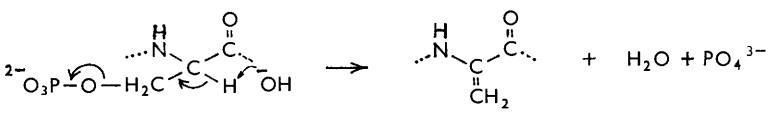
46. *Elimination Reactions and Hydrolysis of Serine Phosphate.*

By David Samuel and Brian L. Silver.

The breakdown of serine phosphate in aqueous solution is due to hydrolysis in strongly acidic media, elimination in basic media, and a mixture of hydrolysis and elimination in the pH range 0—7. The products of hydrolysis are serine and orthophosphate, the products of elimination are ammonia, pyruvic acid, and orthophosphate. By using oxygen-18 as tracer, bond fission was found to be at the P–O ester bond in hydrolysis and at the C–O ester bond in elimination. Substitution of deuterium for the α-hydrogen of serine resulted in a kinetic isotope effect (k_H/k_D) of ~2 for the elimination. The rate of elimination in strongly basic solution may be explained in terms of the H^- function. The rates of hydrolysis in strongly acid solution and of elimination in the pH range 7—13 are practically independent of pH.

In 1906 Plimmer and Bayliss [1] found that phosphoproteins were dephosphorylated in a few minutes by 0·2N-sodium hydroxide at room temperature, but that no hydrolysis was observable in acid. About 50 years later Anderson and Kelley [2] repeated this experiment in solvent water containing an excess abundance of oxygen-18 and found no isotopic enrichment in the recovered orthophosphate ion. Using previous evidence [3] that most phosphate groups in phosphoproteins are attached to serine residues they proposed a base-catalyzed β-elimination:



Recently, β-elimination of biological phosphates and pyrophosphates has been recognized as of considerable metabolic significance.[4] It was therefore of interest to

[1] Plimmer and Bayliss, *J. Physiol.*, 1906, **33**, 439.
[2] Anderson and Kelley, *J. Amer. Chem. Soc.*, 1959, **81**, 2275.
[3] Aldridge, *Ann. Reports*, 1956, **53**, 294; Gibson, *ibid.*, 1957, **54**, 306; Cohen, Oosterbaan, and Warringa, *Discuss. Faraday Soc.*, 1955, **20**, 114.
Lynen, Eggerer, Henning, and Kesse, *Angew. Chem.*, 1958, **70**, 738; Flavin and Kono, *J. Biol. Chem.*, 1960, **235**, 1109.

determine the relative importance of hydrolysis and elimination in the breakdown of serine phosphate in aqueous solution, and the results of such a study are presented in this paper. Both hydrolysis and elimination probably occur in biological material. The breakdown of serine phosphate has been shown [5,6] to be catalyzed by pyridoxal and metal ions at pH 9, the experimental results being interpreted in terms of the formation of a Schiff base followed by an elimination.

A specific serine phosphatase,[7] which appears to act as a hydrolytic enzyme, has recently been isolated from a number of tissues, and it has been suggested [8] that serine phosphate is the immediate precursor of serine in the biosynthesis of the amino-acid.

Experimental

Materials.—DL-Serine phosphate, synthesized [9] from DL-serine (Eastman) and chlorophosphoric acid, had m. p. 186° [lit.,[10] 185° (Found: P, 16·8%; equiv., 185·8. Calc. for $C_3H_8NO_6P$: P, 16·7%; equiv., 186·1].

DL-[α-^{2}H] Serine phosphate was synthesized by an adaptation of King's serine synthesis: [11] ethyl acetamidomalonate (43·4 g.) was dissolved in water (25 ml.), and 37% formaldehyde solution (17·0 g.) was added. The pH was adjusted to ~7, then a further 0·5 ml. of 0·9N-sodium hydroxide was added. After 2 hr. the solution was filtered, sodium hydroxide (16·8 g. in 120 ml. of water) was added, and the solution left overnight. After evaporation, 1·5 g. of the residue were dissolved in 99·8% D_2O (25 g.). 7·5N-Deuterium chloride (3 ml.) was added and the solution refluxed for 30 min. After evaporation, concentrated hydrochloric acid (7 ml.) was added and the solution refluxed for 1½ hr. Removal of the solvent gave crude deuteroserine, which was phosphorylated with chlorophosphoric acid to give DL-[α-^{2}H]serine phosphate, m. p. 185°. A sample of normal DL-serine phosphate prepared in the same way had m. p. 186° and gave the same rates of dephosphorylation, within experimental error, as serine phosphate prepared from commercial serine.

Methods.—Reaction products. The three products were detected colorimetrically in preliminary experiments: phosphate by the molybdate reagent, ammonia by nesslerization, and pyruvic acid by the 2,4-dinitrophenylhydrazine reagent. Pyruvic acid was identified as the crystalline 2,4-dinitrophenylhydrazone, m. p. 194° (lit.,[12] 192°).

Isotope experiments. ^{18}O-Enriched water was obtained from the distillation plant of this Institute. 99% D_2O was purchased from Norsk Hydroelektrik. The position of bond fission of serine phosphate was determined from pH 1 to 6N-sodium hydroxide. The general procedure was as follows: Serine phosphate (100—200 mg.) was dissolved in aqueous acid, alkali, or buffer containing an excess abundance of oxygen-18 in the solvent water. After incubation at 100° for approximately one half-life, inorganic phosphate was isolated as barium phosphate and converted into carbon dioxide by Anbar and Guttmann's method.[13] Carbon dioxide was analyzed for oxygen-18 with a Consolidated Engineering Corp. model 21-401 mass-spectrometer. The concentration of deuterium in [α-^{2}H]serine was determined by burning the compound to water as described by Keston *et al.*[14] Cylinder oxygen was passed successively through sulphuric acid, a " Drierite " drying tower, and then cupric oxide at 700—800°. Serine phosphate (10—15 mg.) was placed in a porcelain boat in the combustion tube 2—3 cm. before the cupric oxide. The sample was heated with a flame, and the resulting water collected in a trap cooled in acetone–solid carbon dioxide. Analysis for deuterium in the above-mentioned mass-spectrometer showed that 66% of the " hydrogen " on the α-carbon of serine was deuterium. The position of the deuterium is known from the method of synthesis.

[5] Longenecker and Snell, *J. Biol. Chem.*, 1957, **225**, 409.
[6] Metzler and Snell, *J. Biol. Chem.*, 1952, **198**, 353, 363.
[7] Borkenhagen and Kennedy, *Biochim. Biophys. Acta*, 1958, **28**, 222; *J. Biol. Chem.*, 1959, **234**, 849; Neuhaus and Byrne, *Biochim. Biophys. Acta*, 1958, **28**, 223; *J. Biol. Chem.*, 1959, **234**, 113; Schramm, *ibid.*, 1958, **223**, 1169.
[8] Ichihara and Greenberg, *J. Biol. Chem.*, 1959, **224**, 331.
[9] " Biochemical Preparations," Vol. VI, p. 75, John Wiley and Sons Inc., New York, 1958.
[10] Lapidot, personal communication.
[11] King, *J. Amer. Chem. Soc.*, 1947, **69**, 2738.
[12] Campbell, *Analyst*, 1936, **61**, 391.
[13] Anbar and Guttmann, *J. Appl. Rad. Isotopes*, 1959, **3**, 233.
[14] Keston, Rittenberg, and Schoenheimer, *J. Biol. Chem.*, 1937, **122**, 227.

Colorimetric analysis of phosphate ion, pyruvic acid, and ammonia. Phosphate ion was determined by Allen's modified method,[15] the concentration of the blue phosphomolybdate complex being measured in a Beckman spectrometer calibrated at 650 μ. Strongly acid or alkaline samples were neutralized with aqueous sodium hydroxide or hydrochloric acid before addition of the molybdate reagent.

Pyruvic acid was determined with the 2,4-dinitrophenylhydrazine reagent.[5]

Ammonia was determined by nesslerization.[5] The optical density was measured at 420 μ in a Beckman spectrometer.

Kinetic procedure. The kinetics of dephosphorylation were followed by colorimetric estimation of phosphate, and in selected runs by simultaneous colorimetric determination of pyruvic acid and ammonia. Hot concentrated alkali attacks glass, to form soluble silicates which interfere with the determination of phosphate by the molybdate method. Consequently, runs at pH >10 were carried out in 30 ml. Polythene containers supported in metal cylinders. At regular time intervals the containers were opened for 20—30 sec. and aliquot parts were removed for analysis.

Runs at pH <10 were carried out in sealed Pyrex ampoules. In a typical run, 20 ml. of 0·02M-serine phosphate and 50 ml. of 0·2M-potassium hydrogen phthalate buffer were made up to 100 ml. with distilled water. Portions (~9 ml.) were sealed in Pyrex ampoules and kept at 100° ± 0·5°. After 10 min. allowed for thermal equilibration, the first ampoule was removed and dropped into liquid air, and a stop-watch was started. Ampoules were allowed to come to room temperature, then opened, and a 2-ml. sample was taken for phosphate analysis, and 3-ml. samples each for pyruvate and ammonia analysis.

Optical densities were measured in 1-cm. cells against a control consisting of the reagents. In these conditions, the optical density was a linear function of concentration of phosphate, pyruvic acid, or ammonia. First-order rate constants for liberation of inorganic phosphate may therefore be calculated from the formula

$$k_P = (2{\cdot}303/t)\log_{10}[(D_\infty - D_0)/(D - D_t)],$$

where D_0, D_t, and D_∞ are optical densities for samples taken at zero time, time t, and 100% reaction, respectively. The concentration of phosphate at infinity, calculated from the initial concentration of serine phosphate, agreed within experimental error (±3%) with that found experimentally. It is not possible to measure accurately the ammonia and pyruvic acid produced by the breakdown of serine phosphate since serine itself is slowly deaminated in experimental conditions, giving ammonia and pyruvic acid. However, this deamination proceeds at a rate less than about 5% of that of the breakdown of serine phosphate. Hence measurements of the rate of formation of ammonia and pyruvate over the first 40—50% of the decomposition of serine phosphate show first-order kinetics within experimental error. Values of k_{Amm} and k_{Pyr}, (the first-order rate constants for production of ammonia and pyruvic acid) may therefore be derived from the ratio of inorganic phosphate to ammonia (or pyruvate) at any time during the early part of the reaction. This follows from the relations: $[PO_4]/[NH_3] = k_P/k_{NH_3}$, and

TABLE 1.

Deamination of 0·01M-DL-serine and DL-serine phosphate at 100·0° ± 0·5°.

pH *	1·0	2·2	4·1	6·0	9·6	2N-NaOH
10^3k (hr.$^{-1}$) (deamin.) serine	2·0	1·8	1·0	0·5	0·2	— 2·4
10^3k (hr.$^{-1}$) (elim.) serine phosphate	25	33	26	12	10	80

* For buffers see Tables 3 and 4.

$[PO_4]/[\text{Pyruvate}] = k_P/k_{Pyr}$. The rate of deamination of serine was followed by the same procedure as for serine phosphate. Since the reaction was slow it was not followed beyond 30—40% and the rate constants are therefore not as accurate as for serine phosphate, but even after allowance for an estimated error of ±20% it may be seen from Table 1 that the experimental error of ±4% in the rate constants for serine phosphate covers any corrections necessary for the concurrent deamination of serine.

[15] Allen, *Biochem. J.*, 1940, **34**, 858.

Results

Kinetics.—The main aim of the present work was to establish the mechanism by which serine phosphate breaks down under alkaline conditions. However, for comparison with other monoesters of phosphoric acid the pH–rate profile was determined over the acid range.

It will be shown that the breakdown of serine phosphate proceeds by two main mechanisms: (1) Hydrolysis giving serine and orthophosphate ion and (2) elimination giving ammonia, pyruvic acid, and orthophosphate ion. It follows that the observed first-order rate constant for the production of orthophosphate k_H is the sum of two terms.

$$k_P = k_{Hydrol} + k_{Elim}$$

Since k_{Elim} is given by either k_{Amm} or k_{Pyr} (as defined above) and k_P is an experimentally observed quantity, k_{Hydrol} may be calculated from the difference, $k_P - k_{Elim}$.

Table 2.

Hydrolysis of 0·004M-serine phosphate in strongly acid solution at 100·0° ± 0·5°.

$HClO_4$ (M)	1·0	2·0	4·0	4·0 *	6·0	7·0	8·0	10·0
k_P (hr.$^{-1}$)	0·035	0·040	0·051	0·050	0·065	0·062	0·060	0·070

* [Serine Phosphate] = 0·001M.

Table 3.

Hydrolysis and elimination of serine phosphate at pH 1—6 and 100·0° ± 0·5° (unless specified otherwise).

pH	k_P (hr.$^{-1}$)	k_{Hydrol} (hr.$^{-1}$)	k_{Elim} (hr.$^{-1}$)	Buffers *
1·0	0·120	0·095	0·025	0·1M-$HClO_4$
2·2	0·145	0·112	0·033	0·1M-HCl–KCl
2·6	0·150	0·117	0·033	0·1M-KHP–HCl
3·2	0·154	0·123	0·031	0·1M-KHP–HCl
3·6	0·156	0·127	0·029	0·1M-KHP–HCl
4·1	0·149	0·123	0·026	0·1M-KHP
4·1	0·148	0·120	0·028	0·16M-KHP
5·0	0·105	—	—	0·1M-KHP–NaOH
5·4	0·095	0·076	0·019	0·1M-KHP–NaOH
6·0	0·067	—	—	0·1M-KHP–NaOH
4·1 †	0·0134	0·0099	0·0035	0·1M-KHP
4·1 ‡	0·0393	0·031	0·0083	0·1M-KHP

* Buffers as given by Stene (*Rec. Trav. chim.*, 1930, **49**, 1133); KHP = potassium hydrogen phthalate. † At 80·0° ± 0·2°. ‡ At 90·0° ± 0·2°.

The results are given in Tables 2—4 and 7. At selected pH values runs were carried out with [α-^{2}H]serine phosphate as substrate. The results obtained are given in Table 6. For comparison the equivalent runs with normal serine phosphate are also given. In calculating

Table 4.

Hydrolysis and elimination of 0·004M-serine phosphate at pH 7—14 at 100·0° ± 0·5° (unless specified otherwise).

pH	10^2k_P (hr.$^{-1}$)	10^2k_{Hydrol} (hr.$^{-1}$)	10^2k_{Elim} (hr.$^{-1}$)	Buffers *	pH	10^2k_P (hr.$^{-1}$)	10^2k_{Hydrol} (hr.$^{-1}$)	10^2k_{Elim} (hr.$^{-1}$)	Buffers *
7·0	2·2	0·8	1·4	0·1M-Borate	9·65	1·2	—	1·2	0·025M-Borate
7·5	1·0	0·15	0·85	,,	13·7	2·3	—	2·3	0·5N-NaOH
9·6	1·4	—	1·4	,,	9·6 ‡	0·13	—	0·13	0·1M-Borate
9·6 †	1·4	—	1·4	,,	9·6 §	0·45	—	0·45	,,

* See Table 3. † [Serine phosphate] = 0·001M. ‡ At 80° ± 0·2°. § At 90° ± 0·2°.

Table 5.

Elimination of serine phosphate in aqueous sodium hydroxide at 100·0° ± 0·5°.

[NaOH]	1·0	2·0	3·0	4·0	5·0	6·0	6·0 *	6·0 †	6·0 ‡
k_P (hr.$^{-1}$)	0·037	0·080	0·152	0·260	0·405	0·636	0·625	0·115	0·275
k_P/[NaOH]	0·037	0·040	0·051	0·065	0·081	0·106	0·104	—	—

* [Serine phosphate] = 0·001M. † At 80·0° ± 0·2°. ‡ At 90·0° ± 0·2°.

the rate constants for the deuterated compound in sodium hydroxide solution, account was taken of the presence of 34% of the normal compound, by treating the overall reaction as the sum of two simultaneous non-competing reactions.

TABLE 6.

Deuterium effects on the hydrolysis and elimination of serine phosphate at 100·0° ± 0·5°.

	[α-^{2}H]Serine phosphate			Serine phosphate				
Medium *	k_P^D	k_{Hydrol}^D (hr.$^{-1}$)	k_{Elim}^D	k_P^H	k_{Hydrol}^H (hr.$^{-1}$)	k_{Elim}^H	$(k_H/k_D)_{Elim}$	$(k_H/k_D)_{Hydrol}$
6·0N-$HClO_4$	0·065	0·065	—	0·065	0·065	—	—	1·0
pH 4·0, 0·1M-KHP	0·138	0·124	0·014	0·149	0·123	0·026	1·85	1·0
pH 9·6, 0·1M-Borate	0·010	—	0·010	0·014	—	0·014	1·40	—
pH 9·65, 0·025M-Borate	0·008	—	0·008	0·012	—	0·012	1·50	—
2·0N-NaOH	0·042	—	0·042	0·080	—	0·080	1·90	—
6·0N-NaOH	0·350	—	0·350	0·625	—	0·625	1·83	—

* Cf. Table 3.

TABLE 7.

Energies and entropies of activation of serine phosphate.

	E^* (kcal. mole^{-1})		ΔS^* (e.u.)	
Medium	Hydrol.	Elim.	Hydrol.	Elim.
6·0N-NaOH	—	22·2	—	−18·7
pH 9·7, 0·1M-Borate buffer	—	31·1	—	+2·29
pH 4·1, 0·1M-KHP	32·0	25·5	+4·39	−16·2

TABLE 8.

Positions of bond fission of serine phosphate at 100·0° ± 0·5°.

Medium †	Solvent: atom % excess ^{18}O	Orthophosphate: atom % excess ^{18}O	C–O Bond fission (%)	Elimination (%) *
pH 1·0, 0·1M-$HClO_4$	2·56	0·512	20	20
pH 2·2, 0·1M-KHP–HCl	1·74	0·327	25	23
pH 4·1, 0·1M-KHP	2·15	0·405	25	18
pH 5·4, 0·1M-KHP–NaOH	2·37	0·440	26	20
pH 7·5, 0·1M-Borate	2·92	0·133	82	85
pH 9·6, 0·1M-Borate	2·74	0·002	100	100
pH 13·7, 0·5N-NaOH	3·21	0·008	99	100
2·0N-NaOH	5·45	0·003	100	100
6·0N-NaOH	5·58	0·005	100	100

* Calc. from results in Tables 3—5. † See Table 3.

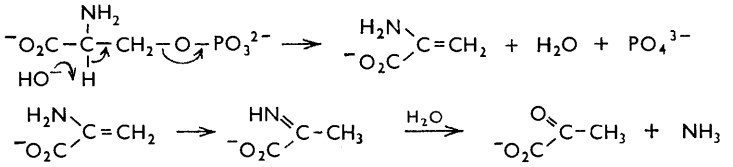
In the weakly acid range, where elimination and hydrolysis are concurrent, correction for the presence of the normal compound is not simple and a different approach was used in determining the kinetic isotope effects. The substrate concentration was increased ten-fold, to 0·04M, and the sample size for analysis was reduced ten-fold. Rate constants for the deuterated species were derived from the last 6—7% of the reaction. At this stage of the reaction most of normal compound has broken down. This procedure can only give a lower limit for the ratio k_H/k_D since a small percentage of the normal compound remains, however far the reaction proceeds.

Bond fission. Positions of bond fission are summarised in Table 8.

DISCUSSION

Strongly Alkaline Range.—The results obtained in this range indicate the occurrence of β-elimination. It is safe to assume that at pH >14 the carboxyl and phosphate groups

are completely ionized and that the nitrogen atom is completely unprotonated. The mechanism may therefore be as illustrated.



This mechanism is compatible with the experimental results in that it demands faster dephosphorylation with increasing hydroxide-ion concentration, C–O bond fission, ammonia and pyruvic acid as products, and a decrease in rate on substitution of deuterium for the α-hydrogen atom. The observed kinetic isotope effect of 1·85 is too large to be explicable as a secondary isotope effect on a hydrolytic mechanism involving the β-carbon atom as the reaction centre. In comparing the rather low value of $(k_H/k_D)_{Elim}$ in Table 6 with values of the analogous ratio for similar reactions it should be noted that the theoretical maximum value [16] of k_H/k_D at 100° is 4·7. Saunders and Edison [17] have given values of k_H/k_D as low as 2·98 at 50° for elimination from trimethylphenethylammonium

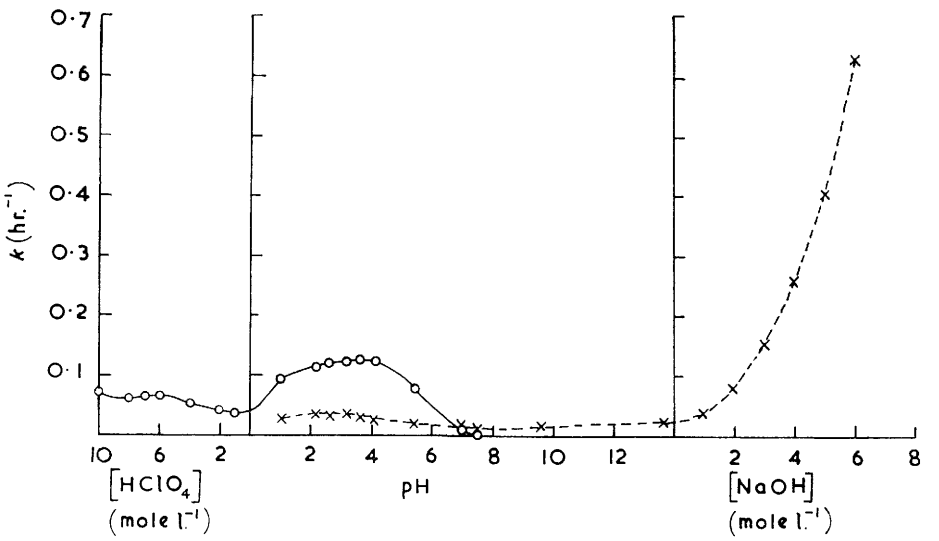


FIG. 1. Dependence of rate constants of (○) hydrolysis and (×) elimination of serine phosphate on pH at 100°.

bromide in aqueous solution. As seen from Table 6 for the range pH 1·0 to 6N-NaOH, the amount of elimination (estimated from the amounts of ammonia and pyruvic acid) parallels the amount of C–O bond fission (determined by the use of oxygen-18).

Fig. 1 shows that k_{Elim} in strongly basic solution increases faster than does the stoicheiometric hydroxide-ion concentration. Behaviour of this kind has been observed for a number of reactions and attempts have been made [18] to correlate rate constants with the H_- function.[19] A recent theoretical analysis [20] shows that for rate-determining proton abstraction by hydroxide ion the logarithm of the observed first-order rate constant

[16] Wiberg, *Chem. Rev.*, 1955, **55**, 713.
[17] Saunders and Edison, *J. Amer. Chem. Soc.*, 1960, **82**, 138.
[18] Allison, Bamford, and Ridd, *Chem. and Ind.*, 1958, 718.
[19] Schwarzenbach and Sulzerberger, *Helv. Chim. Acta*, 1948, **27**, 348.
[20] Anbar, Bobtelsky, Samuel, Silver, and Yagil, *J. Amer. Chem. Soc.*, in the press.

should be proportional to (H_- + log C_W), where C_W is the concentration of free water, not bound to hydroxide ion. A plot of log k_{obs} against (H_- + log C_W) is linear, with a slope of 0·98. As explained in ref. 20, such a correlation is consistent with a rate-determining proton abstraction, but it does not distinguish between this mechanism and a pre-equilibrium between the substrate SH and its conjugate base, S^-, the species S^- being subsequently converted into products in a bimolecular rate-determining interaction with a molecule of solvent. However, the latter reaction is ruled out in the case of serine phosphate by the deuterium isotope results which show that proton abstraction is rate-determining.

The postulated mechanism is similar to that suggested for the pyridoxal-catalyzed breakdown of serine phosphate.[5] In the presence of Cu^{2+}, pyridoxal forms a Schiff

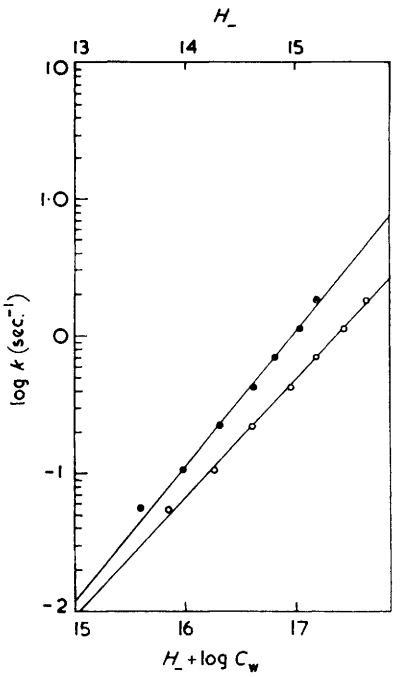


FIG. 2. Plot of log k_{Elim} for serine phosphate in aqueous NaOH against (○) H_- function and (●) H_- + log C_W.

base with serine phosphate, the consequent weakening of the α-C–H bond of serine resulting in a ready elimination of the orthophosphate group. The intermediate imine $HN{:}CMe{\cdot}CO_2^-$ postulated in both cases is not expected to accumulate in detectable quantities since these compounds are known [21] to be hydrolysed readily under the experimental conditions.

The β-elimination from serine phosphate is several orders of magnitude slower than the presumably analogous alkaline dephosphorylation of phosphoproteins.[1] The large difference in rate probably reflects differences in the strength of the α-C–H bond of free and substituted serine phosphate. Above pH 14 there is a negative charge on the carbonyl group of serine phosphate and a lone pair of electrons on the nitrogen atom. The resulting inductive effects will tend to strengthen the α-C–H bond, with respect to the corresponding bond in peptides.

The relative lability of the α-hydrogen atom in polypeptides is confirmed by the finding [22] that benzyloxy-D-serylglycyl-L-alanine methyl ester is racemized readily to benzyloxycarbonyl-DL-serylglycyl-L-alanine methyl ester by trimethylamine in methanol,

[21] Roger and Neilson, *Chem. Rev.*, 1961, **61**, 179.
[22] Schnabel, *Z. physiol. Chem.*, 1959, **314**, 114.

the reaction almost certainly proceeding by the removal of the α-hydrogen atom by the comparatively weak organic base.

Weakly Alkaline Range (pH 7·0—13·5).—The observed first-order rate constant k_P appears to be practically constant over this pH range (Table 4, Fig. 1). The products, ammonia, pyruvic acid, and orthophosphate, are produced in equimolar amounts throughout the reaction. Bond fission is 100% C–O and there is a kinetic effect k_H/k_D of 1·8 ± 0·1 when deuterium is substituted for the α-hydrogen atom. The evidence indicates that β-elimination is the sole mechanism occurring in this range. An explanation for the apparent constancy of k_P over such a wide range of hydroxide-ion concentration cannot be confidently formulated on the basis of the present evidence. Above pH 7 the phosphate group will carry two negative charges and below 12—13 the amino-group will be protonated. A conceivable mechanism in this range is the water-catalyzed *E*2 reaction of the ion, $^{+}H_3N{\cdot}CH(CO_2^{-}){\cdot}CH_2{\cdot}O{\cdot}PO_3^{2-}$.

Another conceivable mechanism is the intramolecular attack by the phosphate group on the α-hydrogen atom. An attempt is being made to elucidate the mechanism in this range by a study of the reactions of substituted serine phosphates.

The Acid Range.—The pH–rate profile of serine phosphate in acid media is quantitatively and qualitatively similar to that of ethanolamine phosphate,[23] the " bell-shaped " curve now being a familiar feature of the pH–rate profile of monoesters of phosphoric acid, explicable in terms of the relatively high reactivity of the monoanion.[24]

As indicated by the formation of ammonia and pyruvic acid, about 20% of the total rate at pH 4 appears to be due to elimination. This figure agrees fairly well with the value of 26% of C–O bond fission (Table 8). As may be seen from Table 6, substitution of deuterium for the α-hydrogen atom reduces k_{Elim}, the rate constant for the formation of ammonia and pyruvic acid. The value of 1·85 for $(k_H/k_D)_{Elim}$ is equal to that found in the weakly and strongly alkaline range, but the exact agreement is probably fortuitous in view of the larger errors in the determination of k_{Elim} at pH 4. A secondary isotope effect on hydrolysis was not observed, the deuterium atom being too far removed from the P–O bond to produce an observable effect on the hydrolysis. The kinetic isotope effect indicates that the elimination is an *E*2 reaction; a more exact description of the reaction is precluded by the fact that the detailed mechanism of hydrolysis of the monoanion of phosphate esters is still a matter of conjecture. It is hoped that work on substituted serine phosphate derivatives will clarify this matter.

Strongly Acid Range.—In strongly acid solution, the absence of acid-catalysis parallels the behaviour of ethanolamine phosphate,[23] as opposed to that of methyl phosphate.[25] In 4·3N-perchloric acid, dephosphorylation appears to be due solely to hydrolysis, the rate of formation of ammonia being at most 1% of the rate of orthophosphate formation. Consistently there is no deuterium isotope effect on the rate (see Table 6). Acid-catalysis is probably associated with protonation of the ester-oxygen atom. In the present case the positive charge of the protonated amino-group will reduce the basicity of the ester-oxygen by an inductive effect. A similar argument would explain the absence of acid-catalysis for phenyl phosphate.[26]

This investigation was supported in part by a grant from the U.S. Public Health Service.

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[24] Barnard, Bunton, Llewellyn, Oldham, Silver, and Vernon, *Chem. and Ind.*, 1955, 760.
[25] Bunton, Llewellyn, Oldham, and Vernon, *J.*, 1958, 3574.
[26] Vernon, *Chem. Soc. Special Publ.*, No. 8, 1957, 17.