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Charge Description of Base-Catalyzed Alcoholysis of Aryl Phosphodiesters: A Ribonuclease Model

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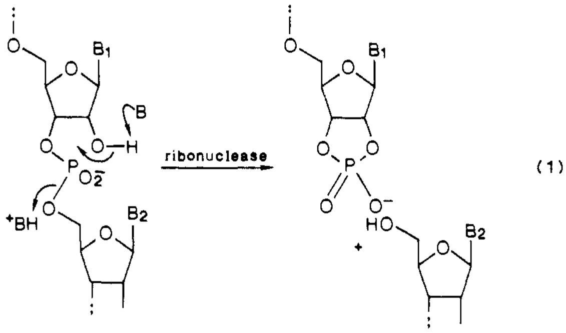
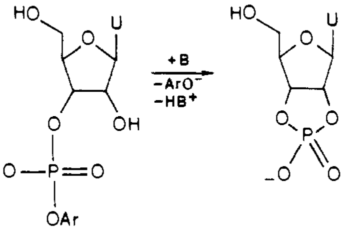
Abstract: The release of substituted phenol from aryl uridine-3′-phosphates is catalyzed by bases and involves cyclization to form the 2′,3′-cyclic nucleotide. The rate constants for imidazole and hydroxide ion catalysis (k_{im} and k_{OH}, respectively) obey the Brønsted equations (25 °C and 0.25 M ionic strength) log $k_{im} = -0.59$ p$K^{ArOH} + 1.40$ ($n = 7$, $r = 0.955$) and log $k_{OH} = -0.54$ p$K^{ArOH} + 6.68$ ($n = 9$, $r = 0.967$). General-base-catalyzed release of 4-nitrophenol from the 4-nitrophenyl ester (k_B) obeys the Brønsted relationship log $k_B = 0.67$ p$K^{BH} - 7.50$ ($n = 7$, $r = 0.989$). Charge changes on base and leaving group atoms as determined from the corresponding β and β_{eq} values do not balance. Comparison with data in the literature indicates that the difference in charge may be assigned to the attacking oxygen (2′-hydroxyl) rather than to the phosphoryl oxygens in the O···PO$_2$··· group of atoms. Both P–O bond forming and bond fission components of the reaction are considered to be only weakly advanced in a transition state that lies on a concerted pathway.

Fission of the phosphodiester bond has considerable relevance with regard to the chemistry of nucleotides and related biochemical reactions. Ribonucleases catalyze the transfer of the nucleotidyl group from the 5′-hydroxy function of an adjacent nucleotide to the neighboring 2′-hydroxyl group (eq 1).[1] This reaction has been studied in models by Brown,[2] Usher,[3] Breslow,[4] and Inoue.[5,6] Both the base and acid catalytic components of the proposed enzymatic mechanism have been demonstrated.



(1)

We have investigated the effect on kinetics of change of substituent in the leaving group and of the variation of the base in the reaction of the substituted phenyl uridine-3′-phosphate esters (eq 2) to determine charge change. Application of simple β values is not appropriate if we require to know how far bond formation or fission has progressed from ground to transition state compared with the overall bond change.[7] Recent studies from our laboratory[8,9] have provided values for β_{eq} for transfer of phosphoryl groups from aryl oxide nucleophiles. This gives us the opportunity of measuring the calibrated charges on reacting oxygen atoms in the transition state of the rate-limiting step of eq 2. Since we are in a position to vary both catalytic base and leaving group for the reaction we should then be able to describe the overall charge distribution of the transition state from which we could discuss how polar groups could affect catalysis in the enzymatic reaction.



(2)

The reaction under investigation (eq 2) is a good model of the first step of ribonuclease action except that the leaving group is activated inductively and mesomerically; proton transfer to the leaving oxygen, the proposed source of part of the activation in the enzyme,[1] is not provided in the model as its presence makes it difficult to monitor charge on the leaving oxygen during fission of the P–O bond.

Experimental Section

Materials. Aryl 2′,5′-ditetrahydropyranyluridine-3′-phosphates were prepared according to the following general procedure for the 4-nitrophenyl ester. The syntheses are based on the methods of Moffatt,[10] Sierakowska,[11] and Reese.[12] Uridine (Aldrich Chemical Co.) was benzoylated specifically at the 3′-hydroxyl position by use of the dibutyltin cyclic ether to direct the esterification. The 2′- and 5′-hydroxyl groups were then protected as their tetrahydropyranyl ethers. Deprotection of the 3′-hydroxyl ester with ammonia and aryl phosphorylation gave the protected phosphate ester. Yields throughout were not optimized.

Methods for the preparation of 3′-*O*-benzoyluridine and 2′,5′-ditetrahydropyranyluridine are given as supplementary material.

4-Nitrophenyl 2′,5′-Di-*O*-tetrahydropyranyluridine-3′-phosphate. 1,2,4-Triazole (0.88 g) was mixed with 4-nitrophenyl phosphorodichloridate (1.25 g) in acetonitrile (5 mL). Triethylamine (1.3 mL) was added and the reactants stirred at room temperature. After 15 min, the 2′,5′-protected nucleoside (0.7 g) was added followed by the addition of pyridine (40 mL). The mixture was stirred for 2 h and then a solution of triethylamine (1.3 mL) and water (0.6 mL) in pyridine (4 mL) added; after being stirred for 20 min, the reaction mixture was shaken with a saturated aqueous solution of NaHCO$_3$ (250 mL). The mixture was extracted with CHCl$_3$ (3 × 50 mL), and the CHCl$_3$ layers were dried with MgSO$_4$ and evaporated in vacuo.

The material was passed through a silica gel column (ethyl acetate/methanol (3:1, v/v)) to yield crude product as its triethylammonium salt. The crude salt in a solution of 50 mL of water and 50 mL of methanol was adjusted to pH 9.5 with ammonia and then loaded onto an ion-exchange column (Fractogel TSK:DEAE 650, 2 × 30 cm^3) in its carbonate

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Table I. Fission of Substituted Phenyl Uridine-3′-phosphates Catalyzed by Imidazole at 25 °C and 0.25 M Ionic Strength[b,c]

subst[a]	pK^{ArOH}	$k_{im} \times 10^5$,[h] M^{-1} s^{-1}	pH[g]	$k' \times 10^4$,[d,e] s^{-1}	λ,[c] nm
4-Cl	9.38	5.2	7.80	4.7–8.1	240[f]
2-Cl	8.48	11	7.70	5.3–12.5	241
3-NO_2	8.35	65	7.69	40–82	235
3,5-Cl_2	8.18	57	7.74	38–76	241
4-NO_2	7.14	229	7.68	160–300	400
		217	7.22	100–520	
2,4,5-Cl_3	6.72	210	7.70	101–237	241
2-Cl-4-NO_2	5.45	1200	7.58	4700–12300	400

[a] Concentrations of substrates in all cases ~0.1 mM. [b] Ionic strength maintained with Na_2SO_4. [c] Wavelength employed for kinetics; molar extinction coefficients are given in Table III. [d] Rate constant range observed. [e] Number of data points not including duplicates was in all cases 4 over the range of concentrations of imidazole. [f] A higher wavelength than that employed for other bases was necessary because of imidazole absorption. [g] Average pH; individual values did not deviate by more than 0.02 pH units. [h] Error no greater than 5% in all cases. [i] Average 2.3×10^{-3} M^{-1} s^{-1}.

Table II. Fission of 4-Nitrophenyl Uridine-3′-phosphate Catalyzed by Nitrogen Bases at 25 °C and 0.25 M Ionic Strength[d]

base[a]	pK^{BH}	$k_B \times 10^3$,[g] M^{-1} s^{-1}	pH[f]	$k' \times 10^3$,[b,c] s^{-1}
β-alanine	10.19	570	9.21	29–51
glycine	9.60	180	9.68	81–110
serine	9.15	64	9.15	29–42
morpholine	8.36	26	8.42	7.3–13
Tris[e]	8.10	29	8.75	12–22
2-methylimidazole	7.75	6.2	7.78	1.7–2.9
imidazole	6.95	2.3	from Table 1	

[a] Buffer concentration ranged from 0.1 to 0.5 M; substrate concentration 0.1 mM. [b] Rate constant range. [c] Number of data points not including duplicates was 5. [d] Ionic strength maintained with Na_2SO_4; wavelength for kinetics 400 nm. [e] Tris(hydroxymethyl)aminomethane. [f] Average pH; individual values did not differ by more than 0.02 pH units. [g] Error not greater than 5% for all the rate constants.

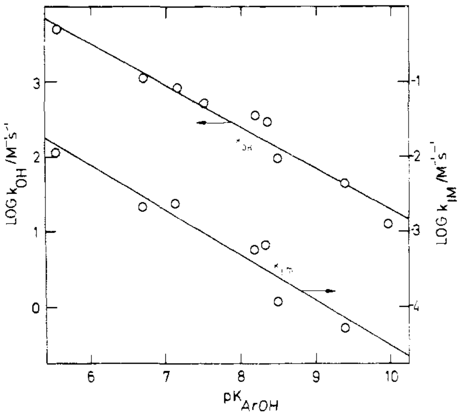

Figure 1. Brønsted dependencies of k_{OH} and k_{im} for fission of aryl uridine-3′-phosphates; conditions as in Tables I and III, which also indicate the identities of the points. Lines are calculated from eq 4 and 5.

form prepared by washing with ammonium carbonate solution followed by a water rinse until a stable UV base line (279 nm) was achieved in the UV recorder. Elution was effected with triethylammonium bicarbonate solution at pH 7.5. Fractions containing product were pooled and evaporated, and the residual solution was freeze-dried to yield the phosphodiester as a pale yellow hygroscopic powder. The material was shown to have a single TLC spot (ethyl acetate/methanol, 3:1, v/v), and TLC was used in subsequent preparations to identify the peaks from the column ion-exchange procedure. R_f values of the phosphodiesters were independent of the aryl function. Elemental analyses are given in Table I, supplementary material. The proton NMR spectra for the compounds are very complex owing to the protons of the tetrahydropyranyl residues swamping signals from the other protons and also because of the induced chirality at C_5' and C_2'. Integration of the triethylammonium methyl triplet absorption and the aromatic absorptions of the aryl group gave the expected ratios for the esters.

Yields of protected aryl uridine phosphates from the aryl phosphorodichloridate coupling reactions were low compared with those normally obtained with the deoxyribose series where yields in excess of 90% are common.[13]

Better yields were often obtained for the esters of the more acidic phenols, possibly due to the greater overall reactivity of the corresponding dichloridates.

Aryl phosphorodichloridates were obtained from Aldrich in the case of the parent and the 4-chloro-, 2-chloro-, 2,5-dichloro-, and 4-nitro-substituted species. The other dichloridates were prepared by the method exemplified for the 3-nitrophenyl phosphorodichloridate. A mixture of NaCl (0.15 g) and $POCl_3$ (30 g) was kept at 90 °C and 3-nitrophenol (5.4 g) added portionwise over a period of 20 min. The reaction mixture was heated to 105–110 °C and heating continued until HCl evolution had subsided (ca. 2 h). The excess $POCl_3$ was removed by evaporation under reduced pressure and the product isolated by vacuum distillation. Comparison of melting points and boiling points with literature values (Table II, supplementary material) indicated the identity of the products.

Melting points were determined with a Kofler Thermospan instrument, and elemental analyses were carried out by A. J. Fassam with a Carlo Erba instrument. Infrared spectra (Perkin-Elmer 297) and NMR spectra (JEOL 100-MHz JNM PS) were employed routinely to check identity and purity of intermediates and products.

Kinetic Methods. The substrates were stored as their 2′,5′-ditetrahydropyranyl derivatives, and these were deprotected as required by the following technique. A stock solution of the protected ester (2 mL, 10 mM) was prepared in water to which was added 50 mg of "wet" swelled Amberlyst 15 strong acid ion-exchange resin; the mixture was stirred at room temperature for 30 min. The resin was removed by filtration and the solution used directly. The stock solution after deprotection was about pH 2.5, and the solution was stable at this pH over a few days when kept at 4 °C.

Reaction rates were followed from the changes in UV absorption (A_t) with time on a Perkin-Elmer Lambda 5 double-beam UV-vis spectrometer. Pseudo-first-order rate constants were calculated graphically from plots of $A_t - A_\infty$ against time on semilogarithmic graph paper. For a typical run, 20 μL of the stock solution (10 mM) was injected into a silica cell containing a solution of buffer (2.5 mL) containing base buffer species at the appropriate concentration. Repetitive scanning of the UV spectrum during reaction indicated the best single wavelength to use in fixed wavelength studies of the kinetics. The wavelengths employed, together with their respective extinction coefficient changes, are given in Tables I–III. The pH of the solution was measured in the silica cell after the experiment with a Radiometer PHM 26 instrument standardized to ±0.01 pH units with EIL standard buffers.

Results

Release of phenols from the phosphodiester substrates exhibited excellent pseudo-first-order kinetics up to 90% of the total reaction. The observed rate constants (k') for reactions of diesters in imidazole-containing buffers are linearly dependent on the concentration of total imidazole. The apparent second-order rate constant ($k'' = dk'/d[\text{imidazole}_{tot}]$) for the 4-nitrophenyl ester is proportional to the fraction of imidazole present as free base; including a background term, the rate law is given by the general eq 3. It

$$k' = k_{bkgrd} + k_B[B_{free}] \tag{3}$$

is assumed that the rate law for other leaving groups is eq 3, and the derived values of k_{im} are collected in Table I together with the conditions.

The reaction of the 4-nitrophenyl ester is catalyzed by bases, and plots of pseudo-first-order constants versus base concentration were analyzed according to eq 3. Data for k_B are recorded in Table II with the conditions.

Reaction of hydroxide ion with the aryl esters was measured by studying the rates in bicarbonate ion buffers adjusted to the appropriate pH. Doubling the bicarbonate buffer concentration at the same pH (0.05–0.1 M in buffer) had no effect on the rate constant. The second-order rate constants (k_{OH}) were derived from the slope of the plot of k' versus the hydroxide ion concentration computed from the pH, and these are listed in Table III.

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Table III. Fission of Substituted Phenyl Uridine-3′-phosphates Catalyzed by Hydroxide Ion at 25 °C and 0.25 M Ionic Strength[h]

subst[a]	pK^{ArOH}	k_{OH},[j] M^{-1} s^{-1}	pH[b,c]	$k' \times 10^4$,[d,i] s^{-1}	N[e]	λ,[f] nm	Δϵ,[g] cmM^{-1}
parent	9.95	12	9.20–10.40	2–26	4	230	3700
4-Cl	9.38	45	9.24–10.50	6.4–99	4	230	4000
2-Cl	8.48	95	8.50–9.60	2.8–30	4	241	2000
3-NO_2	8.35	300	9.32–10.47	78–860	5	235	3400
3,5-Cl_2	8.18	370	8.55–9.72	17–200	4	241	1600
2,5-Cl_2	7.51	520	8.70–9.20	14–87	3	240	3700
4-NO_2	7.14	840	9.31–10.53	0.2–3.4	5	400	16000
2,4,5-Cl_3	6.72	1100	9.27–10.50	38–210	3	240	5000
2-Cl-4-NO_2	5.45	3600	8.66–9.71	290–1970	4	400	11000

[a] Substrate concentrations ~0.1 mM. [b] Bicarbonate buffer at 0.05 M. [c] Range of pH. [d] Range of observed rate constants. [e] Number of data points not including duplicates. [f] Wavelength for kinetics. [g] Average exctinction coefficient. [h] Ionic strength maintained with Na_2SO_4. [i] Slight curvature in plots of k' against hydroxide ion concentration was observed at higher pH's owing to incursion of nucleotide base ionization. [j] Error no greater than 5%.

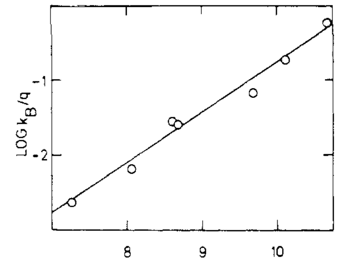


Figure 2. Brønsted dependence of the fission of 4-nitrophenyl uridine-3′-phosphate catalyzed by general bases. The correlation is statistically corrected (Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969), and the identities of the points are given in Table II. The line is calculated from eq 6, and the conditions are recorded in Table II.

The parameters k_{im} and k_{OH} obey Brønsted type relationships (Figure 1) quantified by eq 4 and 5.

$$\log k_{im} = (-0.59 \pm 0.08)pK^{ArOH} + 1.40 \pm 0.42 \ (n = 7, r = 0.955) \quad (4)$$

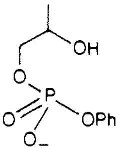
$$\log k_{OH} = (-0.54 \pm 0.05)pK^{ArOH} + 6.68 \pm 0.42 \ (n = 9, r = 0.967) \quad (5)$$

$$\log k_B = (0.67 \pm 0.05)pK^{BH} - 7.50 \pm 0.42 \ (n = 7, r = 0.989) \quad (6)$$

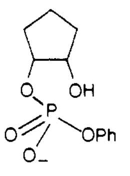
The release of 4-nitrophenol from the phosphodiester catalyzed by bases obeys a linear Brønsted eq 6 illustrated in Figure 2 (standard deviations are given in the equations).

Discussion

Reaction Catalyzed. The rate constant for attack of hydroxide ion on the phenyl ester (12 M^{-1} s^{-1} at 25 °C) is very much larger than that reported by Brown and Usher[2] for the hydrolysis of phenyl 2-hydroxypropyl phosphate (I, 0.98 × 10^{-3} M^{-1} s^{-1} at 24.6 °C) but is closer to those of II and III (0.03 and 2.17 M^{-1} s^{-1}, respectively).[3]



I



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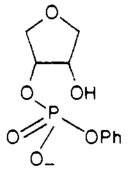
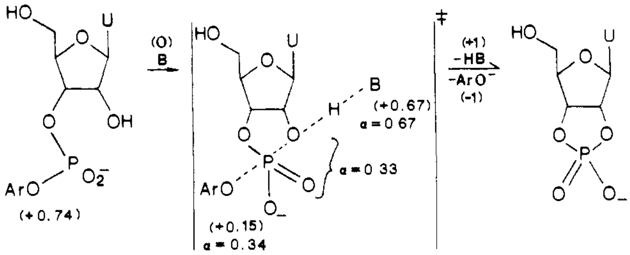


III

Scheme I. Effective Charges (in Parentheses) and Leffler α Values for Cyclization of Aryl Uridine-3′-phosphates



Simple attack of nucleophiles on the phosphodiester can be excluded as a reaction path because the rate constants are far in excess of those expected for such a reaction. Kirby and Younas[14] showed that release of 2,4-dinitrophenol from 2,4-dinitrophenyl methyl phosphate by direct attack of hydroxide ion has the rate constant 4.7 × 10^{-4} M^{-1} s^{-1} at 39 °C. The rate constant for release of 2,4-dinitrophenol from the corresponding uridine ester may be calculated to be 2.9 × 10^4 M^{-1} s^{-1} at 25 °C by using eq 5. The formation of an intermediate is indicated from the time traces of UV absorbance of the aryl esters in the aqueous buffer solutions, which exhibited an initial rapid first-order change (due presumably to cyclization) followed by a very slow first-order decay; the rate constant for the decay was very much smaller than that of the initial change and did not interfere with the kinetics. In most cases the kinetics were not pursued after the initial change. The rate constant for the decay in the case of the 2-chlorophenyl ester at pH 11 was 1.2 × 10^{-4} s^{-1} at 25 °C which is close to that for decomposition of the cyclic 2′,3′-uridine monophosphate (5.7 × 10^{-3} s^{-1}) at 50 °C.[15] Previous studies[16] have argued persuasively that nucleophilic displacement of a good leaving group from pentavalent phosphorus by oxyanions is concerted or involves an intermediate in a very shallow energy well at the top of a barrier. In the present case (eq 2) a putative intermediate would possess two full negative charges that could act as internal nucleophiles,[16c] thus favoring a concerted mechanism in the expulsion of good leaving groups.

Conservation of Effective Charge. When component values of β may be determined for the transition state of a reaction involving more than one bonding change then the corresponding changes in effective charge are not necessarily conserved. Imbalance of changes in effective charge has little meaning unless the individual changing bonds are similar[7,16,17] and the component calibrating substituent effects (β_{eq}) are the same. In order to study charge balance in a reaction with dissimilar component bond changes, we can use a modification of Leffler's approach[18] where normalized substituent effects are employed ($\alpha = \beta/\beta_{eq}$) for the *component* bond changes in a reaction. The substituent effect for a rate is "calibrated" by the substituent effect for the overall equilibrium. When the substituent effect monitors a *single* bond change in the

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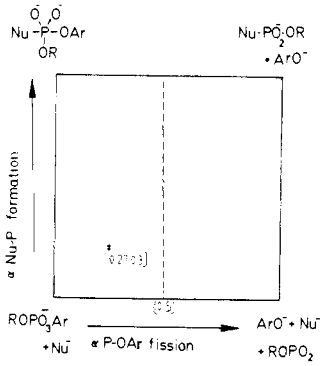


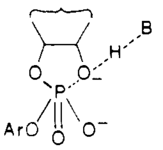
Figure 3. Schematic reaction map for the reaction of nucleophiles with phosphodiester. The proton-transfer component cannot be illustrated, but its coordinate will be at its minimum energy for each position in the diagram. The coordinates for the transition state of the base-catalyzed reaction of aryl uridine-3′-phosphate are given, and the transition state of the benzyl guanosine-3′-phosphate has coordinates at an unspecified point on the dotted line (corresponding to $\alpha = 0.5$).

reaction (for example the substituent is coupled to the bond via either leaving atom or attacking atom) the maximal value of α will be unity.

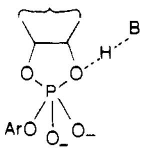
An imbalance in Leffler's α values for component bonding changes in a reaction will indicate whether charge is building up or being depleted.[19]

Phosphodiester Alcoholysis. The transition state for the transfer of the phosphoryl group from phenolate ion nucleophile to the neighboring 2′-hydroxyl function (eq 2) can be described by the "effective charge" and "Leffler" map given in Scheme I. The effective charge for the aryl oxygen in the aryl phosphodiester is reasonably assumed to be the same as that (+0.74)[8] on the aryl oxygen of the species $HOPO_2^-OAr$.

The imbalance of the Leffler indices on nucleophilic and leaving groups of atoms (0.43) indicates buildup of negative charge on the central ($O_2^-P\cdots O$) group of atoms (Scheme I). The present data do not distinguish between the possibilities of where most of this charge resides: on the attacking oxygen (IV), phosphoryl oxygens (V), or delocalized. Both of these extreme structures could be consistent with concerted to stepwise processes. The data of Kirby and Younas[14] for displacement of 2,4-dinitrophenol by phenolate anions from methyl 2,4-dinitrophenyl phosphate indicates a β_{nuc} of about 0.47; this result coupled with a β_{eq} of 1.74 for the overall reaction gives a Leffler α index of 0.27 for formation of the Ar–O–P bond (VI). Since there is considerable similarity between the above intermolecular reaction and the intramolecular case studied here, we assign only weak bonding to the forming O–P bond in the cyclization reaction and the excess charge will therefore reside mainly on the attacking oxygen as in IV. The bonding distribution is illustrated in the reaction map (Figure 3), where the imbalance of 0.33 in α is identified with charge buildup on the attacking oxygen and only little P–O bond formation occurs.



IV



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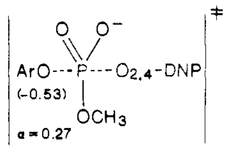

VI

2,4-DNP = 2,4-dinitrophenyl

Work of Satoh and Inoue[6] has indicated that substituted benzyl esters of guanosine-3′-phosphoric acid possess k_{OH} values that exhibit a ρ value of 1.24 for variation of the aromatic substituent. The parent benzyl ester has k_{OH} (2.2×10^{-4} M^{-1} s^{-1} at 35 °C, which is of the order of magnitude expected for extrapolation from Figure 1. The nature of the nucleotide base is not expected to significantly affect the reactivity. There is no consistent set of data for the ionization of substituted benzyl alcohols, but a value of ρ^* (1.42) has been reported for the ionization of RCH_2OH.[21] The Brønsted β_{lg} would then be −0.87; this value and the β_{eq} for the transfer reaction give a Leffler index for the breaking P–O bond of 0.5, indicating more charge development on the leaving oxygen atom than in the aryl ester case. This is consistent with the much weaker leaving ability of the benzyloxy anion, and the change in β_{lg} is probably consistent more with a Hammond effect on a concerted reaction coordinate than on a stepwise process.

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Supplementary Material Available: Preparative details and tables of analytical and physical data (3 pages). Ordering information is given on any current masthead page.

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[19] The apparent exceptions to the Leffler hypothesis discovered by Bordwell and discussed by Pross and Shaik[20] refer to substituents directly coupled to *two* bonding changes via an atom central to both bonds. Confusion has arisen in the application of Leffler's hypothesis and of uncalibrated β (or ρ) values to reactions that normally have significant bonding changes in at least two bonds. The original Leffler assignment was to a hypothetical reaction involving only one bond change. The overall structure of a transition state in a reaction involving more than one bond change cannot be measured from a single substituent effect since this does not monitor bonds other than that to which the substituent is coupled. A single β (or Leffler α) value is thus not completely helpful in describing transition-state structure, and it is better to monitor all the changing bonds. Most reactions involve more than one bonding change.

[20] (a) Bordwell, F. G.; Boyle, W. J. *J. Am. Chem. Soc.* **1972**, *94*, 3907. (b) Pross, A.; Shaik, S. S. *Acc. Chem. Res.* **1983**, *16*, 363.

[21] (a) Barlin, G. B.; Perrin, D. D. *Q. Rev., Chem. Soc.* **1966**, *20*, 75. (b) Ballinger, P.; Long, F. A. *J. Am. Chem. Soc.* **1960**, *82*, 795.