Studies in Sulfate Esters. V. The Mechanism of Hydrolysis of Phenyl Phosphosulfate, a Model System for 3′-Phosphoadenosine 5′-Phosphosulfate

S. J. Benkovic and R. C. Hevey

Contribution from the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received January 19, 1970*

Abstract: Phenyl phosphosulfate has been synthesized as a model for 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and adenosine 5′-phosphosulfate. The pH–rate profile for the nonenzymic hydrolysis of the analog indicates the dianion and monoanion to be hydrolytically labile. Hydrolysis of the monoanion is viewed as an acid-catalyzed unimolecular mechanism with elimination of sulfur trioxide on the basis of predominant S–O bond cleavage, the small negative value of $\Delta S^{\neq}$, the sulfating properties of the monoanion in mixed alcohol–water solvents, and a deuterium oxide solvent isotope effect typical of an A-1 mechanism. A comparison with the mechanistic mode of hydrolysis of acetyl sulfate is developed in order to delineate the unique characteristics of the phosphosulfate bond. These results are interpreted with regard to the significance of the structure which evolved biologically to transport active sulfate.

The importance of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfate donor in the formation of chrondrointin sulfate, cerebrosulfatides, and phenolic sulfates is well established.[1-3] PAPS and adenosine 5′-phosphosulfate (APS) have also been shown to act as intermediates in the reduction of sulfate to sulfite.[4] Yet since the discovery and elucidation of the structure of PAPS[5,6] little has been accomplished in regard to the chemistry of the phosphosulfate linkage. Both of the sulfuryl adenylates (PAPS and APS) are known to undergo rapid hydrolysis in dilute aqueous acid at room temperature[7] and in the case of PAPS to transfer the sulfuryl moiety [SO_3] in the presence of charcoal to a hexoseamine to give a 6-O-sulfate derivative.[8]

In this paper we report synthesis of a model compound, phenyl phosphosulfate, and the mechanism of its nonenzymic hydrolysis. Moreover, a comparison with the mechanistic mode of acetyl sulfate hydrolysis is developed in order to delineate the unique characteristic of the phosphosulfate bond.

Experimental Section

Phenyl Phosphosulfate Diammonium Salt (PPS). To a slurry of 2.77 g (0.0159 mol) of phenylphosphoric acid and 1.26 g (0.0159 mol) of anhydrous pyridine in a 35-ml round-bottomed flask was added 2.53 g (0.0159 mol) of pyridine–sulfur trioxide complex. The flask was sealed with parafilm and placed in a preequilibrated 55° oil bath for 12 hr. The mixture was stirred constantly by means of a magnetic stirring apparatus throughout this reaction time. After the reaction period the viscous mixture was chilled at 0° for 15 min, neutralized with a saturated solution of sodium bicarbonate to a pH of 7, and extracted with four 50-ml portions of ether. The extent of reaction was estimated by descending paper chromatography (Whatman No. 4: *n*-propyl alcohol–ammonia–water, 6:3:1). The sodium salts of phenyl phosphate and inorganic sulfate served as internal standards. Inorganic sulfate was determined by the method of Burma[9] and phosphate esters by the method of Hanes and Ischerwood[10] as modified by Bandurski.[11] Phenyl phosphate and phenyl phosphosulfate (PPS) could also be located directly by short wave ultraviolet light quenching. The pertinent R_f values are given in Table I. The solution was diluted to 500 ml with water,

Table I. R_f Values at 25° in Solvent System *n*-Propyl Alcohol–Ammonia–Water[a]

Compound	R_f
Phenyl phosphosulfate (disodium salt)	0.62
Phenyl phosphate (disodium salt)	0.45
Sodium sulfate	0.24
Sodium phosphate	0.05

[a] 6:3:1, on Whatman No. 4 paper.

and applied to an ECTEOLA column which had been preconditioned according to the procedure of Balasubramanian.[12] The phosphate esters were eluted by a linear gradient formed *via* a two-chamber system comprised of 1-l. mixing and reservoir cylinders filled initially with 0.02 and 0.75 *M* NH_4HCO_3, respectively. Fractions (10 ml) were collected and monitored at 260 mμ for ultraviolet absorption. Inorganic sulfate was located by its immediate precipitation as barium sulfate with 0.5 *M* barium chloride which remains after the fractions have been acidified to a pH < 1. In the case of fractions containing PPS, addition of barium chloride solution followed by acidifying to a pH < 1 gives initially a clear solution from which the time-dependent precipitation of barium sulfate can be observed. The fractions containing PPS were combined and stripped to dryness under vacuum. The white material that remained was then heated to 45–50° under vacuum for 8 hr to decompose residual ammonium bicarbonate: yield, 0.46 g; mp 176–179° dec. Descending paper chromatography showed the material to be homogeneous. Chromatography and hydrolysis of a sample to inorganic sulfate by the technique of Schneider and Lewbart[13] gave a positive sulfate test coincident with a prior ultraviolet quenching spot of R_f corresponding to phenyl phosphosulfate. Hydrolysis of a sample of PPS in 0.1 *N* HCl for 1 hr at 55° and chromatography with the above solvent system gave two spots corresponding to inorganic sulfate and to phenyl phosphate. The uv spectrum of PPS (pH 11.84, μ = 1.0) shows absorption at 262 mμ (ϵ 383). The ir spectrum (KBr) shows peaks at 1570 (sharp),

(1) J. D. Gregory and F. Lipmann, *J. Biol. Chem.*, **229**, 1081 (1957).
(2) E. Meezan and E. A. Davidson, *ibid.*, **242**, 1685 (1967).
(3) A. S. Balasubramanian and B. K. Bachhawat *Indian J. Exp. Biol.*, **1**, 179 (1963).
(4) A. Roy, *Advan. Enzymol.*, **22**, 205 (1960), and references therein.
(5) P. Robbins and F. Lipmann, *J. Amer. Chem. Soc.*, 78, 2652 (1956).
(6) F. Lipmann, *Science*, **128**, 575 (1958).
(7) P. Robbins, *Methods Enzymol.*, **6**, 766 (1963).
(8) J. Adams, *Biochem. Biophys. Acta*, **62**, 17 (1962).
(9) R. J. Block, E. L. Durrum, and G. Zweig, "Manual of Paper Chromatography and Paper Electrophoresis," Academic Press, New York, N. Y., 1958, p 428.
(10) G. S. Hanes and F. Ischerwood, *Nature*, **164**, 1107 (1949).
(11) R. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(12) A. S. Balasubramanian, *Anal. Biochem.*, **21**, 23 (1967).
(13) J. J. Schneider and M. L. Lewbart, *J. Biol. Chem.*, **222**, 787 (1956).

1490 (sharp), 1390 (sharp), 1280–1220 (triplet, broad), 1125 (strong), 948 (sharp, weak), 860–835 (strong, doublet), 780–750 (broad triplet), 725 (broad), and 690 (weak) cm^{-1}. After complete hydrolysis in 0.1 *N* HCl for 1 hr the amount of inorganic sulfate, determined by the method of Jaselskis and Vas,[14] and the amount of phenyl phosphate determined by optical density measurements at 262 mμ (pH 6.36), gave a ratio of phenyl phosphate:sulfate of 1.0:0:0.94.

Pyridine-$^{35}SO_3$ Sulfation. Phenyl phosphosulfate was synthesized by the above procedure employing pyridine-$^{35}SO_3$ and was chromatographed with the above solvent system. Kodak single-coated, blue sensitive X-ray film was placed on the chromatogram for 3 days in an X-ray exposure holder. After film development a spot of R_f 0.62 was located which corresponded to the unlabeled phenyl phosphosulfate salt prepared previously. Pyridine-$^{35}SO_3$ was furnished through the courtesy of Dr. R. O. Mumma.

Acetyl sulfate was prepared as the monopotassium salt by the method of Tanghe and Brener[15] and used without further purification. The infrared spectrum (Nujol) showed peaks at 1555 (sharp), 1550 (sharp), 1260 (broad multiplet), 1098 (sharp), 1069–1052 (sharp doublet), 1005 (sharp), 900 (broad), 875 (broad multiplet), 730 (quartet broad), and 645 (broad multiplet) cm^{-1}.

Oxygen-18 Incorporation. Phenyl phosphosulfate was completely hydrolyzed in 2.5 atom % $H_2{}^{18}O$ at pH 1.43 and 3.71, μ = 1.0. Addition of 0.5 ml of saturated barium chloride solution precipitated barium sulfate which was removed by centrifugation. The pH of the supernatant was adjusted to pH 8.0 with 1.0 *N* KOH. Addition of 2 vol of ethanol to the supernatant which was now chilled and maintained at 5° precipitated barium phenyl phosphate which was isolated by centrifugation. The precipitate was washed twice with 95% ethanol and dried over P_2O_5 in a vacuum dessicator overnight. Potassium dihydrogen phosphate was obtained from barium phenyl phosphate by the method of Haake and Westheimer.[16]

The oxygen of potassium dihydrogen phosphate was converted to CO_2 by heating with dry guanidine hydrochloride according to the method of Boyer, *et al.*,[17] and analyzed in a Nuclide mass spectrometer by the isotope ratio method. The ^{18}O content of the enriched water was determined by the same procedure. A control of phenyl phosphate was conducted to check for any incorporation of ^{18}O into phenyl phosphate during the time of the run.

Incorporation of ^{18}O into the acetic acid produced from acetyl sulfate by hydrolysis in 1 atom % $H_2{}^{18}O$ in the pH range 4.70–5.20 and 0.9–1.5 was determined by converting the acetic acid to CO_2 according to the method of Bentley.[18] Spectra were furnished through the courtesy of Dr. F. Lampe and Dr. P. Potzinger of this department.

Materials. Methanol (Baker reagent grade), D_2O (99.8% Diaprep), $H_2{}^{18}O$ (5 atom %, BioRad), pyridine (purified by refluxing over barium oxide, distilling, and storing over calcium hydride), ammonium bicarbonate (Fisher, reagent grade), and twice-distilled deionized water were employed. Pyridine–sulfur trioxide complex (Aldrich) was stored in a desiccator over P_2O_5. ECTEOLA was a product of Gallard Schlessinger. All other buffer materials were reagent grade (Baker, Fisher). Metal nitrates (reagent grade, Baker) were utilized.

Apparatus. Instrumentation used in this study has been previously described.[19] Kinetic runs were carried out in Kimax screw cap tubes (No. 45066A) with Teflon-lined caps (no. 9447-83) maintained at constant temperature (±0.1°) by a circulating water bath.

Kinetics. The hydrolysis of phenyl phosphosulfate was followed at 55°, μ = 1.0 with KCl, by several monitoring techniques dependent on the pH region under investigation. At low pH values (0–1.6) the release of phenyl phosphate from a substrate concentration of 3–7 × 10^{-3} *M* in HCl buffer was monitored at 262 mμ utilizing a Gilford uv spectrophotometer (Model 2000). The observed first-order rate constants for hydrolysis in this pH region were generally calculated by the method of Guggenheim.[20] Several constants were calculated by the integrated form of the first-order rate equation utilizing OD_∞. Very low acid rates in this pH region were also obtained by quenching 1-ml aliquots in 1 ml of 0.5 *M* potassium phosphate buffer, pH 11.80, at appropriate time intervals and reading at 262 mμ. Duplicate runs for each method agreed within ±5 and ±9%. The quenching procedure was employed at high and intermediate pH's except that OD_∞ was assumed equal to the measured OD_{262} for a phenyl phosphate standard at the corresponding pH values because of interference from phenol owing to phenyl phosphate hydrolysis. In the pH region 1.5–3.6 the rate of hydrolysis was monitored by a pH stat discussed below. In the pH region 3.5–8 the hydrolysis of phenyl phosphosulfate was determined by two other procedures. Inorganic sulfate release was determined by the barium chloranilate method of Spencer[21] or Wainer and Koch[22] after quenching aliquots in acetate buffer, 0.5 *M*, pH 4.00 at appropriate time intervals. The chloranilate absorption at 327.5 or 530 mμ was compared to a standard curve to determine the amount of inorganic sulfate present. The observed first-order rate constants were calculated from the slopes of plots of log $(OD_\infty - OD_0/OD_\infty - OD_t)$ against time for kinetic runs to at least two half-lives. Where applicable, rates were also determined by the method of Bunton[23] for consecutive reactions by monitoring the release of inorganic phosphate from phenyl phosphate by utilizing the analytical method of Martin and Doty.[24] The rate of hydrolysis of phenyl phosphate disodium salt was determined concommitantly. Rate constants calculated from either of the two methods (Wainer and Koch[22] and Bunton[23]) agreed within ±15%.

Buffers employed were HCl (pH < 2.5), formate (0.2 *M*, pH 2.8–3.8), acetate (0.2 *M*, pH 4.0–5.4), Tris (0.2 *M*, pH 6.8–7.8), and carbonate (0.2 *M*, pH 9.0–10.0) adjusted to μ = 1.0 with KCl. Observed rates were invariant with changing buffer concentrations (acetate 0.06–0.6 *M*) or ionic strength (0.1–1.0). The pH of all buffers was determined at 55°. Kinetic runs with a pH variation greater than ±0.03 during the course of the run were not utilized.

In the pH region 1.2–3.6 the rate of hydrolysis of phenyl phosphosulfate was followed at constant pH at 55 ± 0.1° by titration employing a Radiometer pH Stat unit as described by Bruice and Bradbury.[25] The instrument was modified such that the three-necked Metrohm cell accommodated the buret tip, a Metrohm type XEA125 combination electrode, and an air tight nitrogen inlet. Carbonate free, 0.1 *N* KOH was prepared by the method of Albert and Serjeant.[26] The base (10 ml) adjusted to μ = 1.0 with KCl was diluted to 50 ml with decarbonated, ion-free distilled water. The reaction was initiated by addition of 5.0–7.0 mg of substrate to 2.0 ml of preequilibrated 1 *M* KCl (pH *ca.* 6), passing nitrogen through the cell for approximately 5 min, and then bringing the solution to the titrating pH. The amount of base added per unit time, to maintain constant pH, was recorded on a precalibrated titrigraph recorder. The system was standardized at pH 4.08 with Fisher Scientific pH 4.00 buffer. The sensitivity and linearity of the electrode was checked periodically according to Albert and Sergeant.[26] Observed first-order rate constants were determined by the method of Guggenheim.[20] The observed first-order rate constants for the hydrolysis in deuterium oxide, μ = 1.0 at 55°, were calculated using the corrected pD calculated from the formula of Fife and Bruice.[27]

The hydrolytic rates of acetyl sulfate were also determined at constant pH at 35° and μ = 1.0 by the above procedure. A value of pK_w 13.8 was employed to calculate $[OH^-]$.

Kinetic runs utilizing metal ions were standardized by the appropriate EDTA procedure of Flaschka[28] or Schwarzenbach.[29] Metal ion rates in runs maintained at μ = 1.0 with KNO_3 were followed spectrophotometrically by quenching with phosphate buffer as stated above or by the method of Wainer and Koch after first removing cations from the quenched sample by ion exchange with Rexyn 101 $(NH_4)^+$ resin.

(14) B. Jaselskis and S. Vas, *Anal. Chem.*, **36**, 1965, (1964).
(15) L. Tanghe and R. Brewer, *ibid.*, **40**, 350 (1968).
(16) P. Haake and F. Westheimer, *J. Amer. Chem. Soc.*, **83**, 1102 (1961).
(17) P. D. Boyer, D. J. Graves, C. H. Suelter, and M. E. Dempsey, *Anal. Chem.*, **33**, 1906 (1961).
(18) R. Bentley, *J. Amer. Chem. Soc.*, **71**, 2765 (1949).
(19) S. J. Benkovic and P. A. Benkovic, *ibid.*, **88**, 5504 (1966).
(20) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).
(21) B. Spencer, *J. Biochem.*, **75**, 435 (1960).
(22) A. Wainer and A. Koch, *Anal. Biochem.*, **3**, 457 (1962).
(23) C. A. Bunton, M. M. Mhala, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3293 (1960).
(24) J. B. Martin and D. M. Doty, *Anal. Chem.*, **21**, 965 (1949).
(25) T. C. Bruice and W. C. Bradbury, *J. Amer. Chem. Soc.*, **87**, 4838 (1965).
(26) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," John Wiley & Sons, Inc., New York, N. Y., 1962.
(27) T. H. Fife and T. C. Bruice, *J. Phys. Chem.*, **65**, 1079 (1961).
(28) H. A. Flaschka, "EDTA Titrations. An Introduction to Theory and Practice," Pergamon Press, Elmsford, N. Y., 1959.
(29) G. Schwarzenbach, "Complexometric Titrations," Interscience, New York, N. Y., 1959.

Table II. Rate Constants for Hydrolysis of Phenyl Phosphosulfate and Acetyl Sulfate

Compd	Solvent	k_{H^+}, sec^{-1} M^{-1}	k, sec^{-1}	k_{obsd}, sec^{-1} [b]	k_0, sec^{-1}	k_{OH^-}, sec^{-1} M^{-1}
Phenyl phosphosulfate, 55° [a]	H_2O	2.78×10^{-2}	4.06×10^{-4}	1.73×10^{-4}	4.19×10^{-7}	
	D_2O			3.44×10^{-4}		
Acetyl sulfate, 35°	H_2O	6.10×10^{-1}			1.03×10^{-3}	3.98×10^{1}
	D_2O				7.62×10^{-4}	

		37°	55°	75°
Phenyl phosphosulfate	k [c]	5.30×10^{-5} sec^{-1}	4.06×10^{-4} sec^{-1}	2.96×10^{-3} sec^{-1}
		$E_a = 21.9 \pm 1.3$ [d] kcal mol^{-1}	$\Delta S^{\ddagger} = -9.5 \pm 4$ eu [d]	

[a] $pK_a = 2.37$ estimated from kinetic data at 55°, $\mu = 1.0$. [b] Values of k_{obsd} at pH = 2.65. [c] The pK_a utilized to calculate k is assumed to be invariant with temperature. This is the case for the pK_a associated with monoionization of phosporic acid.[26] [d] Calculated from the equation $\Delta H^{\ddagger} = E_a - RT$; $\Delta S^{\ddagger} = \Delta H^{\ddagger} - \Delta F^{\ddagger}/T$; $\Delta F^{\ddagger} = -RT \ln kh/kT$ at 55°. E_a obtained from a plot of log k *vs.* $1/T$.

For the solvolysis of phenyl phosphosulfate in mixed methanol-water solvent, inorganic sulfate was determined by the method of Wainer and Koch and the amount of methyl sulfate calculated *via* a mass balance relationship. The recovery of standard inorganic sulfate in a control run was quantitative, the accuracy being ±2.6%. For acetyl sulfate solvolysis the oscillometric procedure of Benkovic and Benkovic[30] was employed.

Results

The pH–rate profile for the hydrolysis of phenyl phosphosulfate in water at 55° is shown in Figure 1. Values of k_{obsd}[31] may be calculated from eq 1 where

$$k_{obsd} = k_{H^+}[a_H^2/(a_H + K_a')] + k[a_H/(a_H + K_a')] + k_0 \quad (1)$$

k_{H^+} is the second-order rate constant associated with hydronium ion catalyzed hydrolysis of the neutral species, k and k_0 are the first-order rate constants for

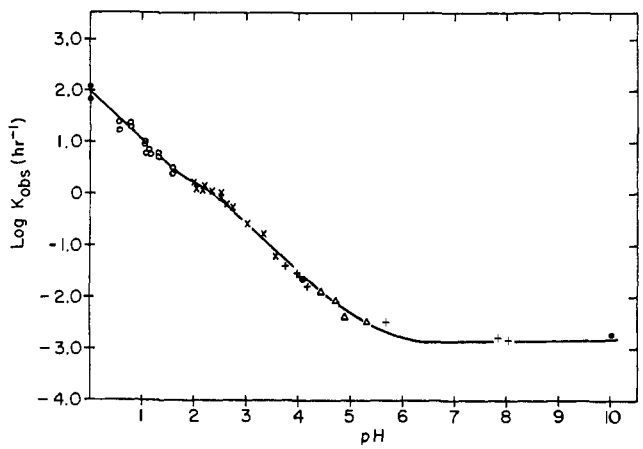


Figure 1. The log k_{obsd}–pH rate profile (55°, $\mu = 1.0$) for the hydrolysis of phenyl phosphosulfate. Solid line is theoretical curve calculated from values listed in Table II and eq 1. Points designated (●) were obtained by quenching, (○) by automatically recording phenyl phosphate release, (×) with an auto titrator, (+) employing barium chloranilate, and (Δ) determining phosphate release.

the hydrolysis of the mono- and dianion, respectively, and a_H is the activity of hydrogen ion as measured by the glass electrode. The acid dissociation constant K_a' is kinetically determined. The pH–rate profile may be divided into three regions: (1) at pH > 6.00 log k_{obsd} is independent of pH; (2) at pH 2–6 log k_{obsd} is independent and then dependent on pH with a slope of −1; (3) at pH < 2 log k_{obsd} is dependent on hydronium ion catalysis with a slope of −1.

The pH–rate profile for hydrolysis of acetyl sulfate in water at 35° is shown in Figure 2. Calculated

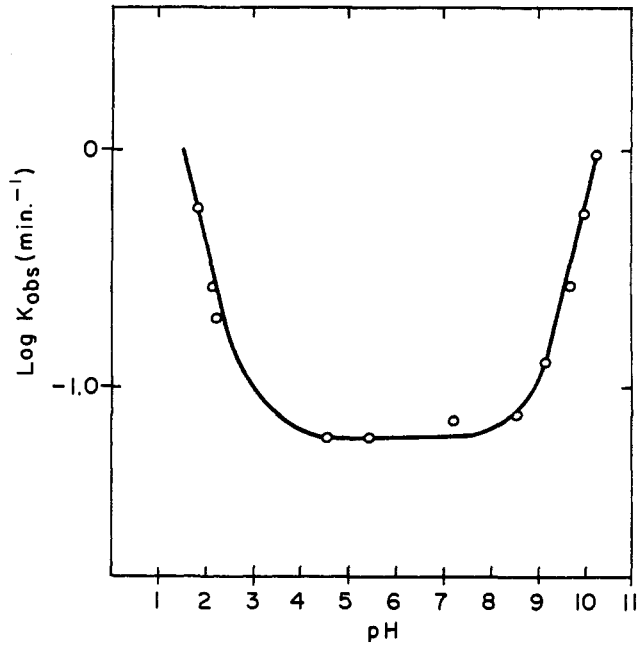


Figure 2. The log k_{obsd}–pH rate profile (35°, $\mu = 1.0$) for the hydrolysis of acetyl sulfate. Solid line is theoretical curve calculated from values listed in Table II and eq 2.

values of k_{obsd} may be obtained from eq 2 where

$$k_{obsd} = k_{H^+}[a_H] + k_0 + k_{OH^-}[a_{OH^-}] \quad (2)$$

k_{H^+}, k_{OH^-}, and k_0 are the second- and first-order rate constants for acid, base, and spontaneous-catalyzed hydrolysis, respectively.

Pertinent kinetic data for phenyl phosphosulfate and acetyl sulfate are summarized in Table II. Included in Table II are the rate constants for hydrolysis of phenyl phosphosulfate at three temperatures and the computed activation energy and entropy for hydrolysis. A change of ionic strength from 1 to 0.1 M and buffer concentration from 0.2 to 0.6 M showed the rates to be invariant to buffer or ionic strength effects.

(30) S. J. Benkovic and P. A. Benkovic, *J. Amer. Chem. Soc.*, **90**, 2646 (1968).

(31) A satisfactory fit of the data is also obtained if the plateau rate is assumed to be a bimolecular process, $k'a_H[K_a'/(K_a' + a_H)]$ where $k' = 9.51 \times 10^{-2}$ sec^{-1} M^{-1}, *i.e.*, hydronium ion catalysis of the dianion species.

The data for hydrolysis of phenyl phosphosulfate and acetyl sulfate in $H_2{}^{18}O$ are summarized in Table III. Hydrolysis was carried out to at least five half-lives for both phenyl phosphosulfate and acetyl sulfate. A control experiment on phenyl phosphate showed that no ^{18}O exchange occurred with the solvent during the time of hydrolysis.

The results of the solvolysis of phenyl phosphosulfate at 55° and acetyl sulfate at 35° in mixed methanol-water are recorded in Table IV. Total hydrolysis of phenyl phosphosulfate and acetyl sulfate at each pH showed theoretical (±2.5%) and (±2.8%) inorganic sulfate recovery. The data for sulfur trioxide are taken from Benkovic and Benkovic.[30]

Table III. Hydrolysis of Phenyl Phosphosulfate and Acetyl Sulfate in $H_2{}^{18}O$

Phenyl phosphosulfate diammonium salt[a]				
	Atom % ^{18}O			Atoms ^{18}O/ molecule[c]
		Phenyl phosphate		phenyl
pH	Water	Calcd	Found[b]	phosphate
1.4–2.0	2.50	0.625	0.233 ± 0.005	0.061
3.71	2.50	0.625	0.249 ± 0.003	0.089
2.1	Normal water		0.198 ± 0.004	

Phenyl phosphate disodium salt[a]				
	Atom % ^{18}O			Atoms ^{18}O/ molecule
		Phenyl phosphate		phenyl
pH	Water	Calcd	Found	phosphate
3.5	2.50	0.625	0.197	0.00

Acetyl sulfate potassium salt[d]				
	Atom % ^{18}O			Atoms ^{18}O/ molecule[e]
		Acetic acid		acetic
pH	Water	Calcd	Found[b]	acid
4.7–5.2	1.00	0.500	0.631	1.08
	1.00	0.500	0.661	1.15
0.9–1.5	1.00	0.500	0.580	0.95
	Normal water		0.199	

[a] Temperature 55°, μ = 1.0. [b] Values not corrected for natural abundance $H_2{}^{18}O$. [c] Atoms ^{18}O/phenyl phosphate = 1.0 for 100% P–O bond cleavage. [d] Temperature 35°, μ = 1.0. [e] Atoms ^{18}O molecule acetic acid = 1.0 for 100% C–O bond cleavage.

Table IV. Mixed Solvent Studies. Solvolysis of Phenyl Phosphosulfate, Acetyl Sulfate, and Sulfur Trioxide in Aqueous Methanol

	Mole fraction		
Substrate	Methanol	Methyl sulfate	Ratio
PPS[a]	0.309	0.573	1.85
PPS[b]	0.309	0.459	1.49
PPS[c]	0.229	0.231	1.01
Acetyl sulfate[d]	0.307	0	0
Sulfur trioxide[e]	0.303	0.57	1.88

[a] pH = 1.79, T = 55°, μ = 1.0, t = 3 hr. [b] pH = 2.28, T = 55°, μ = 1.0, t = 18 hr. [c] pH = 3.81, T = 55°, μ = 1.0, t = 48 hr. [d] pH = 4.30, T = 35°, t = ∞. [e] T = 35°, μ = 0.05–0.16; taken from S. J. Benkovic and P. A. Benkovic, *J. Amer. Chem. Soc.*, **90**, 2646 (1968).

Data for metal ion studies are found in Table V. Aluminum(III) catalyzes the reaction threefold at pH 2.96 while magnesium, calcium, and manganese are only slightly acceleratory. Zinc, cobalt, and copper, meanwhile, can be seen to have a twofold inhibitory effect on the hydrolysis of phenyl phosphosulfate at pH 3.00.

Table V. Metal Ion Catalyzed Hydrolysis of Phenyl Phosphosulfate[a]

Metal ion	pH	k, hr^{-1}	$M \times 10^{-2}$	Rate effect
None	3.10	0.224		
None	3.15	0.199		
None	3.05	0.251		
Magnesium(II)	3.17	0.250	7.0	1.26 faster
Calcium(II)	3.13	0.300	7.0	1.5 faster
Zinc(II)	3.10	0.109	7.0	2.05 slower
Manganese(II)	3.09	0.187	2.0	1.2 slower
Cobalt(II)	3.05	0.136	2.0	1.85 slower
Copper(II)	3.08	0.101	(5.96×10^{-4})	2.21 slower
Aluminum(III)	2.96	0.978	7.0	3.16 faster

[a] Substrate concentration about 5×10^{-3} M; at 55°; μ = 1.0 with KNO_3.

Discussion

The synthesis and purification of phenyl phosphosulfate (PPS), the first synthetic analog of APS and PAPS, was dependent on two critical findings. First, in order to maximize the yield of PPS a 1:1 ratio of pyridine to phenyl phosphoric acid is utilized and second in order to achieve component separation of the reaction mixture the mixed anion exchange resin, ECTEOLA, is vastly superior to other anion exchange resins. Polystyrene strongly basic anion exchange resins lead to extensive decomposition of PPS during the elution process.

The rate of hydrolysis of phenyl phosphosulfate follows the rate law

$$\frac{d(SO_4^{2-})}{dt} = k_0[\text{dianion}] + k[\text{monoanion}] + k_{H^+}[\text{monoanion}][H^+] \quad (3)$$

which indicates the dianion and monoanion to be hydrolytically labile. The latter also undergoes acid-catalyzed hydrolysis. Only the dianion is labile if one employs the kinetically indistinguishable rate law

$$\frac{d(SO_4^{2-})}{dt} = k_0[\text{dianion}] + k[\text{dianion}][H^+] + k_{H^+}[\text{dianion}][H^+]^2 \quad (4)$$

The pK_a of phenyl phosphosulfate (2.37), estimated from kinetic data, is in reasonable agreement with other polyphosphates, *e.g.*, pyrophosphoric acid,[32,33] $H_3P_2O_7^-$. In order to elucidate the point of bond cleavage, PPS was hydrolyzed in enriched $H_2{}^{18}O$ water (Table III). In the pH region (1–2) one observes incorporation of ~0.07 atom of ^{18}O/molecule of phenyl phosphate and at pH 3.71 approximately 0.09 atom of ^{18}O/molecule of phenyl phosphate. This corresponds to 93 and 91% sulfur–oxygen bond cleav-

(32) S. M. Lambert and J. I. Watters, *J. Amer. Chem. Soc.*, **79**, 4262 (1957).

(33) J. D. McGilvery and J. P. Crowther, *Can. J. Chem.*, **32**, 174 (1954).

age, respectively. The small but significant incorporation of ^{18}O into phenyl phosphate is understandable, since phenyl phosphosulfate, a diester of phosphoric acid, might be expected to hydrolyze *via* a bimolecular displacement on phosphorus, a mechanism postulated for tetraethyl pyrophosphate ester hydrolysis.[34,35] It is of considerable interest that the rate of P–O bond cleavage in tetraethyl pyrophosphate relative to phenyl phosphosulfate monoanion—comparable conditions of pH and temperature—is only fourfold slower. A similar approximate result is obtained with pyrophosphoric acid monoanion. It is obvious, however, that S–O bond cleavage is the main reaction pathway. Two alternate mechanisms may then be postulated for overall PPS hydrolysis: (1) a unimolecular sulfur–oxygen bond fission with elimination of SO_3 or a solvated species resembling SO_3 or (2) hydrolysis proceeding *via* bimolecular nucleophilic attack of water on sulfur.

Recently Fendler and Fendler[36] found that a plot of log k for a series of substituted arylsulfate monoanions against pK_a of the corresponding phenol correlated linearly with a slope of -1.2, while the corresponding slope for acid-catalyzed hydrolysis (k_{H^+}) was found to be between -0.22 and -0.26. The last value is similar to the result obtained by Burkhardt[37,38] who showed that the rate of acid-catalyzed hydrolysis of a series of substituted phenyl sulfates increases with increasing electron withdrawal in the leaving group where $\log k_{H^+} = -1.53 - 0.25pK_a$.[39] Since the evidence for a unimolecular elimination of SO_3 in sulfate ester hydrolysis is well documented in both the acid-catalyzed hydrolysis and spontaneous hydrolysis of substituted aryl sulfates monoanions,[36–38,40] the magnitude of the slope of log k_{H^+} and k_0 *vs.* pK_a, for these hydrolytic reactions, suggests a protonated and anionic leaving group, respectively. Thus the magnitudes of the slopes are identical with the well-established effect of leaving group on phosphate monoester monoanion and dianion hydrolysis where the mechanisms are presumably the unimolecular expulsion of the isoelectronic monoanionic metaphosphate. Extrapolating the experimental data of Fendler and Fendler for substituted aryl sulfate monoanion and acid-catalyzed hydrolysis to 55° yields the linear free-energy expressions

$$\log k_0 = 1.80 - 1.20pK_a \quad (5)$$

$$\log k_{H^+} = -1.54 - 0.23pK_a \quad (6)$$

Applying eq 5 (see Figure 3) to the spontaneous hydrolysis of phenyl phosphosulfate dianion yields, on employing a pK_a for phenyl phosphate monoanion of 5.88,[41] a predicted value of log $k_0 = -5.26$. The experimental value for phenyl phosphosulfate dianion hydrolysis is log $k_0 = -6.38$. One may attribute the 13-fold deviation in the spontaneous dianion rate of

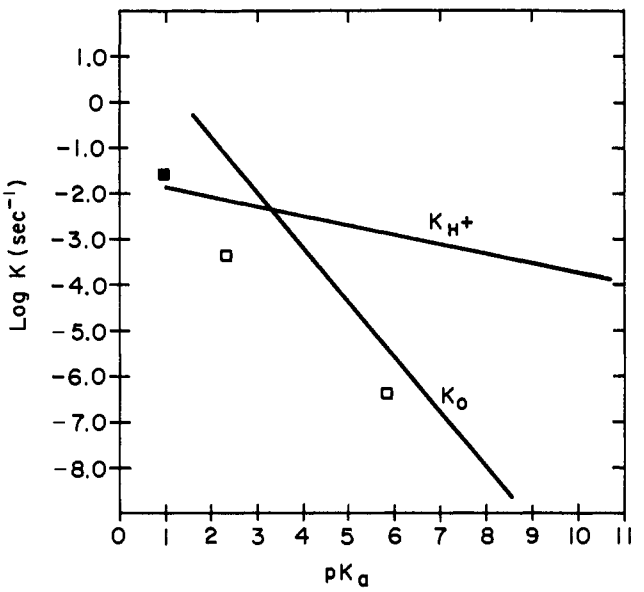
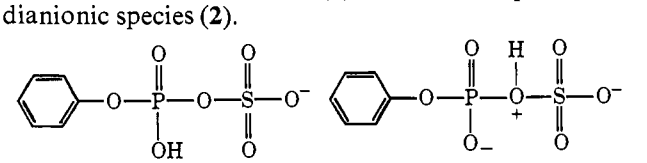


Figure 3. Plot of log k_{H^+} (top line) and log k_0 (bottom line) for the hydrolysis (at 55°) of a series of substituted phenyl sulfates *vs.* the pK_a of the corresponding phenol.[36–38] Points designated [□] are for monoanion and dianion hydrolysis of phenyl phosphosulfate. The point designated [■] is for the acid-catalyzed hydrolysis of phenyl phosphosulfate.

hydrolysis of phenyl phosphosulfate from the linear free-energy relationship (eq 5) to such factors as either ground-state destabilization or transition-state stabilization by resonance interaction in the substituted aryl sulfate esters[42] (all bear nitro groups), relative to the insulation of the sulfate moiety in PPS by the intervening phosphoryl group.[43]

Whatever the cause, let us assume that a 13-fold slower rate for PPS hydrolysis may be considered characteristic for an alkyl or aryl phosphate leaving group. If these same factors are operative in the hydrolysis of phenyl phosphosulfate monoanion, the 230-fold slower rate calculated on the basis of eq 5 by employing the kinetic pK_a' is reduced to a factor of eighteen. In applying eq 5 we are assuming that the monoanion and not a hydronium ion plus the dianionic species is the correct description for the reaction. This argument is based on the finding that the rate of unsymmetrical diethyl P_1P_1 pyrophosphate is nearly identical with the rate of hydrolysis of the dianion of pyrophosphoric acid. The near identity of the rate constants mitigates against the kinetically indistinguishable mechanism involving a hydronium ion and trianionic species of pyrophosphoric acid. Consequently we treat our system as involving the thermodynamically more favorable monoanion (**1**) rather than a protonated dianionic species (**2**).

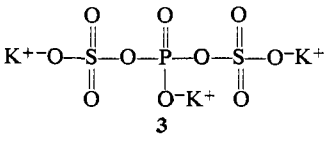


1 PPS monoanion

2 PPS dianion

(34) D. M. Brown and N. K. Hamer, *J. Chem. Soc.*, 1155 (1960).
(35) D. O. Campbell and M. L. Kilpatrick, *J. Amer. Chem. Soc.*, **76**, 893 (1954).
(36) E. J. Fendler and J. H. Fendler, *J. Org. Chem.*, **33**, 3852 (1968).
(37) G. N. Burkhardt, W. G. Kenneth Ford, and E. Singleton, *J. Chem. Soc.*, 17, (1936).
(38) G. N. Burkhardt, A. G. Evans, and F. Warhurst, *ibid.*, 5 (1936).
(39) S. J. Benkovic and L. K. Dunikoski, *Biochemistry*, **9**, 1390 (1970).
(40) S. J. Benkovic and P. A. Benkovic, *J. Amer. Chem. Soc.*, 88, 5504 (1966).
(41) J. D. Chanley and E. Feageson, *ibid.*, 77, 4002 (1955).
(42) J. F. Kirsch, W. Clewell, and A. Simon, *J. Org. Chem.*, **33**, 127 (1967).
(43) R. F. Hudson, "Structure and Mechanisms in Organo-phosphorus Chemistry," Academic Press, New York, N. Y., 1965, Chapter 8.

That this negative deviation is probably general for substrates possessing the phosphosulfate linkage is substantiated by the data of Von Lampe[44] for the hydrolysis of potassium disulfatomonophosphate (**3**).



3

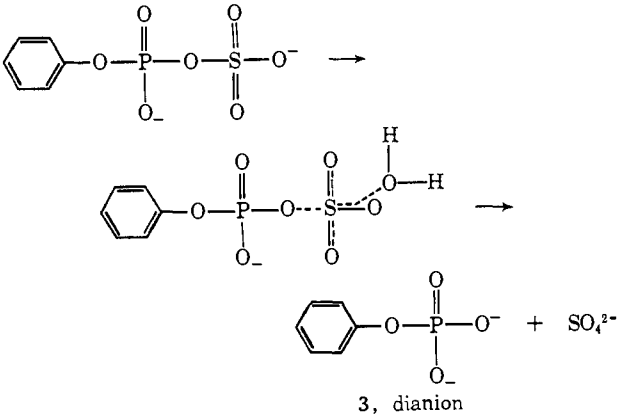
A comparison of the latter's rate with the rate of hydrolysis of phenyl phosphosulfate, after statistically correcting the rate constant by a factor of two, discloses that at pH 3.8 only a sixfold difference exists between k_{obsd} for the two anhydrides. Moreover, it is interesting that attempts to correlate P_1P_1 diethyl pyrophosphate hydrolysis[45a] with the linear free-energy relationship found by Kirby and Varvoglis[45b] for phosphate ester dianion hydrolysis reveals that this rate also is *ca.* 200-fold slower than predicted. One may, therefore, conclude that the same effect is acting in the hydrolysis of both phosphosulfate monoanion and pyrophosphate dianion. This additional factor may be a stabilizing hydrogen bond between adjacent oxygens on phosphorus and sulfur in the monoanion or the destabilizing juxtaposition of charge in the dianion.

The deuterium solvent isotope effect (k^{H_2O}/k^{D_2O} = 0.5) for the monoanion of PPS, however, would favor greater involvement of solvent than is manifested by a pure unimolecular elimination reaction. The kinetic deuterium solvent isotope effect as classified by Bunton and Shriner[46] would for reactions for the A-2 type have a range of 0.6–0.7 and for A-1 a value less than 0.5. The k^{H_2O}/k^{D_2O} value for phenyl phosphosulfate, which contains approximately a 12% contribution from the acid-catalyzed mechanism, would reflect a borderline behavior between an A-1 and A-2 solvolysis reaction. Indeed, support for the above interpretation of the deuterium isotope effect may be seen in the estimated entropy of activation ($\Delta S^{\ddagger} \approx -10 \pm 4$ eu). This value of $\Delta S^{\ddagger}$ for phenyl phosphosulfate monoanion is in agreement with other sulfate esters containing good leaving groups, where negative entropies of activation in the range of -10 to -18 eu have been found.[36,40,47] In general, the more negative entropy of activation is a consequence of the restriction of the degrees of freedom of solvent molecules in the transition state. The greater involvement of solvent in a bimolecular process relative to a unimolecular process is characterized by lower values -20 to -30 eu for entropies of activation.[48] By employing the data of Von Lampe[44] for the hydrolysis of potassium disulfatomonophosphate a value of approximately -4.0 eu for the entropy of activation may be estimated for the hydrolysis of this phosphosulfate. The conclusion of Benkovic and Benkovic[40] that the low β = 0.20 and presumed high ρ values for amine nucleophilic reactions with *p*-nitrophenylsulfate is characteristic of reactions kinetically unimolecular but featuring some bimolecular dependency is seen as extending the reaction to nucleophiles other than water.

In Table IV are tabulated the results of methanol–water solvolysis. From the data it may be seen that the solvolysis of PPS yields a product ratio that is indicative of selection for methanol. The mole ratio of methylsulfate to methanol is seen to decrease steadily as one proceeds from low to high pH's, *i.e.*, through the inflection point of the pH–rate profile. These results are suggestive of a transition state which generates solvated sulfur trioxide monomer but with a lower selectivity for methanol than found for sulfur trioxide.[30] The pH dependency of methyl sulfate production may be indicative of partial bond formation between the SO_3 moiety and the leaving group in the product forming transition state. The behavior of PPS in the partitioning of the sulfuryl moiety between alcohol and water is in sharp contrast to that observed for the phosphate anhydride, pyrophosphoric acid. Attempts to trap metaphosphate ions[49] as alkyl phosphate have proven unsuccessful for pyrophosphate hydrolysis at pH 4. This finding does not fit the other experimental probes which imply a metaphosphate mechanism but at present the underlying reasons for this behavior are not known.

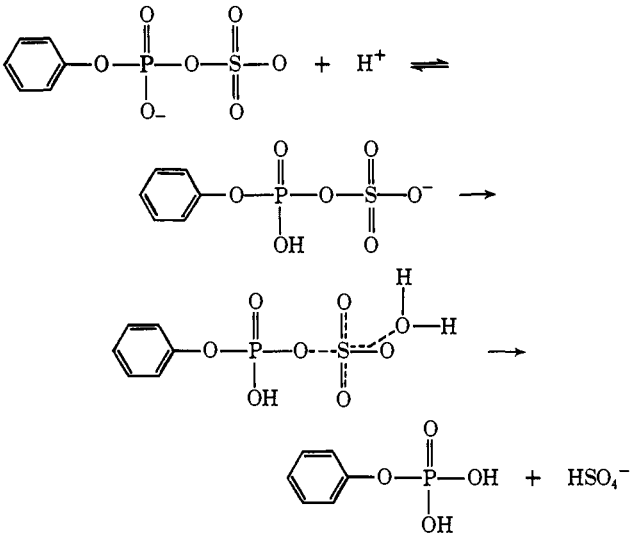
By applying eq 6 in a similar fashion it can be shown that the rate of acid-catalyzed hydrolysis of phenyl phosphosulfate is in good agreement with the predicted value (calcd -1.50; found -1.72). Thus it is suggested that the mechanism for acid-catalyzed hydrolysis of aromatic sulfate esters and phenyl phosphosulfate are identical, *i.e.*, an A-1 mechanism involving preequilibrium protonation of substrate followed by rate determining unimolecular decomposition. Only upon complete protonation which effectively eliminates resonance and electrostatic contributions to the stability or instability of ground or transition state, does the behavior of the phosphosulfate resemble an aryl sulfate ester.

The accumulated evidence for the dianion and monoanion hydrolysis of PPS, therefore, appears to be in agreement with a unimolecular elimination of sulfur trioxide involving a molecule of solvent in the transition state, where the "activation" for the monoanion occurs by a preequilibrium protonation of the dianion.



3, dianion

(44) F. Von Lampe, *Z. Anorg. Allg. Chem.*, **367**, 170 (1969).
(45) (a) D. L. Miller and T. Ukena, *J. Amer. Chem. Soc.*, **91**, 3050 (1969); (b) A. J. Kirby and A. G. Varvoglis, *ibid.*, **89**, 415 (1967).
(46) C. A. Bunton and V. J. Shriner, *ibid.*, **83**, 3207 (1961).
(47) B. E. Fleischfresser and I. Lauder, *Aust. J. Chem.*, **15**, 242 (1962).
(48) F. A. Long, J. G. Pritchard, and F. E. Stafford, *J. Amer. Chem. Soc.*, **79**, 2363 (1957).
(49) C. A. Bunton and H. Chaimovich, *Inorg. Chem.*, 4, 1763 (1965).



4 monoanion

Hydrolysis of Acetyl Sulfate

The pH profile for hydrolysis of acetyl sulfate is given in Figure 2. The solid line was calculated using eq 7. The hydrolysis is catalyzed by hydronium and

$$k_{obsd} = k_{H^+}[a_{H}] + k_0 + k_{OH}[a_{OH^-}] \qquad (7)$$

hydroxide ions, with an appreciable spontaneous pH independent rate. Characteristically, the shape of the pH–rate profile resembles that observed for other activated acyl ester hydrolyses.[50-52] The results of hydrolysis of acetyl sulfate in $H_2{}^{18}O$ and in the mixed solvent system methanol–water are given in Table II and Table III. An average incorporation of 1.1 atoms of ^{18}O into the acetate residue, and the complete lack of sulfate partitioning in the mixed solvent methanol–water dictate a mechanism which proceeds through C–O bond cleavage. This mechanism might be a nucleophilic reaction of water with the acyl group of the ester since there is no appreciable deuterium isotope effect (k^{H_2O}/k^{D_2O}) = 1.4. This contrasts with k^{H_2O}/k^{D_2O} ratios of >2 for the water-catalyzed hydrolysis of acyl-activated esters where the mechanism presumably is general base catalysis.[53,54]

For comparison one may calculate the pH-independent rate of hydrolysis of acetyl sulfate by S–O bond cleavage from the linear free-energy equation of Fendler and Fendler, extrapolated to 35°. One observes that the actual rate of hydrolysis of acetyl sulfate (pK_a = 4.76) *via* C–O bond cleavage is 63 times faster than theoretically predicted for S–O fission, a rate difference readily explicable through the acyl group displacement mechanism.

The results of the above study allow one to speculate on the significance of the structure chosen biologically to transport active sulfate. Although many "high-energy" compounds found in biochemical systems are acid anhydrides, for example ATP, PAPS, their chemical make-up constitutes a unique group. Whereas, anhydrides like acetic anhydride and phosphoryl chloride are readily hydrolyzed by water at physiological pH's the biologically important inorganic anhydrides are relatively stable to hydrolysis until enzymatically activated. The structure of the phosphate–sulfate anhydride PAPS allows such an enzymatic "triggering device" to be operative. At physiological pH's, PAPS would be expected to exist as a dianion, a form known to be stable. When activation is required, complexation with an enzyme and "activation" by either protonation of the phosphoryl oxygen or complexation with metal ion should produce a reactive sulfate ester capable of carrying out the desired sulfation step. A similar mechanistic process has been postulated by Westheimer and Miller[55] for monoprotonated γ-phenylpropyl diphosphate hydrolysis.

The choice of the phosphate anhydride PAPS instead of an acyl anhydride, *e.g.*, acetyl sulfate as the biological sulfating agent, illustrates the complications of dual functionality. Thus, even though the pK_a's of acetic acid and phosphoric acid are reasonably close to allow for a rather facile SO_3 elimination process to occur, the presence of the electrophilic carbonyl group induces almost exclusive C–O bond cleavage rather than S–O bond cleavage. The sulfating ability of this PPS is solely dependent on the reduced reactivity to nucleophilic attack of the phosphate group relative to the acyl group. However as discussed previously a very small margin of reactivity, no more than a factor of 10, designates PPS as a sulfating rather than phosphorylating agent.

To test the possibility that activation might occur by chelation with a metal ion, the catalytic effect of a number of metal ions was tested (Table IV). It may be seen that rate enhancement is at most only threefold and that zinc, cobalt, and copper actually slightly retard the rate of hydrolysis. Although not of catalytic importance in aqueous solution the results do not exclude metal ion catalysis for a PAPS–enzyme–metal ion complex.

In conclusion, it may be stated that the chemical structure chosen by biological systems to carry active sulfate may have been dictated by the process of activation. In fact, the susceptibility of the phosphosulfate linkage to reduction[44] may indicate yet another unique chemical feature of the phosphate sulfate anhydride not observed in other biological anhydrides. This is being pursued in our laboratory at present.

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(50) D. E. Koshland, Jr., *J. Amer. Chem. Soc.*, 74, 2286 (1952).
(51) G. DiSabato and W. P. Jencks, *ibid.*, 83, 4400 (1961).
(52) C. A. Bunton and J. H. Fendler, *J. Org. Chem.*, 31, 2307 (1966).
(53) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, New York, N. Y., 1966, Chapter 1.
(54) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, Chapter 10.
(55) D. L. Miller and F. H. Westheimer, *J. Amer. Chem. Soc.*, 88, 1407 (1966).