzed hydrolysis dominates over the spontaneous reaction as the leaving groups become poorer. It is possible that the dialkyl diester is not hydrolyzed through water attack on the monoanion but through the kinetically equivalent mechanism of hydroxide attack on the neutral.
diester (Fig. 4). We can estimate the rate of the latter reaction by assuming that the methyl triester of Np₂P is a reasonable model for the neutral diester; at 25°C, the rate of reaction of Np₂P with the hydroxide ion is \(1 \times 10^{-13} \text{ M}^{-1} \text{s}^{-1}\). Taking into account the unfavorable equilibrium \(K = K_w/K_a \approx 3 \times 10^{-13}\); Fig. 4) involved in transferring a proton from water to the diester anion, the predicted rate for the reaction through this mechanism is \(3 \times 10^{-19} \text{ s}^{-1}\). These comparisons and the associated errors do not allow a clear distinction to be made between the two mechanisms using our data, although recent calculations (11) give very good agreement with the triester-like mechanism.  

Comparing the rate of reaction of the methyl triester and corresponding diester with hydroxide, we note that the effect of neutralizing the phosphoryl group is to accelerate this reaction by \(10^9\) fold. That effect is substantially greater than the effect (\(\sim 10^6\)-fold) of the analogous change in RNA models that undergo transesterification with an intramolecular nucleophile (14). Interestingly, like the diaryl diesters and in contrast to the observations reported here, RNA cleavage is dominated by the base-catalyzed reaction from pH 5 with a minimal contribution from any pH-independent reaction (15).

It is of interest to consider these observations in relation to the lifetime of the backbone of DNA. Because C–O cleavage competes effectively with P–O cleavage (Table 1), it seems reasonable to suppose that the predominant mode of phosphodiester hydrolysis of DNA might involve C–O cleavage by water (or hydroxide) attack at the relatively unhindered 5′-carbon atom of the nucleoside to which the phosphoryl group is attached. The possibility of confirming that conjecture by experiment is clouded, however, by the likelihood that other modes of decomposition transpire far more rapidly than ester hydrolysis, as discussed below.

A surprisingly rapid rate of internucleotide hydrolysis \(k = 6 \times 10^{-7} \text{ s}^{-1}\) has been reported for TpT at 80°C (5). At pH 14 at 80°C, TpT was found to decompose \(\sim 5\) orders of magnitude more rapidly than would be expected from the behavior of Np₂P (6) or the present findings for Np₂P. We found, however, that under both neutral and basic conditions, thymidine itself is unstable, and that both thymidine and TpT decompose at the same rate in such a way as to obscure any cleavage of the phosphodiester bond of TpT. In 1 M KOH, both thymidine and TpT decompose at 80°C with approximately the same pseudo-first-order rate constants and activation parameters, to a similar set of products, as indicated by proton NMR analysis (see Figs. 6 and 7, which are published as supporting information on the PNAS web site). UV analysis indicates that opening of the pyrimidine ring proceeds with the same rate constant. Because both thymidine and TpT decompose at similar rates in 1 M KOH at 80°C, whereas Np₂P and Np³P are hydrolyzed 5 orders of magnitude more slowly, we infer that ring-opening in base opens pathways for further decomposition (e.g., by elimination) that were not available before ring-opening and are not available to a simple phosphodiester. Thus, the spontaneous cleavage of DNA is likely to be dominated by pathways that occur through the formation of abasic sites, rather than by P–O bond breaking as catalyzed by many phosphodiesterases. From these results, it is evident that DNA cleavage can be initiated in a variety of ways, suggesting the need for caution in attributing the decomposition (e.g., by novel artificial catalysts) of long strands of DNA to any particular mechanism.

If the rate constant for the uncatalyzed hydrolysis of the Np₂P anion, extrapolated to 25°C \(\left(7 \times 10^{-16} \text{ s}^{-1}\right)\) is compared with \(k_{\text{cat}}\) for staphylococcal nuclease at 25°C (95 s⁻¹) (7), the rate enhancement produced by staphylococcal nuclease is found to be \(4 \times 10^{17}\)-fold, corresponding to a 23.2 kcal/mol reduction in \(\Delta G_f^\ddagger\). That value is comparable in magnitude with the rate enhancements produced by orotidine 5′-monophosphate decarboxylase \(\left(1.4 \times 10^{12}\text{-fold}\right)\) (16) and \(\beta\)-amylase \(\left(7 \times 10^{12}\text{-fold}\right)\) (17) and is presently exceeded only by the values that have been recorded for fructose 1,6-bisphosphatase \(\left(1.1 \times 10^{21}\text{-fold}\right)\) (10) and arginine decarboxylase \(\left(7 \times 10^{19}\text{-fold}\right)\) (18).

Comparison of \(\Delta H_f^\ddagger = 27.8\) for water attack on the Np₂P anion (Table 1) with \(\Delta H_f^\ddagger = 10.8\) for reaction of the enzyme–substrate complex of staphylococcal nuclease indicates that most of the 23.2 kcal/mol reduction in \(\Delta G_f^\ddagger\) by staphylococcal nuclease is accounted for by a reduction in the heat of activation for phosphodiester hydrolysis. That tendency, also observed in the other slow reactions mentioned in the previous paragraph, may be understandable in view of the fact that most of the very high activation barrier to product formation is enthalpic rather than entropic in these reactions (19). A major reduction in enthalpy

### Table 1. Kinetics of phosphate ester hydrolysis

<table>
<thead>
<tr>
<th>Ester</th>
<th>Ion</th>
<th>Ref.</th>
<th>Cleavage</th>
<th>(k_{25^\circ C} \text{ s}^{-1} \text{ or M}^{-1} \text{ s}^{-1})</th>
<th>(\Delta G^\ddagger \text{ 25°C}) kcal/mol</th>
<th>(\Delta H^\ddagger) kcal/mol</th>
<th>(T_{a^\ddagger}) 25°C kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoester</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H_2O + MeP^-)</td>
<td>Monoanion</td>
<td>9</td>
<td>P–O</td>
<td>(2.4 \times 10^{-10})</td>
<td>+30.6</td>
<td>+30.0</td>
<td>–0.6</td>
</tr>
<tr>
<td>(H_2O + MeP^-)</td>
<td>Dianion</td>
<td>10</td>
<td>P–O</td>
<td>(2 \times 10^{-20})</td>
<td>+44.3</td>
<td>+47.0</td>
<td>+2.7</td>
</tr>
<tr>
<td>Diester</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H_2O + Me₂P)</td>
<td>Neutral</td>
<td>1</td>
<td>C–O</td>
<td>(6 \times 10^{-10})</td>
<td>+30.0</td>
<td>+25.0</td>
<td>–5.0</td>
</tr>
<tr>
<td>(H_2O + Me₂P)</td>
<td>(or (H_2O^+ + Me₂P))</td>
<td>4</td>
<td>C–O</td>
<td>(1.6 \times 10^{-13})</td>
<td>+34.9</td>
<td>+25.9</td>
<td>–9.0</td>
</tr>
<tr>
<td>(HO^- + MeP^-)</td>
<td>Anion (neutral)</td>
<td>2</td>
<td>C–O</td>
<td>(3 \times 10^{-11})</td>
<td>+31.7</td>
<td>+27.6</td>
<td>–4.1</td>
</tr>
<tr>
<td>(HO^- + NpP^-)</td>
<td>Anion</td>
<td>1</td>
<td>C–O</td>
<td>(7 \times 10^{-16})</td>
<td>+38.1</td>
<td>+29.5</td>
<td>–8.6</td>
</tr>
<tr>
<td>(HO^- + Np³P^-)</td>
<td>Anion</td>
<td>1</td>
<td>C–O</td>
<td>(1.4 \times 10^{-15})</td>
<td>+37.7</td>
<td>+29.5</td>
<td>–8.0</td>
</tr>
<tr>
<td>(HO^- + EtP^-)</td>
<td>Anion</td>
<td>6</td>
<td>P–O</td>
<td>(1 \times 10^{-15})</td>
<td>+37.9</td>
<td>+30.8</td>
<td>–7.1</td>
</tr>
<tr>
<td>Triester</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>(H_2O + MeP)</td>
<td></td>
<td>11</td>
<td>C–O</td>
<td>(2 \times 10^{-8})</td>
<td>+28.1</td>
<td>+22.6</td>
<td>–5.5</td>
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<tr>
<td>(HO^- + MeP)</td>
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<td>12</td>
<td>P–O</td>
<td>(1.4 \times 10^{-4})</td>
<td>+22.7</td>
<td>+15.4</td>
<td>–7.3</td>
</tr>
<tr>
<td>(HO^- + EtP)</td>
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<td>11</td>
<td>P–O</td>
<td>(9 \times 10^{-6})</td>
<td>+24.3</td>
<td>+14.1</td>
<td>–10.2</td>
</tr>
<tr>
<td>Diesterase</td>
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<td>P–O</td>
<td>95</td>
<td>+14.7</td>
<td>+10.8</td>
<td>–3.9</td>
</tr>
</tbody>
</table>

### Fig. 4.  
Equilibrium between alternate reactants in the apparently uncatalyzed hydrolysis of the Np₂P anion.
of activation might be expected for a reaction in which polar forces of attraction, especially ionic H-bonds (20, 21), are at work between an enzyme and the altered substrate in stabilizing the transition state. In the particular case of staphylococcal nuclease (7), ionized functional groups have been shown to line the active site cavity (22).

Materials and Methods

Dimethyl phosphate was synthesized by the alkaline hydrolysis of trimethyl phosphate, and Np₂P was synthesized by the alkaline hydrolysis of 4-nitrophenyl dioxepinyl phosphate. Methyl distyryl triesters were synthesized from methyl dichlorophosphate and inorganic phosphate without significant accumulation of products. The integrated intensities of the resonances of the reactants and products were compared with those of pyrazine, a known integration standard. Each of these reactions yielded the alcohol and inorganic phosphate without significant accumulation of monoester. In contrast to Np₂P, dimethyl phosphate undergoes hydrolysis in 1 M KOH much more rapidly than does methyl phosphate, except at very high temperatures (compare the first two entries in Table 1). As a practical consequence of the present observations, either trimethyl or dimethyl phosphate can be converted entirely to the monoester overnight in 1 M KOH at 150–170°C, with no trace of contaminating dimethyl phosphate or inorganic phosphate. The reactions of Np₂P and methyl Np₂P were measured by monitoring the changes in concentration of starting diester and product alcohols relative to an internal standard of 4-methylbenzoic acid by HPLC analysis.

For the reactions of Np₂P at 250°C, the reaction mixtures were divided between several small polytetrafluoroethylene-lined stainless steel bombs that were completely immersed in a vigorously circulating bath containing silicon oil. The temperature was continuously monitored with an electronic contact thermometer, and individual samples were withdrawn at various time intervals. When using the circulating oil bath, thermal equilibration was achieved in ~15 min.

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