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CHEMISTRY

KINETICS OF THE FORMATION OF THE PEPTIDE BOND. HYDROLYSIS AND AMINOLYSIS OF CARBOBENZYLOXY-LEUCYL-DIBENZYL-PHOSPHATE I

BY

A. M. VAN DE VEN, V. V. KONINGSBERGER AND J. TH. G. OVERBEEK

(Communicated at the meeting of December 29, 1956)

1. *Introduction*

Nucleic acids, acylated with aminoacids occupy a central place in the hypothesis on the biosynthesis of the peptide bond, put forward by two of us [1]. In a kinetic study on the reaction between phenyl benzoyl phosphate (P.B.P.) and glycine, P.B.P. was used as a model substance for acylated nucleic acid. Obviously this model is deficient in at least two respects. The acyl group belongs to benzoic acid, not to an aminoacid and one of the three acid functions of the phosphate is free, whereas in the acylated nucleic acids all three are bound. Carbobenzyloxy-leucyl-dibenzyl-phosphate (C.L.D.P.) recently prepared by KATCHALSKY and PAECHT [2], is a much better model substance, especially as it was pointed out recently [3] that the structural resemblance between dibenzylphosphate and the corresponding phosphate ester groupings in nucleic acids is quite close.

Moreover recent investigations (HOAGLAND [4], NOVELLI c.s. [5], LIPMANN c.s. [6]) indicate that esterified acylphosphate compounds probably are intermediates in the enzymatic activation of amino acids.

SHEEAN and FRANK [7] have already used di-esterified acylphosphates in the synthesis of dipeptides but a detailed kinetic study has not been made.

In this paper we present a kinetic analysis of the hydrolysis and aminolysis by glycine of C.L.D.P. at 25° and 37° C.

2. *Experimental methods and calculation of concentrations*

2.1. *C.L.D.P.*

Since it proved to be very hard to redissolve C.L.D.P., once it had been obtained in pure form this compound was prepared and used in the form of a concentrated (about 1,5 molar) solution in carbontetrachloride. One series of experiments was performed with a sample obtained from Mrs PAECHT and prepared by the method of KATCHALSKY and PAECHT [2]. For subsequent experiments, C.L.D.P. was prepared by a modified method

based in part upon the original procedure and in part on a method for the preparation of acylphosphates as described by CHANTRENNE [8].

A solution of dibenzylchlorosphosphonate in waterfree carbontetrachloride was prepared according to ATHERTON and TODD [9]. The content of chlorophosphonate in this solution was determined by means of the formation of the anilido-compound [9] in a sample. Equivalent amounts of crystalline carbobenzyloxyleucine [10] and waterfree pyridine could then be added. Pyridine is functioning as HCl-acceptor in this process. After standing overnight at 4° C the precipitate of pyridine-HCl was filtered off. The resulting clear and completely pyridine free [1]) solution of C.L.D.P. was then concentrated to about 1.0 molar by careful evaporation at low pressure and at a temperature of 25° C. This product was directly used in the reactionvelocity measurements.

A microanalysis gave the values 3.4% for nitrogen and 5.8% for the phosphorous content of our preparation after complete evaporation of the solvent. Calculated N 2.7%, and P 5.9%. The somewhat high value for nitrogen was due to traces of free leucine as was shown by paper chromatography. The different preparations used were about 95% pure. Their behaviour in kinetic experiments at 25° C was identical.

Reactions were carried out at 25° C and at 37° C in 0.1 m acetic acid-sodium acetate buffers in 70% ethanol – 30% water (by volume). For each experiment a few drops of the concentrated C.L.D.P. solution was added. C.L.D.P. was determined at different times by the method of LIPMANN and TUTTLE [12], with the modification that all the reactants were dissolved in the ethanol-water mixture.

An appropriate amount of the reaction mixture was added to a neutralized 2 m hydroxylamine solution and kept 45 minutes at room temperature, the hydroxamic acid formation then being completed. The colour of the ferric carbobenzyloxyleucyl hydroxamate was measured at an average wavelength of 530 mμ against a blank containing all the reagents without C.L.D.P.

The relative percentages of C.L.D.P. left after increasing times of incubation were calculated from the extinctions measured. Pseudo first order rate constants were obtained by plotting the logarithm of the concentrations of C.L.D.P. left against the time of incubation. These constants showed a slight dependency on the concentration of the acetic acid-sodium acetate buffers. Measurements, however, with added sodiumchloride to compensate for the loss of ionic strength indicated that this was not a simple salt effect. Therefore precisely 0.1 m buffers were used throughout all the experiments.

2.2. *pH and concentration of OH^--ions*

For the calculation of the pH and for the conversion of pH-values into OH^--ion concentrations a reference point, activity factors and the ioniza-

[1]) The very sensitive identification reaction [11] for pyridine with 2,4-dinitrochlorobenzene was completely negative.

tion constant of water had to be determined in ethanol-water solutions.

The reference point was found by measuring the E.M.F. of the cell $Pt(H_2)$ | dilute HCl solution | AgCl, Ag and extrapolating to infinite dilution. In this way the pH of 0.001 N hydrochloric acid was found to be 3.05 at 25° C and 3.03 at 37° C. These values were taken as the reference points for other measurements.

By measuring the E.M.F. of the above cell for solutions of 0.001 N HCl with added sodium nitrate activity coefficients of HCl could be calculated (table 1). These activity coefficients should be a reasonable approximation for any monovalent ions at the same ionic strength. In none of our experiments did the ionic strength rise above 0.1. Multivalent ions were not present.

TABLE 1

Activity coefficients at 25° and 37° C of HCl in the presence of $NaNO_3$ in 70% ethanol — 30% water (by volume)

concentration HCl (moles/l)	concentration $NaNO_3$ (moles/l)	activity coefficient $f_\pm$ 25°	37°
0.001	0	0.90	0.93
0.001	0.0015	0.83	0.88
0.001	0.0040	0.77	0.85
0.001	0.0065	0.74	0.84
0.001	0.0090	0.69	0.82
0.001	0.0240	0.65	0.77
0.001	0.0490	0.61	0.74
0.001	0.0740	0.58	0.70
0.001	0.0990	0.55	0.68

pK water was calculated from the pH of 0.001 N NaOH assuming again 0.90 and 0.93 for the activity coefficients at 25° and 37° C. The values found were:

$$pK_{water}\ (25°) = 15.08;\ pK_{water}\ (37°) = 14.75$$

The accuracy of this method is reasonable, as can be proved by comparison of the pK values of acetic acid, determined by GRUNWALD and BERKOWITZ [13]: $pK_{\text{acetic acid in 70\% ethanol-30\% water}} = 6.2_9$ with the value we found $pK_{\text{acetic acid}} = 6.20$.

2.3. *Glycine*

Electrometric titrations of glycine showed that in the region between pH = 4 and pH = 7 where the reaction velocity experiments were carried out nearly all the added glycine is present in the uncharged or dipolar form (RNH_3^+). The empirical titration constant $K_{NH_3^+}$ [14] varies only very slightly with the glycine concentration as well as with pH. According to electrometric data the values of $pK_{NH_3^+}$ found by extrapolation to

small [OH] are

$$pK_{NH_3^+}\ (25°) = 9.56;\ pK_{NH_3^+}\ (37°) = 9.37.$$

For the interpretation of the kinetic results the values of

$$(1) \qquad K_{base} = \frac{[RNH_3^+][OH]}{[RNH_2]} = \frac{K_{water}}{K_{NH_3^+}}$$

are needed. Concentrations are indicated by symbols between square brackets. RNH_2 represents glycine in the glycinate form. The values for K_{base} are:

$$K_{base}\ (25°) = 3.0 \times 10^{-6}\ (\text{mol}\ l^{-1});\ K_{base}\ (37°) = 4.2 \times 10^{-6}\ (\text{mol}\ l^{-1})$$

3. *Hydrolysis*

At all pH's investigated the rate of hydrolysis was first order with respect to C.L.D.P. The results could therefore be expressed by the pseudo first order rate constant K_{obs}:

$$(2) \qquad K_{obs} = -\frac{\frac{d[C.L.D.P.]}{dt}}{[C.L.D.P.]}$$

TABLE 2

Hydrolysis at 25° and at 37° C of C.L.D.P. solutions in 0.1 M acetate buffers in 70 vol % ethanol — 30 vol % water

Exp. no.	Temp. ° C	pH	[OH] × 10^{10} (mol l^{-1})	K_{obs} × 10^4 (sec^{-1})
27	25	4.76	0.60	1.8
19	25	5.10	1.5	2.3
17	25	5.56	4.7	3.8
14	25	5.98	13.0	5.0
18	25	6.20	22.4	6.3
15	25	6.83	100	8.3
15A	25	6.86	107	9.4
22	25	7.40	382	11.5
40	37	4.61	0.84	2.8
42	37	4.93	1.8	3.6
44	37	5.45	6.6	5.6
45	37	5.78	14.6	7.2
41	37	6.16	37	9.4
43	37	6.74	142	13.0
73	37	6.79	195	17.3
74	37	7.13	353	18.8
75	37	7.27	488	21.1

It appears from table 2, that at low pH the rate is nearly proportional to the concentration of OH^--ions, but extrapolation to [OH] = 0 leaves a finite reaction rate, indicating some spontaneous non-catalyzed hydrolysis.

The reaction rate does not grow indefinitely with increasing pH, but seems to level off to a constant value. However, semiquantitative measurements around pH = 10 have shown that the reaction velocity continues to increase, although at a slower rate. Figure 1 (section 4) illustrates these facts.

The observed phenomena can be explained by a stepwise mechanism at low and middle pH and a direct OH^- catalyzed hydrolysis that prevails at high pH. In the stepwise mechanism the first step is a monomolecular activation of C.L.D.P. or more likely a reaction of this compound with water. The second step is either a spontaneous decomposition or an OH^- catalyzed reaction.

The following reaction scheme can thus be set up:

$$\text{C.L.D.P.} + H_2O \underset{K_1}{\overset{K_2}{\rightleftharpoons}} X$$

$$X \xrightarrow[K_3]{} \text{carbobenzyloxyleucine (CL)} + \text{dibenzylphosphoric acid (DPA)}$$

$$X + OH^- \xrightarrow[K_4]{} \text{CL} + \text{dibenzylphosphate (DP)} + H_2O$$

$$\text{C.L.D.P.} + OH^- \xrightarrow[K_5]{} \text{CL} + \text{DP}$$

Straightforward analysis of this reaction scheme [15] leads to the following rate equation

$$(3) \quad \begin{cases} K_{obs} = -\dfrac{1}{[\text{C.L.D.P.}]} \dfrac{d[\text{C.L.D.P.}]}{dt} = \\ \qquad = K_1' \dfrac{K_3}{K_2 + K_3 + K_4[OH]} + K_1' \dfrac{K_4}{K_2 + K_3 + K_4[OH]} [OH] \end{cases}$$

in which $K_1' = K_1[H_2O]$ whereas K_5 has been neglected.

For high concentrations of OH^--ions eq. (3) can be approximated by

$$(4) \quad K_{obs} = K_1' - \frac{K_1' K_2}{K_4} \cdot \frac{1}{[OH]}$$

and plotting of K_{obs} against $1/[OH]$ leads to values for K_1' and K_2/K_4.

For low concentrations of OH^--ions eq. (3) can be written as

$$(5) \quad K_{obs} = K_1' \cdot \frac{K_3}{K_2 + K_3} + \frac{K_1' K_4}{K_2 + K_3} [OH]$$

and by plotting K_{obs} against $[OH]$ (K_3/K_2) and (K_4/K_2) can be found, K_1' being known already. The results of this procedure are given in table 3.

TABLE 3

Rate constants for the hydrolysis of C.L.D.P.

	25°	37°
K_1' (sec^{-1})	11.5×10^{-4}	21×10^{-4}
K_3/K_2	0.15	0.12
K_4/K_2 (1 mol^{-1})	5.1×10^8	3.8×10^8
K_5 (1 $mol^{-1} sec^{-1}$)		10^4 (order of magnitude)

4. *Simultaneous hydrolysis and aminolysis*

The results of our experiments are summarized in table 4. Again, all reactions are first order with respect to C.L.D.P. and can be expressed by means of K_{obs}, the observed rate constant, defined by eq. (2).

TABLE 4

Simultaneous hydrolysis and aminolysis by glycine at 25° C and at 37° C of C.L.D.P. solutions in acetate-buffers of various pH. In the pH-range investigated, glycine exists almost completely as $^{+}H_3N-CH_2-COO^{-}=RNH_3^{+}$.

Exp. no.	Temp. ° C	pH	[OH] × 10^{10} (mol l^{-1})	[RNH_3^{+}] × 10^{2} (mol l^{-1})	$K_{obs} \times 10^{4}$ (sec^{-1})
21	25	4.52	0.36	1	1.8
12	25	5.21	1.9	1	3.8
11	25	5.86	9.7	1	12.7
16	25	6.08	16.6	1	21.5
36	25	6.53	49	1	44.5
35	25	6.76	86	1	71
31	25	6.89	116	1	83
32/32A	25	7.06	178	1	120
28	25	4.55	0.36	2	2.0
20	25	4.59	0.41	2	2.7
8	26	5.04	1.3	2	3.8
9	25	5.57	4.8	2	12.1
9A	25	5.59	5.0	2	13.8
10	25	5.98	13.0	2	35
30	25	6.48	44	2	68
34	25	6.60	58	2	91
25	25	6.86	107	2	141
25A	25	6.87	111	2	149
47	37	4.69	1.02	1	3.8
57	37	4.98	2.07	1	6.3
48	37	5.65	10.7	1	18.9
91	37	5.71	12.5	1	24
82	37	5.86	17.7	1	31
49	37	6.00	25	1	37
83/88	37	6.18	38	1	48
89	37	6.17	38	1	50
60	37	6.41	66	1	71
59	37	6.86	117	1	109
54	37	4.59	0.78	2	4.2
55	37	4.98	2.07	2	8.8
56	37	5.52	7.8	2	25
85	37	5.67	11.1	2	36
61	37	5.82	16.4	2	46
86	37	5.92	20.6	2	54
90	37	6.05	28	2	67
52	37	6.17	37	2	82
53	37	6.30	51	2	101

Evidently, the rate constants contain contributions from hydrolysis as well as from aminolysis, as can be shown by comparison with table 2. In the lowest pH region a proportionality to OH^--ion concentrations can be observed, but not to the glycine concentration. The rate constant still retains a positive value after extrapolation to $[OH] = 0$. At higher OH^--ion concentrations the rate constant is proportional again to [OH]; moreover, proportionality to the glycine concentration can now be observed. A plot of K_{obs} against [OH] (fig. 1) illustrates these facts.

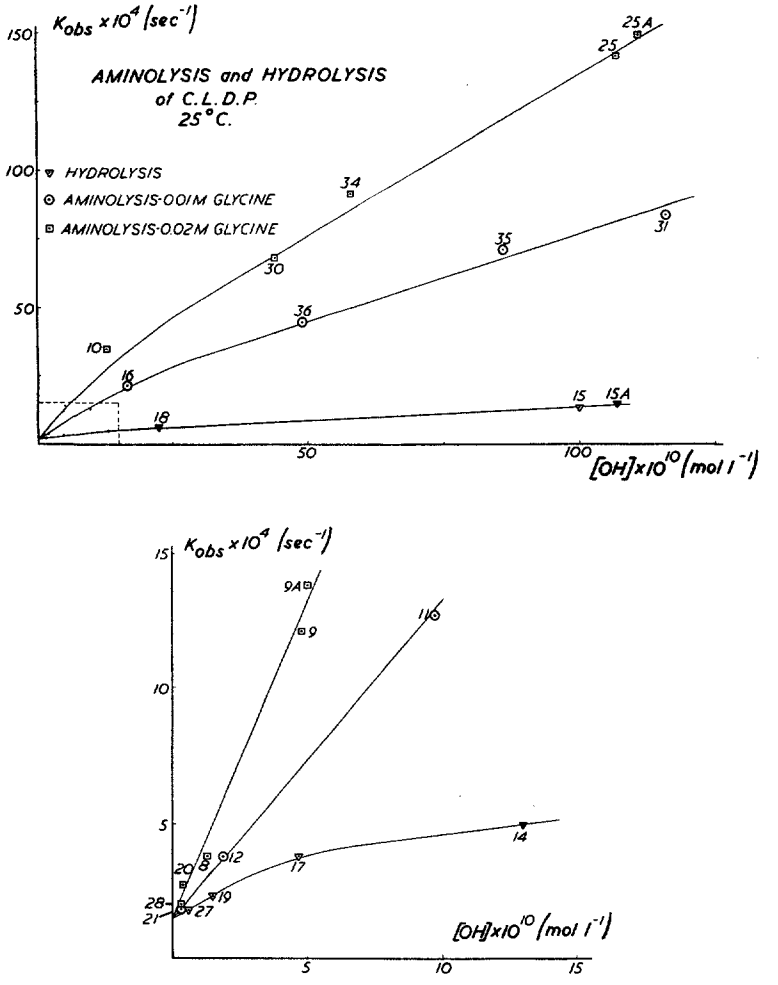

Fig. 1. Hydrolysis and simultaneous hydrolysis and aminolysis of C.L.D.P. at 25° C under the experimental conditions as mentioned in tables 2 and 4. The curves represent the data of hydrolysis and simultaneous hydrolysis and aminolysis with 0.01 and 0.02 molar glycine respectively.

For the analysis of these data the same procedure is used as was applied by OVERBEEK and KONINGSBERGER [14] to the analysis of the simultaneous hydrolysis and aminolysis of ethylthioacetate.

The phenomena mentioned above can then be fully explained by assuming the following reactionscheme:

Hydrolysis: As mentioned in § 3.

Aminolysis: $X + RNH_2 \underset{K_6}{\rightarrow}$ either hydrolysis, or formation of carbobenzyloxyleucylglycine.

$C.L.D.P. + RNH_2 \underset{K_7}{\rightarrow}$ carbobenzyloxyleucylglycine + DP

This implies that rates of the reactions 6 and 7 are found to be proportional to $[RNH_3^+]$ and [OH], which fact, using the dissociation equation (1) is interpreted as a first order mechanism with respect to the glycinate-(RNH_2) concentration.

Whether hydrolysis or animolysis products are formed in the reaction of the complex X with glycinate is not certain.

The assumed scheme is in partial agreement wtih the data of other authors on the aminolysis of thioesters [14, 16] and is in perfect harmony for the second aminolysis reaction with the schemes of KOSHLAND [17] and of KONINGSBERGER and OVERBEEK [1] for the aminolysis of acylphosphates, involving a nucleophilic attack of the carboxyl carbon by the free electron pair of the amino group nitrogen.

A reaction kinetic analysis of the given scheme [14] leads to the rate equation:

$$(6)\quad \begin{cases} K_{obs} = -\dfrac{1}{[C.L.D.P.]} \cdot \dfrac{d[C.L.D.P.]}{dt} = \\ = K_1' \dfrac{(K_3/K_2) + (K_4/K_2)\,[OH] + (K_6/K_2)\,[RNH_2]}{1 + (K_3/K_2) + (K_4/K_2)\,[OH] + (K_6/K_2)\,[RNH_2]} + K_7\,[RNH_2] \end{cases}$$

After some rearrangement the following conclusions can be drawn.

For small OH^--ion concentrations the rate constant can be written as:

$$(7)\quad K_{obs} = K_1' \frac{K_3}{K_2 + K_3} + \left\{ \frac{K_1' K_4}{K_2 + K_3} + \left(\frac{K_1' K_6}{K_b (K_2 + K_3)} + \frac{K_7}{K_b} \right) [RNH_3^+] \right\} [OH]$$

At higher pH values, however, the following equation is valid:

$$(8)\quad K_{obs} = K_1' + \frac{K_7}{K_b} [RNH_3^+]\,[OH]$$

By plotting K_{obs} against [OH] (K_7/K_b) can be found and with the values of K_b as determined in § 2.3, K_7 can be calculated. Use of the same plot can be made to obtain the value of $K_6/(K_2K_b)$ from eq. (7).

The value of the factor

$$\frac{K_1' K_4}{K_2 + K_3} + \left(\frac{K_1' K_6}{K_b (K_2 + K_3)} + K_7 \right) [RNH_3^+]$$

can be determined in the lowest pH regions, the other factors and constants being known from the preceding hydrolysis experiments. The value $K_6/(K_2K_b)$ leads then in the same way as mentioned above to the constant K_6/K_2.

Table 5 gives the results of the calculations described in this section.

TABLE 5

Rate constants for the aminolysis by glycine of C.L.D.P.

	25°	37°
K_6/K_2 (1 mol^{-1})	5.4×10^4	10.5×10^4
K_7 (1 $mol^{-1}sec^{-1}$)	198	357

The rate constants were finally used in calculating values of K_{obs} by means of the theoretical eq. (6), which were compared with the experimental data of tables 2 and 4. They have been submitted to some trial and error in order to obtain the closest possible fit. Their accuracy is not high but may be estimated to be within 20% of the actual values. Some representative results of the final calculations are given in table 6.

TABLE 6

Values of K_{obs} as calculated with the constants of tables 3 and 5 from eq. (6) and as determined experimentally.

Exp. no.	Temp. °C	$[OH] \times 10^{10}$ (mol l^{-1})	$[RNH_3^+] \times 10^2$ (mol l^{-1})	$K_{obs} \times 10^4$ experimental (sec $^{-1}$)	$K_{obs} \times 10^4$ calculated (sec $^{-1}$)
19	25	1.5	–	2.3	2.4
15A	25	107	–	9.4	9.7
11	25	9.7	1	12.7	11.8
31	25	116	1	83	87
8	25	1.28	2	3.8	4.0
30	25	44	2	68	67
44	37	6.6	–	5.6	5.6
43	37	142	–	13.0	17.7
73	37	159	–	17.3	17.9
57	37	2.07	1	6.3	6.1
49	37	25	1	37	35
59	37	117	1	109	118
54	37	0.78	2	4.2	4.6
61	37	16.4	2	46	41
52	37	37	2	82	79

KINETICS OF THE FORMATION OF THE PEPTIDE BOND. HYDROLYSIS AND AMINOLYSIS OF CARBOBENZYLOXY-LEUCYL-DIBENZYL-PHOSPHATE II

BY

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5. *Isolation of the product of aminolysis*

The dipeptide carbobenzyloxyleucylglycine is to be expected as the product of the second aminolysis reaction (K_7), in agreement with SHEEAN and FRANK's observation [7] that dipeptides are formed during reactions of this type of diesterified acylphosphate compounds. A chromatogram was made of the reaction products of C.L.D.P. in acetate buffer of pH = 7 and ample excess of glycine after two hours incubation at 25° C. The development of the chromatogram was done according to the method of STARK, GOODBAN and OWENS [18] for chromatography of organic acids. An authentic sample of carbobenzyloxyleucylglycine was used as a reference. The results indicate that the reaction yields more then 80% of the theoretical amount of dipeptide (reaction K_7).

6. *Activation energies and probability factors*

Rate constants as determined at 25° C and at 37° C (table 3 and 5) were substituted into equation (9)

$$K_T = P\,Z\,e^{-E/RT} \tag{9}$$

to calculate the energies of activation (E) and probability factors P of the various hydrolysis and aminolysis reactions. For the reactions 3, 4 and 6, involving the intermediate compound X, this procedure only leads to differences of activation energies, $E_3 - E_2$, etc. and to the corresponding ratios of probability factors P_3/P_2 etc. The results are presented in table 7 and 8.

With respect to these data the following remarks can be made:

1. All activation energies lie within the known range; most of them are rather small.

2. Undoubtedly the complex formation of C.L.D.P. with water (reaction 1) is a "slow" reaction. The P-factor is within the range GLASSTONE, LAIDLER and EYRING [20] have calculated for the reaction between two polyatomic molecules ($P = 10^{-5}$–10^{-10}). The same conclusion can be

TABLE 7

Activation energies and probability factors of the hydrolysis and aminolysis by glycine of C.L.D.P. The collisionfrequency Z for bimolecular reactions was assumed to be 10^{11} l mol^{-1} sec^{-1} [19].

Reaction	$K_{25°}$	$K_{37°}$	E (cal/mol)	PZ (1 mol^{-1}. sec^{-1})	P
1. C.L.D.P. $+H_2O \rightarrow X$ $K_1' = K_1/[H_2O]$ (l mol^{-1} sec^{-1})	6.4×10^{-5}	11.8×10^{-5}	9300	4.5×10^{2}	4.5×10^{-9}
7. C.L.D.P. $+RNH_2 \rightarrow$ aminolysis-products K_7 (1 mol^{-1} sec^{-1})	198	357	9000	8×10^{8}	8×10^{-3}

TABLE 8

Differences of activation energies and ratios of probability factors between reaction A and reaction 2. The collision frequency Z was assumed to be $Z=10^{11}$ l mol^{-1} sec^{-1} for bimolecular reactions and $Z=10^{13}$ sec^{-1} for monomolecular reactions. In all cases reaction 2 is $X \rightarrow$ C.L.D.P. $+H_2O$.

Reaction A	K_A/K_2 25°	K_A/K_2 37°	E_A-E_2 (cal/mol)	$\frac{P_A Z_A}{P_2 Z_2}$	P_A/P_2
3. $X \rightarrow CL+DPA$ K_3/K_2	0.15	0.12	−3000	9×10^{-4}	9×10^{-4}
4. $X+OH^- \rightarrow CL+DP+H_2O$ K_4/K_2 (1 mol^{-1})	5.1×10^{8}	3.8×10^{8}	−4500	3×10^{5} (1 mol^{-1})	3×10^{7}
6. $X+RNH_2 \rightarrow$? K_6/K_2 (1 mol^{-1})	5.4×10^{4}	10.5×10^{4}	+10000	1×10^{12} (1 mol^{-1})	1×10^{14}

drawn if this reaction is a monomolecular activation reaction C.L.D.P. $\rightarrow$ X. The P-factor would then be $P \approx 10^{-10}$.

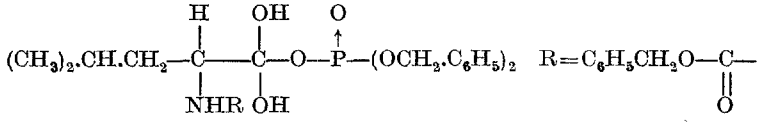
3. Reaction 7 is a "normal" reaction.

4. Any conclusion with respect to the reactions 2, 3, 4 and 6 related with the existence of the complex X, can only be based upon the ratio of the various P-factors of the reactions involved. If it is assumed that reaction 6 is a "fast" reaction ($P \approx 10^{7}$–10^{8}), then the reactions 3, 2 and 4 respectively are "very slow", "slow" and "normal" ($P_3 \approx 10^{-9}$–10^{-10}; $P_2 \approx 10^{-6}$–10^{-7}; $P_4 \approx 1$–10). The activation energies of the reactions 3 and 4 are about the same. The value for the reaction 6 is about 14000 calories higher.

7. *Reaction schemes for the hydrolysis and aminolysis of carbobenzyloxyleucyldibenzylphosphate. Discussion of the results*

Further analysis of the various reactions of the proposed reaction scheme justifies the following suggestions and remarks to be made.

For the first hydrolysis reaction either a monomolecular activation or a bimolecular complex formation could be assumed. If the latter assumption is correct, the following structure can be suggested for the intermediate compound X:



BENDER [21] et al. have proved the intermediate formation of a similar complex between oxygenesters and water, and OVERBEEK and KONINGSBERGER [22] suggested the occurrence of such an intermediate in the hydrolysis of ethylthioacetate.

Considering the speculative character of this asusmption, it does not seem justified to propose further structural reaction schemes for the hydrolysis and aminolysis reactions of C.L.D.P. involving complex X.

A structural reaction scheme for the alkaline hydrolysis reaction of C.L.D.P. by OH^- (K_5) will be similar to the well known scheme for the hydrolysis of oxygenesters.

The aminolysis of C.L.D.P. (K_7) involves a nucleophilic attack on the carboxylcarbon atom of C.L.D.P. by the free electron pair of the amino group nitrogen, as already is proposed for the aminolysis of acylphosphates by KOSHLAND [17] and by KONINGSBERGER and OVERBEEK [1].

Finally, a comparison was made between the results obtained with C.L.D.P. as a diesterified acylphosphate and those described by CHANTRENNE [23] and KONINGSBERGER and OVERBEEK [1] for non-esterified and mono-esterified acylphosphate compounds.

The aminolysis of the non-esterified benzoylphosphate [23] by aqueous glycine does not lead to formation of hippuric acid, probably because hydrolysis is too fast. Estrification of one of the two acidic groups yields phenylbenzoylphoshpate (P.B.P.); the hydrolysis of this product is much slower than that of benzoylphosphate; hippuric acid is formed readily [23, 1] in aminolysis by aqueous glycine according to

$$\text{P.B.P.} + RNH_2 \rightarrow \text{aminolysis products}$$

The activation energy in this case is $E = 7200$ cal/mol, the probability factor $P \approx 10^{-6}$.

In the case of the comparable reaction 7 of the di-esterified compound C.L.D.P.

$$\text{C.L.D.P.} + RNH_2 \xrightarrow[K_7]{} \text{aminolysis products}$$

the activation energy is somewhat higher, $E = 9000$ cal/mol, but the probability factor P is increased: $P \approx 10^{-2}$.

Aminolysis products are formed more readily. Meanwhile the stability of C.L.D.P. is still very reasonable although its rate of hydrolysis is about

1000 × faster than that of P.B.P. In table 9 the reaction rates of the two compounds are compared at 25° C, the only temperature for which all data are available.

TABLE 9

Comparison of the monomolecular rateconstants of hydrolysis and aminolysis by glycine of phenylbenzoylphosphate (P.B.P.) and carbobenzyloxy-leucyl-dibenzyl-phosphate (C.L.D.P.).
The measurements were carried out at 25° C in acetate buffer of pH 6.96 in 70 vol % ethanol − 30 vol % water. [glycine] = 2×10^{-2} (mol l^{-1})

	K_{obs} (sec^{-1})		K_{obs} C.L.D.P. / K_{obs} P.B.P.
	P.B.P.	C.L.D.P.	
Hydrolysis	19.2×10^{-7}	9.4×10^{-4}	500
Aminolysis	109×10^{-7}	141×10^{-4}	1300

These conclusions and observations have some importance for the hypothesis of OVERBEEK and KONINGSBERGER [1], concerning the role of nucleic acids in the biosynthesis of proteins as the supposed intermediates in this process are also diesterified phosphate acyl compounds. These intermediates might be expected to have a very high free energy of hydrolysis and consequently to be too unstable in aqueous surroundings to play the role ascribed to them. The behaviour of the modelsubstance C.L.D.P. seems to invalidate this objection against the theory.

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8. *Summary*

The kinetics of the hydrolysis and the simultaneous hydrolysis and aminolysis by glycine of carbobenzyloxy-leucyl-dibenzyl-phosphate (C.L.D.P.) were investigated at 25° C and at 37° C between pH = 4 and pH = 7. The reactions are first order in C.L.D.P. The dependence on the pH of the hydrolysis reaction is explained by assuming a spontaneous decomposition of hydrated C.L.D.P. molecules and a decomposition catalyzed by OH^--ions. An indication is found for a direct attack of C.L.D.P. by OH^--ions at higher pH. The aminolysis reaction involves both decomposition of the hydrated C.L.D.P. as well as of C.L.D.P. itself by glycine in the glycinate-(RNH_2) form. The aminolysis reaction was demonstrated to yield the dipeptide carbobenzyloxyleucylglycine. Activation energies and, as far as possible, probability factors were calculated from the data on the rate constants at 25° C and at 37° C. For the more complicated reactions the ratios of the P-factors of the separate reaction-

steps were found. Generally, low activation energies and slow and normal reactions proved to be involved in both hydrolysis and aminolysis processes. A discussion of the results is given.

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