366. *Kinetic and Tracer Studies of the Reactions of Carbamoyl Phosphate in Aqueous Solution.*

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The decomposition of carbamoyl phosphate in aqueous solution is due both to hydrolysis, forming carbon dioxide, ammonia, and orthophosphate, and to elimination, forming cyanate and orthophosphate. In solutions of acidity from pH 5 to 2M-perchloric acid, hydrolysis is the main reaction; above pH 6 elimination predominates. In acid solution, the rate of hydrolysis is proportional to the acid concentration. By using the acid dissociation constants for the equilibria between the uncharged molecule and the monoanion and the dianion of carbamoyl phosphate, partial rate constants for the aqueous decomposition of each species have been derived. By using ^{18}O as tracer it was found that in strongly acid solution only C–O bond fission occurs. From pH 1 to pH 5 about 20% of P–O bond fission is found, while the elimination involves only C–O bond fission. Solvolysis in D_2O showed that k_H/k_D is about 1·0 for the conjugate acid and the neutral molecule, 1·3 for the monoanion, 1·0 for the dianion, and 1·3 for the conjugate base. The rate of hydrolysis of the monoanion is not subject to general acid- or base-catalysis; the rates were unaffected by the addition of various nucleophilic reagents but were catalysed by molydate ions. Presence of an excess of sodium azide did not appreciably alter the rate at pH 4 but carbamoyl azide $H_2N{\cdot}CO{\cdot}N_3$ was formed. The dianion ($H_2N{\cdot}CO{\cdot}O{\cdot}PO_3^{2-}$) exchanges its hydrogen atoms rapidly when dissolved in deuterium oxide. The acid dissociation constant of the dianion, K_3, was determined from the kinetic results. The evidence favours a mechanism of unimolecular heterolysis of the undissociated molecule and of the monoanion and the dianion of carbamoyl phosphate, involving formation, in a rate-determining step, of an unstable intermediate $[H_2N{\cdot}C(O)]^+$ which may then (1) react with water to form ammonium carbonate, (2) add azide ions to form carbamoyl azide, or (3) release a proton to a solvent molecule to form the cyanate ion.

CARBAMOYL PHOSPHATE (sometimes called carbamyl phosphate in biochemical literature), $H_2N{\cdot}CO{\cdot}O{\cdot}PO_3H_2$, was first synthesised by Jones, Spector, and Lipmann[1] as the dilithium salt. It is of interest since it is utilised for the enzymic conversion of ornithine into citrulline and of aspartate into carbamoylaspartate, an important intermediate in the biosynthesis of pyrimidines and ultimately of nucleic acids.

The decomposition of carbamoyl phosphate at pH 5 and 38° was briefly examined[2] by measuring the amounts of ammonia, carbon dioxide, and orthophosphate formed after various times: ammonia and carbon dioxide were formed at identical rates under these conditions, but phosphate, measured colorimetrically as phosphomolybdate, was formed more rapidly and hence it was suggested that in the non-enzymic reaction phosphate and free carbamic acid are first produced, the latter then decomposing more slowly. A more recent study[3] of the non-enzymic decomposition in aqueous solution showed that in addition to the hydrolysis (a) to ammonia, carbon dioxide, and phosphate, a reversible elimination (b) gave cyanate and phosphate:

$$NH_3 + CO_2 + H_3PO_4 \xleftarrow{\text{a}} H_2N{\cdot}CO{\cdot}O{\cdot}PO_3H_2 \xrightleftharpoons{\text{b}} HCNO + H_3PO_4$$

The amount of ammonia and cyanate formed in 45 min. at 37° and pH 1—13 was measured, showing that reaction (a) occurred mainly in the acid range and reaction (b) only above pH 6. Details were not elucidated. The present paper presents a mechanistic study of

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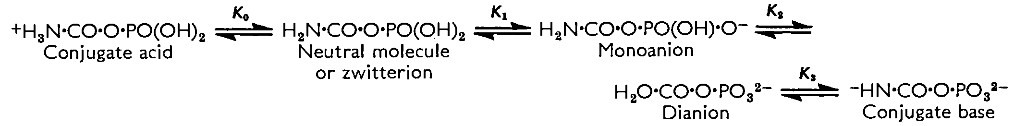
[1] Jones, Spector, and Lipmann, *J. Amer. Chem. Soc.*, 1955, **77**, 819.
[2] Grisola, Grady, and Wallach, *Biophys. Biochim. Acta*, 1955, **17**, 277.
[3] Jones and Lipmann, *Proc. Nat. Acad. Sci. U.S.A.*, 1960, **46**, 1194.

the hydrolysis and " elimination " of carbamoyl phosphate in aqueous solution in the pH range from N-sodium hydroxide to 6N-perchloric acid.

RESULTS

Acid–Base Equilibria of Carbamoyl Phosphate and the Products in Water.—Carbamoyl phosphate has an unusual versatility of charge-state, that depends on the proton-donating and -accepting properties of the solvent. In water the following acid–base equilibria may be postulated:



$$\underset{\text{Conjugate acid}}{^{+}H_3N\cdot CO\cdot O\cdot PO(OH)_2} \overset{K_0}{\rightleftharpoons} \underset{\text{Neutral molecule or zwitterion}}{H_2N\cdot CO\cdot O\cdot PO(OH)_2} \overset{K_1}{\rightleftharpoons} \underset{\text{Monoanion}}{H_2N\cdot CO\cdot O\cdot PO(OH)\cdot O^-} \overset{K_2}{\rightleftharpoons}$$

$$\underset{\text{Dianion}}{H_2O\cdot CO\cdot O\cdot PO_3^{2-}} \overset{K_3}{\rightleftharpoons} \underset{\text{Conjugate base}}{^{-}HN\cdot CO\cdot O\cdot PO_3^{2-}}$$

When dilithium carbamoyl phosphate was titrated with hydrochloric acid at 25°, the acid dissociation constants of the neutral molecule and the monoanion were found to be pK_1 2·70 ± 0·02, $pK_2 = 5{\cdot}23 \pm 0{\cdot}03$ (lit.,[2] $pK_2 = 5{\cdot}3$ at 20°). The equilibrium constants for the formation of the conjugate acid and base could not be measured from titration curves, owing to the rapid decomposition of carbamoyl phosphate in the more strongly acid and basic regions. The acid dissociation constant pK_3 of the dianion was estimated from the pH-dependence of the rate (see Fig. 4).

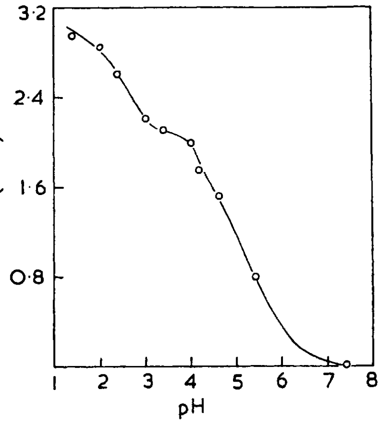


FIG. 1. Dependence of rate constant of hydrolysis of carbamoyl phosphate on pH at 37·0°.

As will be shown below, the data for the rates of decomposition of carbamoyl phosphate at each pH range can be analysed in order to determine the contribution of each charge state to the overall reaction.

Decomposition of the conjugate acid, the neutral molecule, and the monoanion (mainly below pH 6) in water gives only ammonia, carbon dioxide, and orthophosphoric acid (reaction a, above), but decomposition of the dianion and its conjugate base (pH mainly >6) involves only " elimination " to give cyanate and orthophosphate (reaction b).

Kinetics of Hydrolysis.—In the acid range, up to pH 7·5, the rate of hydrolysis of carbamoyl phosphate was determined by following the formation of ammonia colorimetrically. First-order kinetics were observed (see Table 1 and Fig. 1). Fig. 1 shows a plateau at pH 3—4, where almost all the phosphate exists as its monoanion, as found from the titration curve. The result is analogous to that observed for hydrolysis of *N*-phenylphosphoramidates which is also strongly acid-catalysed.[4]

From the equilibrium constants pK_1 and pK_2 described above the concentrations of the various species present have been calculated (see Table 1). Using these and the total rate constants given in Table 1 enabled partial rate constants, k, to be derived for reaction of the

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[4] Chanley and Feageson, *J. Amer. Chem. Soc.*, 1958, **80**, 2686.

TABLE 1.

Hydrolysis of carbamoyl phosphate at 37·0°: first-order rate constants, k_1 (10^{-4} sec.$^{-1}$), and mole fractions of species.

pH	1	1·4	2·0	2·4	3·0	3·4	4·0	4·2	4·6	5·4	7·4
k_{obs}	2·95	2·95	2·85	2·6	2·2	2·1	2·0	1·75	1·51	0·82	0·002
k_{calc}	2·98	2·98	2·81	2·6	2·3	2·1	2·0	1·85	1·65	0·82	0·012
$[H_2A]$	0·98	0·953	0·834	0·666	0·303	0·164	0·045	0·028	0·010	0·007	—
$[HA^-]$	0·02	0·047	0·166	0·334	0·693	0·824	0·95	0·889	0·800	0·400	0·006
$[A^{2-}]$	—	—	—	—	0·004	0·012	0·005	0·083	0·190	0·593	0·994

various charge states; these rate constants and the energies and entropies of activation are given in Table 2.

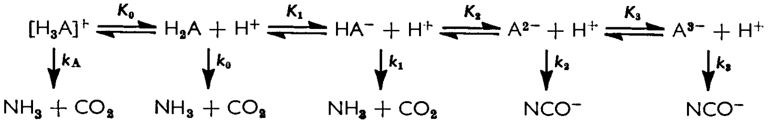

TABLE 2.

Partial rate constants and energies and entropies of activation.

Temp.	25·0°	37·0°	50·0°	ΔE^* (kcal. mole^{-1})	ΔS^* (e.u.)
10^4k_A (l. mole^{-1} sec.$^{-1}$)	3·1	16·1		26	+12·3
10^4k_0 (sec.$^{-1}$)	0·55	1·9		21	−4·1
10^4k_1 (sec.$^{-1}$)	0·505	2·05	8·64	22·8	−3·98

The first-order rate constants for decomposition of undissociated carbamoyl phosphate k_0 and of its monoanion k_1 are seen to be of a similar magnitude, in spite of the difference in charge state.

Acid-catalysis of Hydrolysis.—The hydrolysis of carbamoyl phosphate is strongly catalysed by acids, the rate being proportional to the stoicheiometric concentration of acid up to 2N-acid. The same rate is obtained for equal concentrations of hydrochloric and perchloric acid (see Table 3).

TABLE 3.

Hydrolysis of carbamoyl phosphate in acid solution at 25·0°: (A) no control of ionic strength (solvent H_2O), (B) at constant ionic strength (μ 3).

(A)	$HClO_4$	HCl	$HClO_4$	$HClO_4$	$HClO_4$	HCl	$HClO_4$	$HClO_4$	$HClO_4$
Concn. (M)	0·10	0·12	0·25	0·35	0·50	0·51	0·75	1·00	2·00
10^4k_{obs} (sec.$^{-1}$)	0·91	1·02	1·25	1·50	2·06	2·15	2·9	3·6	6·7

(B)	$DClO_4$	$HClO_4$	$HClO_4$	$HClO_4$	$DClO_4$	$HClO_4$
Concn. (M)	0·415	0·48	0·80	1·25	1·63	1·9
Solvent	D_2O	H_2O	H_2O	H_2O	D_2O	H_2O
10^4k_{obs} (sec.$^{-1}$)	2·40	2·43	3·80	5·9	7·5	8·7

From the intercept of a plot of k_{obs} against acid concentration at zero hydrogen-ion concentration, a value for the rate of the non acid-catalysed reaction of carbamoyl phosphate in the acid region is obtained. This rate, $k_0 = 0·55 \times 10^{-4}$ (sec.$^{-1}$) at 25°, is considered to be the rate of hydrolysis of the neutral molecule of carbamoyl phosphate. The slope, k_A, is the rate coefficient of acid-catalysed hydrolysis. A plot of log ($k_{obs} - k_0$) against the Hammett acidity function of the solvent was found to curve strongly downward; a linear dependence[5] on the logarithm of hydrogen-ion concentration was obtained, with a slope of 1·0 (see Fig. 2). This result will be considered below.

The acid-catalysed hydrolysis of carbamoyl phosphate is subject to a positive salt effect; its rate is increased by addition of sodium perchlorate (see Table 3). At constant ionic strength, the rate of hydrolysis is proportional to the acid concentration up to 3M-perchloric acid. The uncatalysed hydrolysis is not affected by addition of sodium perchlorate, as the linear dependence of rate on acidity at constant ionic strength (μ 3) and without control of ionic

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[5] Paul and Long, *Chem. Rev.*, 1957, **57**, 1; Long and Paul, *ibid.*, p. 940.

strength converge to the same intercept, $k_0 = 0{\cdot}55 \times 10^{-4}$ (sec.$^{-1}$). The observed rate in strongly acid solution is thus the sum of the uncatalysed and the acid-catalysed reactions, $k_{\text{obs}} = k_0 + k_{\text{A}}(\text{H}^+)$. Values of k_{A} (without control of ionic strength) and k_0 for two temperatures have been included in Table 2. In Table 3 values for rates of solvolysis of carbamoyl phosphate in D_2O containing $DClO_4$ are included. These values fall on the same straight line as those for H_2O as solvent.

No general acid-catalysis in the hydrolysis of carbamoyl phosphate was found, as shown by experiments in the presence of acetate and phthalate buffer solutions. Thus no catalytic effect by acids, other than that of hydronium ions, was detected (see Table 4).

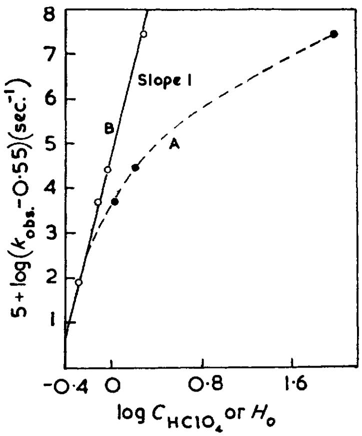

FIG. 2. Dependence of log k_{obs} in the acid-catalysed hydrolysis of carbamoyl phosphate on (A) acid concentration (C_{HClO_4}) or (B) acidity function (H_0).

TABLE 4.

Influence of various buffer solutions on the rate of hydrolysis of carbamoyl phosphate at 37°.

Solvent	Concn. (M)	pH	10^4k_{obs} (sec.$^{-1}$)	Solvent	Concn. (M)	pH	10^4k_{obs} (sec.$^{-1}$)	Solvent	Concn. (M)	pH	10^4k_{obs} (sec.$^{-1}$)
A.B. ...	0·02	4·6	1·55	A.B.	0·02	3·4	2·15	KHP ...	0·2	4·0	2·0
A.B. ...	0·2	4·6	1·51	A.B.	0·2	3·4	2·1	KHP ...	0·05	4·0	2·0

A.B. = Acetate buffer. KHP = Potassium hydrogen phthalate.

Kinetics of Elimination.—In the basic range (above pH 6) carbamoyl phosphate decomposes, forming one equivalent of cyanate.

The net result is consumption of one equivalent of base. The rate of elimination was thus conveniently followed by automatically adding sufficient aqueous sodium hydroxide to keep the pH of the solution constant (see Experimental section). The rate obeyed first-order kinetics. The cyanate ion is not hydrolysed under the conditions of these experiments [6] and no measurable quantity of ammonia was detected on decomposition of carbamoyl phosphate above pH 10. First-order rate constants for the elimination at several temperatures are given in Table 5 and in Fig. 3.

TABLE 5.

Temperature and pH-dependence of elimination from carbamoyl phosphate: first-order rate constants, 10^4k_{obs} (sec.$^{-1}$).

pH	6·5	7·5	8·5	9·2	9·7	10·2	10·7	11·0	11·2	11·5	12·0	12·5
At 37·0°	2·0	2·1	2·3	3·0	4·0	5·5	7·4	10·1				
At 25·0		0·5						3·7	5·5	11·2	29·3	
At 10·0								0·6		1·74	4·4	7·0

At pH 7·4 (see Table 1) 99·4% of the carbamoyl phosphate exists as the dianion. As seen in Fig. 3, the rate of elimination (rate constant k_2) of carbamoyl phosphate is independent of

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[6] Kemp and Kohnstam, *J.*, 1956, 900.

pH in this region but rises rapidly above pH 9. In more basic solutions (10^{-4}M-sodium hydroxide) the rate of elimination becomes approximately proportional to the hydroxyl ion concentration, until the rate levels off again at pH 12 (see Table 6). In the basic range above pH 10 elimination appears to involve a rapid proton-transfer equilibrium between the dianion of carbamoyl phosphate and hydroxide ion to form the conjugate base:

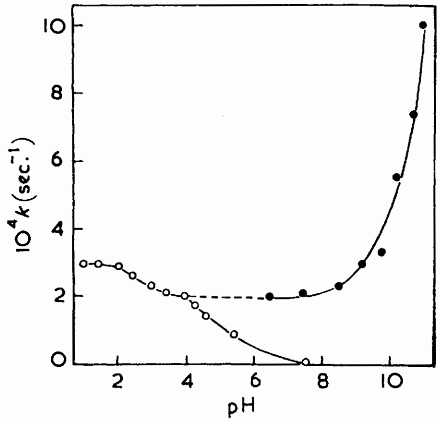


FIG. 3. Dependence of rate constant for (○) hydrolysis of, and (●) elimination from, carbamoyl phosphate on pH at 37·0°.

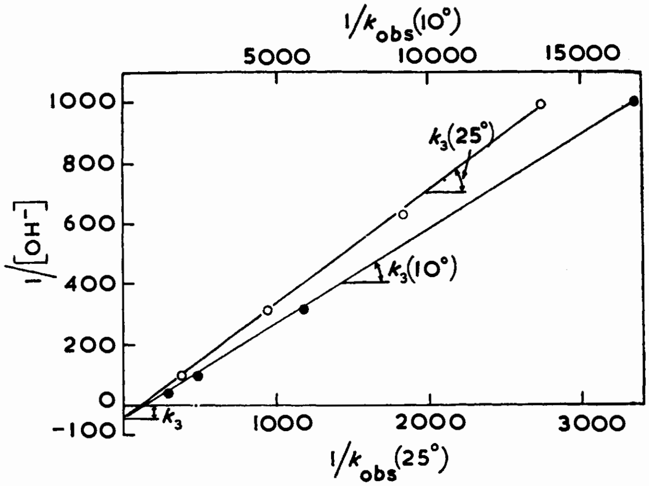
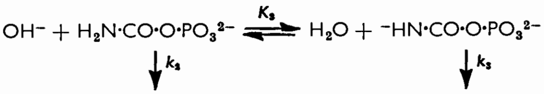


FIG. 4. Elimination from carbamoyl phosphate at pH 11—12·5 at 10° and 25°. k_3 in l. mole^{-1} sec.$^{-1}$.

TABLE 6.

Dependence of rate of elimination of carbamoyl phosphate on (OH^-) concentration at 10·0°.

pH	$10^3(OH^-)$	$10^4 k_{obs}$ (sec.$^{-1}$)	k_{obs}/OH^-	$1/OH^-$	$10^{-3}/k_{obs}$
11	1·0	0·6	0·06	1000	6·7
11·5	3·16	1·7	0·053	316	5·88
12	10	4·4	0·044	100	2·28
12·5	31·6	7·0	0·022	31·6	1·42



$$\begin{array}{ccc} OH^- + H_2N\cdot CO\cdot O\cdot PO_3^{2-} & \overset{K_3}{\rightleftharpoons} & H_2O + {}^-HN\cdot CO\cdot O\cdot PO_3^{2-} \\ \downarrow k_3 & & \downarrow k_3 \end{array}$$

which then breaks up to the products with a rate constant k_3. From the kinetic result and as plot of $1/(OH^-)$ against $1/k_{obs}$ (see Fig. 4) the values of the second-order rate constant k_3 and of the equilibrium constant K_3 were calculated by the method described elsewhere for carboxyamides.[7,8] The results are given in Table 7.

TABLE 7.

Rate constants and energies and entropies of activation for elimination from the dianion and conjugate base of carbamoyl phosphate: equilibrium constant $pK_A = pK_W - pK_3$.

	10·0°	25·0	37·0	ΔE^* (kcal. mole^{-1})	ΔS^* (e.u.)
$10^4 k_2$ (sec.$^{-1}$)		0·5	2·1	21·0	−4·2
k_3 (l. mole^{-1} sec.^{81})	0·062	0·364		20·4	+7·7
pK_A	12·3	12·4			

Deuterium Isotope Effects in Hydrolysis.—A comparison of the solvolysis of carbamoyl phosphate in water and in deuterium oxide was made in order to obtain information on the contribution of fast and of rate-determining proton transfers. Results are given in Tables 3 and 8 for the hydrolysis from strongly acid solutions, up to pH 4·6. As seen in Table 8, the ratio of first-order rate constants in water and deuterium oxide has a noticeable maximum at pH 4·0 at which carbamoyl phosphate exists mainly as the monoanion, with an isotope effect of $k_H/k_D = 1·34$.

TABLE 8.

Deuterium solvent isotope effect in the hydrolysis of carbamoyl phosphate at 37·0°.

Solvent	$HClO_4$	$HClO_4$	H_2SO_4	H_2SO_4	HCl + KCl	A.B.*	A.B.*	A.B.*	KHP *	A.B.*	A.B.*
Acidity (N)	2	0·75	0·071	0·033	0·01	0·2	0·2	0·2	0·05	0·2	0·1
pH			1·15	1·4	2	3·4	3·9	4·0	4·0	4·2	4·6
k_H/k_D	1·00	1·00	1·03	1·02	1·05	1·11	1·34	1·34	1·34	1·29	1·28
	±0·04	±0·04	±0·03	±0·04	±0·005	±0·05	±0·01	±0·005	±0·06	±0·05	±0·03

* A.B. = Acetate buffer. KHP = Potassium hydrogen phthalate.

Deuterium Isotope Effects and Exchange in the Elimination.—The rates of elimination from carbamoyl phosphate in water and in deuterium oxide solvents were compared, with the results given in Table 9. At pH 7, at which most of the carbamoyl phosphate exists as the dianion and decomposes to cyanate, attempts were also made to measure the rate of hydrogen exchange between the amido-group and the solvent deuterium oxide. After partial reaction, the remaining carbamoyl phosphate was isolated from solution and analysed for its deuterium content (see Experimental section). The enrichment corresponded to at least 81% exchange. It is therefore evident that the exchange $D_2O + H_2N{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} \rightleftharpoons H_2O + D_2N{\cdot}CO{\cdot}O{\cdot}PO^{2-}$ is very fast compared with the elimination (k_2) of the dianion.

TABLE 9.

Deuterium solvent effect in the elimination from carbamoyl phosphate.

Species	pH	Temp.	k_H/k_D
Dianion	7·5	37·0°	1·02 ± 0·03
Dianion and conjugate base	11·5	25·0	1·31 ± 0·05

Oxygen Exchange with Solvent during Hydrolysis.—Hydrolysis of carbamoyl phosphate may involve rapid reversible formation of intermediates in which the carbonyl- or the phosphoryl-oxygen atom, or both, become equilibrated with solvent oxygen atoms. By use of labelled water, the formation of such an intermediate would be detected by oxygen exchange accompanying hydrolysis, as has been found for alkyl benzoates [9] and some alkyl phosphates.[10] Carbamoyl phosphate was dissolved in ^{18}O-enriched water at pH 1 and hydrolysed for one half-life. The remaining carbamoyl phosphate was isolated and analysed for its oxygen-isotope content. The results are given in the third column on Table 10.

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[7] Bruylants and Kezdy, *Record Chem. Prog.*, 1960, **21**, 213.

[8] Biechler and Taft, *J. Amer. Chem. Soc.*, 1957, **79**, 4927.

[9] Bender, (*a*) *J. Amer. Chem. Soc.*, 1951, **73**, 1626; (*b*) *Chem. Rev.*, 1960, **60**, 54.

[10] Haake and Westheimer, *J. Amer. Chem. Soc.*, 1961, **83**, 1102.

The carbamoyl phosphate was then hydrolysed in normal water under conditions resulting in C–O bond fission (see below). The orthophosphate was isolated and analysed for its ^{18}O content. It was found to be normal (see last column in Table 10). Thus hydrolysis of carbamoyl phosphate involves a certain amount of oxygen exchange with the carbonyl group but none with the phosphoryl group:

TABLE 10.

Exchange during hydrolysis at pH 1 and 37·0° (atom % excess ^{18}O).

Run no.	*A*, Water initially	*B*, $H_2N{\cdot}CO{\cdot}O{\cdot}PO_3H_2$	*B*/*A*	Orthophosphate
1	63·05	0·824	0·013	0·000
2	5·306	0·066	0·012	—

$$H_2N{\cdot}CO{\cdot}O{\cdot}P{\cdot}O(OH)_2 + H_2{}^{18}O \rightleftharpoons H_2N{\cdot}{}^{18}CO{\cdot}O{\cdot}PO(OH)_2 + H_2O.$$

The exchange of oxygen into the carbonyl group of carbamoyl phosphate was previously investigated in connexion with studies on the biosynthesis of carbamoylaspartic acid. By using water with 1·4 atom % excess of ^{18}O, no measurable excess of isotopic oxygen was found [11] either in the remaining carbamoyl phosphate or in the product, carbamoyl aspartate. This exchange has now been found when water of higher ^{18}O content was used.

Point of Bond Fission during Hydrolysis.—Hydrolysis of carbamoyl phosphate can occur with breakage of the bond between oxygen and carbon or of that between oxygen and phosphorus. The extent of carbonyl–oxygen bond fission may be expected to depend on the charge state of carbamoyl phosphate, which is determined by the pH of the solution. Previous experiments with ^{18}O-enriched water [12] seemed to indicate that in the solution at pH 1·0 about 70% of the ^{18}O expected for exclusive P–O bond fission was found in the phosphate. At intermediate pH values, less ^{18}O transfer from water to phosphate was observed, and very little in strongly alkaline medium. In the present work, measurements were made to cover the entire pH region from strongly acid to strongly basic solution and the results are given in Table 11.

TABLE 11.

Hydrolysis of carbamoyl phosphate in $H_2{}^{18}O$ at 100°.

Buffer	Solution	pH	*A*, Water, atom % excess ^{18}O	*B*, PO^{3-}, atom % excess ^{18}O	*B*/*A*	P–O fission (%)
$HClO_4$	5N		5·0	0·057	0·011	4
$HClO_4$	N		5·55	0·219	0·039	16
$HClO_4$+ .... $NaClO_4$	0·1N 9M	1	4·9	0·208	0·0425	17
A.B.*	0·2M	3·4	4·20	0·265	0·063	25
A.B.	0·2M	4·0	5·17	0·275	0·053	21
A.B.	0·2M	5·0	5·17	0·265	0·053	20
H_2O (1)	Water, no buffer		5·55	0·306	0·055	22
H_2O (2)	,,	,,	4·15	0·213	0·051	20
H_2O (3)	,,	,,	4·15	0·21	0·0505	20

* A.B. = Acetate buffer. ^{18}O-Analysis: by mercuric salt decomposition [13] of (1) barium orthophosphate or (2) trisilver phosphate; or (3) by thermal decomposition of trisilver phosphate.[14]

In these experiments, carbamoyl phosphate in various solutions in ^{18}O enriched water was solvolysed to completion. The resulting orthophosphate was then isolated and analysed for its ^{18}O content by several methods. The remarkable result was that the major reaction in all cases was carbonyl–oxygen fission which, in 5N-perchloric acid and at pH values above 7·5, accounted for practically all the products. However, in the intermediate range, between pH 0 and pH 5, about 20% of the reaction also involved phosphoryl–oxygen fission; in this range, hydrolysis is due to decomposition of un-ionised carbamoyl phosphate or of its monoanion; both these charge states result in equal amounts of C–O bond fission. The 70% of

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[11] Reichard and Hanshoff, *Acta Chem. Scand.*, 1956, **10**, 548.

[12] Allison and Jones, quoted in ref. 3.

[13] Anbar and Guttmann, *Internat. J. Appl. Radiation Isotopes*, 1959, **3**, 233.

[14] Anbar, Halmann, and Silver, *Analyt. Chem.*. 1960, **32**, 841.

P–O bond fission quoted elsewhere [12] for pH 1·0 may have been due to isotopic contamination in the ^{18}O analysis.

Point of Bond Fission during Elimination.—In the elimination from carbamoyl phosphate the orthophosphate molecule is ejected as a whole, $H_2N{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} \longrightarrow HNCO + HO{\cdot}PO_3^{2-}$. Therefore, only C–O bond fission should occur in this reaction. The analytical results have shown that the dianion of carbamoyl phosphate and its conjugate base react by elimination, producing only cyanate and orthophosphate. Hence in the pH regions in which these species are predominant, the decomposition of carbamoyl phosphate in ^{18}O-enriched water results in C–O bond fission, as shown in Table 12. The amount of P–O bond fission observed in these experiments is within the error of isotopic analysis.

TABLE 12.

Decomposition of carbamoyl phosphate in alkaline deuterium oxide.

pH control	pH	*A*, Water, atom % excess ^{18}O	*B*, PO_4, atom % excess ^{18}O	*B*/*A*	P–O fission (%)
pH-stat	7·5	5·75	0·016	0·0028	1·1
N-NaOH	14	5·55	0·011	0·002	0·8

Effect of Added Substances on Rate of Hydrolysis.—Rate measurements in the presence of a variety of added nucleophilic and electrophilic reagents are given in Table 13. The products (ammonia, carbon dioxide, and orthophosphate) are seen to be without effect on the rate, which confirms the conclusions drawn from the strict first-order kinetics of hydrolysis.

Fluoride ion, which is very reactive towards phosphorus,[15] was without effect on the rate of hydrolysis. In addition, the concentration of fluoride ion on completion of hydrolysis was not measurably changed, as determined by titration. This indicates that, at the concentrations used, no fluoride ions are trapped in a stable non-ionic form such as a phosphorofluoridate. Azide, which is a powerful nucleophilic reagent towards carbon,[16] similarly had no effect on the rate of formation of ammonia at 0·01M-concentration. The competition experiments described below were made at higher azide concentrations, where again no increase in the rate of hydrolysis was found. The only reagent found to cause significant catalysis of hydrolysis was sodium molybdate. This result is of practical importance, as it shows that the usual colorimetric method for determination of orthophosphate cannot be used in the presence of carbamoyl phosphate. Previous conclusions [2] that during the hydrolysis of carbamoyl phosphate the rate of liberation of orthophosphate is faster than the rates of liberation of ammonia and carbon dioxide thus have to be reconsidered, since an aqueous molybdate reagent was used for the orthophosphate analysis leading to this result.

TABLE 13.

Effect of various reagents on the hydrolysis of carbamoyl phosphate.

Temp.	pH	$H_2N{\cdot}CO{\cdot}O{\cdot}PO_3H_2$ (mmoles l.$^{-1}$)	Reagent Comp.	mmoles l.$^{-1}$	10^4k_{obs} (sec.$^{-1}$)
25·0°	N-$HClO_4$	7	—		3·6
25·0	N-$HClO_4$	7	NH_4Cl	7	3·6
25·0	N-$HClO_4$	5	Na_2HPO_4	5·5	3·55
25·0	4·0	7	—		0·505
25·0	4·0	7	$Na_2MoO_4,2H_2O$	(1%)	5·05
37·0	4·0	6	—		2·04
37·0	4·0	8	CO_2		2·03
37·0	4·0	6	$CuSO_4$	5·5	2·0
37·0	4·0	7	NaF	12	2·02
37·0	4·0	6	$Me{\cdot}NH_3Cl$	6	2·05
37·0	4·0	7	LiCl	20	2·0
37·0	4·0	5	NaN_3	10	2·0

Cupric sulphate was without effect on the hydrolysis of carbamoyl phosphate, demonstrating that the reaction is not affected by an electrophilic reagent such as cupric ion, which has been found to catalyse strongly the hydrolysis of some phosphate esters.[17] Lithium chloride has

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[15] Dostrovsky and Halmann, *J.*, 1953, 502, 516.

[16] Swain, Scott, and Lohmann, *J. Amer. Chem. Soc.*, 1953, **75**, 136.

[17] Ketelaar, Gersmann, and Beck, *Nature*, 1956, **177**, 392.

also been found to catalyse the hydrolysis and glycinolysis of acetyl phosphate,[18] but has no effect on the rate of hydrolysis of carbamoyl phosphate.

The Effect of Added Substances on the Rate of Elimination.—The rate of the elimination from carbamoyl phosphate was measured in the presence of the reagents shown in Table 14.

TABLE 14.

Effect of various reagents on the rate of elimination from carbamoyl phosphate.

pH	Carbamoyl phosphate (mmoles l.$^{-1}$)	Reagent Comp.	Reagent mmoles l.$^{-1}$	10^4k_{obs} (sec.$^{-1}$)	pH	Carbamoyl phosphate (mmoles l.$^{-1}$)	Reagent Comp.	Reagent mmoles l.$^{-1}$	10^4k_{obs} (sec.$^{-1}$)
At 37°.					*At* 25°.				
8·5	5	—	—	2·3	11·5	6	—	—	10·2
8·5	8	C_6H_5·OH	20	2·25	11·5	5	NaOAc	25	10·6
8·8	4	C_6H_5·OH	9	2·31					
7·5	4	C_6H_5·OH	4	2·05					
7·5	6	NaF	20	2·1					

It is seen that neither acetate nor phenoxide ions affect the rate, indicating that there is no general base-catalysis of the elimination. The constancy of the rate in the presence of fluoride or phenoxide ions shows that no bimolecular reaction of the S_N2 or $E2$ type occurs.

Competition Experiments with Added Sodium Azide.—Kinetic experiments (see above) showed that added azide ion in low concentrations had no effect on the rate of hydrolysis of carbamoyl phosphate at pH 4. Additional information was obtained by measuring the rate of formation of orthophosphate ion by a technique which avoids decomposition of carbamoyl phosphate.[19] Results reported in Table 15 show that even in the presence of 0·01M-sodium azide the overall rate of decomposition of carbamoyl phosphate monoanion is unchanged. However, under fairly similar conditions (see Experimental section), the products were found to be different. Instead of ammonia and carbon dioxide, carbamoyl azide (H_2N·CO·N_3) was isolated by ether-extraction. At pH 4 hydrolysis of carbamoyl phosphate alone produces mainly ammonia and carbon dioxide (only traces of cyanate are formed since at pH 4 only 0·5% of the carbamoyl phosphate exists as dianion; see Table 1) which with azide ions do not produce carbamoyl azide. Azide ions are thus able to change the products of hydrolysis of carbamoyl phosphate, without changing the rate. This observation will be discussed below. As shown in Table 15, the rates of liberation of orthophosphate and of ammonia from the monoanion are equal, in contrast to previous claims.[2]

TABLE 15.

Solvolysis of carbamoyl phosphate at 37·0° in the presence of sodium azide and acetate buffer (0·2M) at pH 4.

H_2N·CO·O·PO$(OH)_2$ (mole l.$^{-1}$)	NaN_3 (mole l.$^{-1}$)	10^4k_1 (sec.$^{-1}$)	Product analysed
$9{\cdot}5 \times 10^{-5}$	10^{-2}	2·15	Phosphate
$4{\cdot}9 \times 10^{-5}$	$3{\cdot}4 \times 10^{-3}$	2·0	,,
$4{\cdot}9 \times 10^{-5}$	$1{\cdot}4 \times 10^{-2}$	2·1	,,
$\sim10^{-3}$	—	2·04	Ammonia

DISCUSSION

A summary of the most important kinetic and isotopic results for the aqueous decomposition of carbamoyl phosphate appears in Table 16. It is useful to discuss the mechanism of reaction in each pH range separately.

The Acid-catalysed Reaction.—Within the range of 0·1—2M-perchloric acid the rate of hydrolysis of carbamoyl phosphate is proportional to the stoicheiometric acid concentration. The Zucker–Hammett hypothesis [5] therefore suggests that a water molecule is involved in the transition state of the rate-determining step. From the equation $k_{obs} = k_A[H^+][H_2A] + k_0[H_2A]$ where $[H_2A]$ is the concentration of undissociated carbamoyl phosphate (or its zwitterion), the values in Table 16 for the rate constants of the

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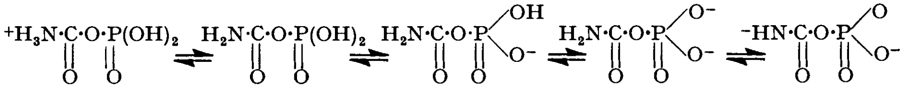
[18] Kurz and Gutsche, *J. Amer. Chem. Soc.*, 1960, **82**, 2175.

[19] Mokrash, *Analyt. Chem.*, 1961, **33**, 432.

TABLE 16.

Summary of kinetic and isotopic results for the decomposition of carbamoyl phosphate in water.



$${}^+H_3N\cdot C(=O)\cdot O\cdot P(=O)(OH)_2 \rightleftharpoons H_2N\cdot C(=O)\cdot O\cdot P(=O)(OH)_2 \rightleftharpoons H_2N\cdot C(=O)\cdot O\cdot P(=O)(OH)O^- \rightleftharpoons H_2N\cdot C(=O)\cdot O\cdot P(=O)(O^-)_2 \rightleftharpoons {}^-HN\cdot C(=O)\cdot O\cdot P(=O)(O^-)_2$$

Charge state	$+1$	0	-1	-2	-3
Rate constant at 25°	$k = 3{\cdot}1 \times 10^{-4}$ (l. mole^{-1} sec.$^{-1}$)	$k_0 = 0{\cdot}55 \times 10^{-4}$ (sec.$^{-1}$)	$k_1 = 0{\cdot}50 \times 10^{-4}$ (sec.$^{-1}$)	$k_2 = 0{\cdot}5 \times 10^{-4}$ (sec.$^{-1}$)	$k_3 = 0{\cdot}364$ (l. mole^{-1} sec.$^{-1}$)
ΔE^* (kcal. mole^{-1})	26	21	22·8	21	20·4
ΔS^* (e.u.)	$+12{\cdot}3$	$-4{\cdot}1$	$-3{\cdot}98$	$-4{\cdot}2$	$+7{\cdot}7$
k_H/k_D	1·0	1·0	1·34	1·0	1·3
P–O splitting (%)	5	15	23	1	1

acid-catalysed (k_A) and the uncatalysed (k_0) reactions were obtained. Results for the acid-catalysed reaction can best be described by a mechanism of the A-2 type. The

$$H_2N\cdot CO\cdot O\cdot PO_3H_2 + H_3O^+ \xrightleftharpoons{\text{Fast}} {}^+H_3N\cdot CO\cdot O\cdot PO(OH)_2 + H_2O$$

$${}^+H_3N\cdot CO\cdot O\cdot PO(OH)_2 + H_2O \xrightarrow{\text{Slow}} NH_4^+ + CO_2 + H_3PO_4$$

intermediate conjugate acid formed has been written with the added proton on nitrogen, although protonation may, in fact, occur on one of the oxygen atoms. By analogy with results of the hydrolysis of carboxylic amides, which are almost completely converted into the conjugate acid [19] in 5M-perchloric acid, it may be assumed that, in this solvent, carbamoyl phosphate exists mainly in the protonated form. This also accounts for the fact that the rate of acid-catalysed hydrolysis is equal, within experimental error, in water and in D_2O.

In 5M-perchloric acid, ^{18}O-tracer experiments showed that practically all the reaction involved carbonyl–oxygen fission. This is analogous to what has been observed in strong acid solutions of acetyl phosphate,[20] and is probably due to the ease of nucleophilic attack by a water molecule on the protonated acyl group.

Reactions of the Neutral Molecule, Monoanion, and Dianion.—Monoalkyl esters [21] and acylamides [22] of phosphoric acid usually exhibit a characteristic maximum in the rate of hydrolysis at about pH 4, in which most of the acid exists as its monoanion. At higher or lower pH values, the rate of hydrolysis decreases, indicating that their dianions and their neutral molecules are less reactive. In carbamoyl phosphate, the rate of hydrolysis increases gradually below pH 4, owing to the very strong contribution of the acid-catalysed reaction. On the other hand, above pH 5 the rate of hydrolysis drops sharply, since the dianion does not contribute to this reaction, but an elimination takes place at a rate which is strikingly similar to that of hydrolysis of the monoanion and of the neutral molecule (see Table 16). This strongly suggests that these reactions involve a similar rate-determining step. This result is somewhat surprising when one considers the difference in charge state of each species, which would also affect their structure and hydration. It appears, therefore, that the change in structure on formation of the transition state is similar for each of the three species, in which the ionisation of the carbamoyl–oxygen bond

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[20] Bentley, *J. Amer. Chem. Soc.*, 1949, **71**, 2765; Koshland, in " Symposium on Phosphorus Metabolism," Vol. I, Johns Hopkins Press, Baltimore, 1951, p. 536; Park and Koshland, *J. Biol. Chem.*, 1958, **233**, 986.

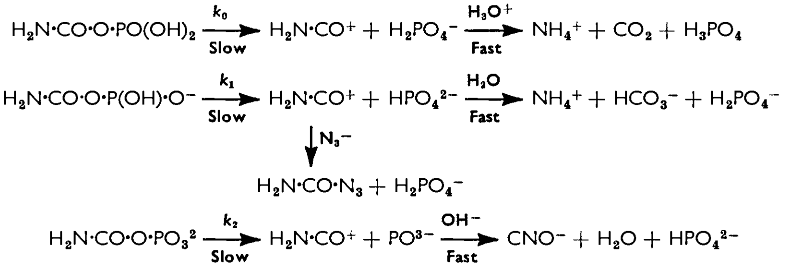
[21] Bunton, Llewellyn, Oldham, and Vernon, *J.*, 1958, 3574.

[22] Halmann and Lapidot, *J.*, 1960, 419; Halmann, Lapidot, and Samuel, *J.*, 1960, 4672; 1961, 3158.

$H_2N{\cdot}CO{-}O{\cdot}P$ is involved. The rate-controlling step in each of the three species appears to be uninfluenced by the charge state at the phosphate end of the molecule. This has been observed in other cases where phosphates are linked by oxygen bridges. Thus, in pyrophosphoric acid, ionisation of one proton does not markedly depress the dissociation of the second proton:[23] $pK_1 = 1{\cdot}0$, and $pK_2 = 2{\cdot}0$. Similarly, from the rate of hydrolysis of various substituted tetra-alkyl pyrophosphates it has been suggested[24] that the transmission of electronic effects along the P–O–P bond is small. The rate of decomposition of carbamoyl phosphate is only affected by reactions which alter the carbamoyl end of the molecule. Thus, acid-catalysis involves protonation (on either the nitrogen or the oxygen atom), and base-catalysis must involve removal of one of the protons of the amido-group.

The equality of the rates of solvolysis of the neutral molecule, monoanion, and dianion and the fact that the rate is unaffected by added reagents (except molybdate, see Table 13) suggest that the rate-determining step involves the breaking of the carbonyl–oxygen bond with the formation of a carbamoyl cation. Evidence for the existence of such transient intermediates has been obtained by the addition of reagents (such as azide) which can react with the intermediate to form a stable product, without altering the rate of disappearance of the substrate.[15] The same technique was applied here to the decomposition of carbamoyl phosphate in water. The rate of hydrolysis of its monoanion was unchanged by addition of an excess of sodium azide but the main product was carbamoyl azide, isolated in high yield. The stability of the carbamoyl cation has been shown[25] in a kinetic study of the hydrolysis of dimethylcarbamoyl chloride ($Me_2N{\cdot}COCl$), in which an S_N1 type of mechanism was demonstrated by the successful competition of amines with water for the dimethylcarbamoyl ion without change in the overall rate.

In aqueous solution the intermediate formed by C–O bond fission in the rate-determining step, can then react in the following ways:



$$H_2N{\cdot}CO{\cdot}O{\cdot}PO(OH)_2 \xrightarrow[\text{Slow}]{k_0} H_2N{\cdot}CO^+ + H_2PO_4^- \xrightarrow[\text{Fast}]{H_3O^+} NH_4^+ + CO_2 + H_3PO_4$$

$$H_2N{\cdot}CO{\cdot}O{\cdot}P(OH){\cdot}O^- \xrightarrow[\text{Slow}]{k_1} H_2N{\cdot}CO^+ + HPO_4^{2-} \xrightarrow[\text{Fast}]{H_2O} NH_4^+ + HCO_3^- + H_2PO_4^-$$

$$\big\downarrow N_3^-$$

$$H_2N{\cdot}CO{\cdot}N_3 + H_2PO_4^-$$

$$H_2N{\cdot}CO{\cdot}O{\cdot}PO_3^{2} \xrightarrow[\text{Slow}]{k_2} H_2N{\cdot}CO^+ + PO^{3-} \xrightarrow[\text{Fast}]{OH^-} CNO^- + H_2O + HPO_4^{2-}$$

This mechanism is also supported by the lack of general acid- or base-catalysis of these reactions (see Table 14).

Reaction of the Conjugate Bases.—Unlike all acyl and alkyl phosphates studied so far, carbamoyl phosphate is also able to lose a proton from nitrogen to form a conjugate base. The existence of a triply ionised species was indicated by the rapid exchange with deuterium from the solvent and by the similarity of the third acid dissociation constant (pK_A) of carbamoyl phosphate to that of carboxylic amides. The latter have been measured directly and are also given in Table 17. The rate of solvolysis of carbamoyl

TABLE 17.

Acid dissociation constants pK_A of amides at 25°.

$CH_2Cl{\cdot}CO{\cdot}NH_2$[7]	$CHCl_2{\cdot}CO{\cdot}NH_2$[7]	$CCl_3{\cdot}CO{\cdot}NH_2$[7]	$C_6H_5{\cdot}NH{\cdot}CO{\cdot}CF_3$[8]	$H_2N{\cdot}CO{\cdot}O{\cdot}PO_3^{2-}$
14·7	13·55	12·42	11·9	12·4

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[23] Shaffer, jun., "The Determination of the Ionization Constants of Polybasic Acids," University Microfilms, Inc., Ann Arbor, Mich., 1960.

[24] Heath, *J.*, 1956, 3796, 3804; Brock, *J. Org. Chem.*, 1957, **22**, 1114.

[25] Hall, jun., *J. Amer. Chem. Soc.*, 1955, **77**, 5993.

phosphate above pH 11 is very great and increases with the concentration of base. The increase is not linear and begins to level off at pH 12 (see Fig. 3). From this it is concluded that a considerable proportion of the substrate is converted into the conjugate base which then reacts by unimolecular elimination (*E*1*cB*) with a rate constant k_3:

$$OH^- + NH_2{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} \underset{\text{Fast}}{\overset{K_3}{\rightleftharpoons}} H_2O + {}^-NH{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} \underset{\text{Slow}}{\overset{k_3}{\longrightarrow}} NCO^- + PO_4^{3-}$$

Reaction with P–O *Bond Fission.*—Hydrolysis with ^{18}O-enriched water shows that in the range of pH 5 to N-perchloric acid some P–O as well as C–O bond fission occurs (see Table 11). In this range, the reacting species are predominantly the neutral molecule and the monoanion. It was suggested in the preceding section, that C–O bond fission in these two species is due to a unimolecular (S_N1) mechanism. However, 23% of P–O bond fission also occurs. Thus a parallel reaction takes place, in which the monoanion is hydrolysed by another mechanism at a rate approximately one-third of that of the unimolecular reaction. The monoanion of acetyl phosphate is also reported [20] to undergo hydrolysis with both C–O and P–O fission, although the precise mechanisms of these reactions have not yet been fully elucidated.

A small amount of P–O fission is also found with both the neutral molecule and the conjugate acid of carbamoyl phosphate. There thus appears to be some competition between nucleophilic attack on carbon (C–O bond fission) and on phosphorus (P–O bond fission). This has been discussed [26] in connexion with the extent of alkyl–oxygen fission in different esters of various oxyacids and was related to the relative strength of the acids. It may here also be significant that the extent of P–O bond fission (S_NX reaction [26]) increases with decreasing acid strength in the order conjugate acid, neutral molecule, monoanion (see Table 16). An analogous systematic variation in the position of bond fission was also found [21] in the hydrolysis of methyl dihydrogen phosphate.

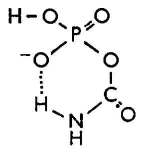
Deuterium Solvent Isotope Effects.—A striking feature of the decomposition of carbamoyl phosphate in water is that a measurable deuterium isotope effect ($k_H/k_D = 1{\cdot}3$) was observed only for the monoanion and the conjugate base. For all other charge states the effect was negligible (see Table 8). A model which seems to fit these observations is that

$H_3^+N{\cdot}CO{\cdot}PO(OH)_2$
Conjugate acid

$HN_2{\cdot}CO{\cdot}PO_3^{2-}$
Dianion

$H_2N{\cdot}CO{\cdot}PO(OH)_2$
Neutral molecule

${}^-HN{\cdot}CO{\cdot}PO_3^{2-}$
Conjugate base


Monoanion

a cyclic hydrogen-bonded structure exists for the monoanion, and open structures for the other charge states. In the monoanion a hydrogen bond can be formed from the amido-group to the negatively charged oxygen atom bound to phosphorus. In the neutral molecule and the conjugate acid, no free negatively charged oxygen atom is available. In the dianion and the conjugate base electrostatic repulsion by the strongly negative phosphate end of the molecule appears to prevent formation of such a cyclic structure. In the monoanion, hydrolysis involves opening of the ring at the O–H and O–C or O–P bonds, and thus may account for the kinetic isotope effect. It is more difficult to account for the isotope effect of $k_H/k_D = 1{\cdot}3$ observed for elimination at pH 11·5. One possibility is that the equilibrium of formation of the conjugate base, $H_2N{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} + OH^- \rightleftharpoons {}^-HN{\cdot}CO{\cdot}O{\cdot}PO_3^{2-} + H_2O$, is more to the left with D_2O as solvent. This is similar to the effect of D_2O on the dissociation constants of acids in solution.[27]

[26] Anbar, Dostrovsky, Samuel, and Yoffe, *J.*, 1954, 3603.
[27] Wiberg, *Chem. Rev.*, 1955, **55**, 713.

EXPERIMENTAL

Dilithium carbamoyl phosphate was prepared as described by Spector *et al.*;[28] it was reprecipitated twice with ethanol. Its purity was determined by hydrolysis in N-hydrochloric acid and analysis of (*a*) ammonia with a Nessler reagent and (*b*) of orthophosphate with a molybdate reagent (Found: N, 9·25; P, 20·0. Calc. for $CH_2Li_2NO_5P$: N, 9·15; P, 20·2%). Its acid dissociation constants were determined from the titration curve with 0·1N-hydrochloric acid, a TTl Radiometer pH-meter being used.

Disodium Carbamoyl Phosphate.—Dilithium carbamoyl phosphate (20—50 mg.) in water (10 ml.) was shaken for 2 min. with the sodium form of Dowex 50-X exchange resin, filtered through paper, and washed with sufficient water to produce a total volume of 25 ml.

Deuterium oxide (99·8%; Norsk Hydro-Elektrisk Kvaelstofaktieselskab) was used to prepare solvents for kinetic experiments. Thus, $DClO_4$ (0·75M) was prepared by dissolving 72% perchloric acid in deuterium oxide. D_2SO_4 (0·75M) was obtained by dissolving 0·1 ml. of D_2SO_4 (96—98%; Fluka) in 20 ml. of D_2O. Water enriched in ^{18}O was obtained from the separation plant of the Weizmann Institute. Its ^{18}O content was determined by equilibration with normal carbon dioxide.

^{18}O *Analysis in Orthophosphate.*—This was (1) by decomposition of silver orthophosphate at 1000° to yield gaseous oxygen[14] or (2) by decomposition of a mixture of barium or silver phosphate with mercuric chloride and mercuric cyanide.[13] Identical results were obtained by the two methods.

Isotopic Oxygen Exchange during the Hydrolysis of Carbamoyl Phosphate.—Carbamoyl phosphate (~150 mg.) was dissolved in ^{18}O-enriched water (5 ml.; 63·06 atom % excess of ^{18}O; brought to pH 1·5 by a few drops of concentrated hydrochloric acid). After 30 min. at 37°, corresponding to 50% hydrolysis, nitrogen was passed through the solution to remove carbon dioxide. (In some experiments normal carbon dioxide was also passed through the solution to dilute any ^{18}O-enriched dioxide remaining; but no different result for the ^{18}O content of carbamoyl phosphate was obtained.) The solution was rapidly brought to pH 9 (phenolphthalein) with N-sodium hydroxide, 2M-barium chloride (2 ml.; previously boiled to remove carbon dioxide) was added, and barium phosphate was centrifuged off. Barium carbamoyl phosphate remaining in the supernatant liquid was precipitated by two volumes of ethanol and washed with 50% ethanol and with dry ethanol. Its ^{18}O content was found by converting a sample into carbon dioxide[13] (Found: 0·824 atom % excess). This barium carbamoyl phosphate was then hydrolysed to completion at pH 1·5 in normal water. The resulting orthophosphate was isolated as the barium salt and analysed for its ^{18}O enrichment. It was found to be of normal isotopic composition within the experimental error.

Deuterium Exchange during the Elimination.—Dilithium carbamoyl phosphate (20 mg.) in deuterium oxide (99·8%; 10 g.) was brought to pH 7 by adding a solution of sodium hydroxide in deuterium oxide. After 6 min. dry dioxan (10 ml.) was added, to precipitate dilithium carbamoyl phosphate, which was washed repeatedly with dioxan to remove deuterium oxide and dried in a vacuum. Its deuterium content was determined by sealing a sample (5—10 mg) with metallic uranium (10 mg.; cleaned with nitric acid, then with acetone, and dried) in an ampoule fitted with a break-seal, for 2 hr. at 400°. The hydrogen formed was analysed by a mass spectrometer; it contained 81% of deuterium.

Determination of the Position of Bond Breakage.—Dilithium carbamoyl phosphate (~100 mg.) was dissolved in the buffer solution (20 ml.). After 20—30 min. at 100° decomposition was complete. The solution was brought to room temperature and pH 1, and nitrogen was bubbled through it to remove carbon dioxide. The solution was brought to pH 9 (phenolphthalein) with N-sodium hydroxide, and M-barium chloride (4 ml.) was added. Barium phosphate was centrifuged off, washed with water, dissolved in N-hydrochloric acid (1 ml.), and reprecipitated by addition of sodium hydroxide to pH 9. After several washings with water and ethanol, the salt was dried in a vacuum and analysed for its ^{18}O content as above.

Kinetic Measurements.—The rate of formation of ammonia was measured by keeping buffer solutions of carbamoyl phosphate (10^{-3} to 10^{-2}M) in a thermostat bath and withdrawing samples at intervals for spectrophotometric analysis of ammonia at 460 μ in conjuction with Nessler's reagent.

[28] Spector, Jones, and Lipmann, in " Methods of Enzymology," Academic Press, New York, 1957, p. 653.

The rate of formation of orthophosphate was determined by a method in which part of the water for the colorimetric molybdate analysis was replaced by *NN*-dimethylformamide.[19] Measurements were made at 335 μ. However, the optical density of solutions was found to be stable only after 30 min. (and not 5—25 min., as described [19]).

The rate of elimination was obtained by measuring the formation of cyanic acid; a Radiometer TTl pH-meter was used to keep the pH constant ($\pm$0·01 unit) by addition of sodium hydroxide solution. The amount of alkali required at given times was recorded. The reaction vessel was double-walled and the temperature was maintained constant by circulating water from a thermostat bath through it. For some of the kinetic experiments above pH 10, disodium carbamoyl phophate was used instead of the dilithium salt in order to prevent the formation of insoluble lithium phosphate, which might coat the electrodes. However, identical results were obtained with the two salts.

Carbamoyl Azide.—A solution of carbamoyl phosphate (0·06M) and sodium azide (M) was brought to pH 4—4·5, by using N-hydrochloric acid and pH-indicator paper. Two volumes of ether were added. After 25 hours' shaking at room temperature, the ether layer was separated, dried (Na_2SO_4), and evaporated. This gave carbamoyl azide (30%), m. p. 96—97° alone or mixed with the azide (m. p. 97°) synthesised from nitrite and semicarbazide by the method of Thiele and Strange.[29] Decomposition of carbamoyl azide under the conditions of isolation was 3%.

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[29] Thiele and Strange, *Annalen*, 1894, **283**, 1.