only about 16–17 g. (88–95% recovery) of starting material.

Acknowledgments.—The authors are grateful to Dr. V. A. Fassel and Mr. R. McCord of the Institute for Atomic Research for the infrared determinations.

AMES, IOWA

[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

A Kinetic Study of the Leuchs Anhydrides in Aqueous Solution. I[1]

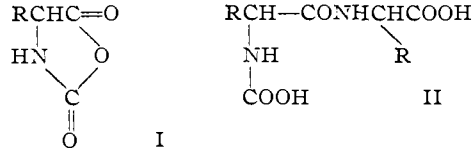
BY PAUL D. BARTLETT AND RICHARD H. JONES

RECEIVED SEPTEMBER 7, 1956

The reaction of an anhydro-N-carboxy-amino acid (Leuchs anhydride) with water is subject to general basic catalysis and is not catalyzed by acids. The reaction with the amino group of an amino acid anion is so rapid that it can compete successfully with hydrolysis. The reactions of anhydro-N-carboxyglycine, anhydro-N-carboxy-D- and L-alanine and anhydro-N-carboxy-α-aminoisobutyric acid with water and some amino acids have been followed kinetically by carbon dioxide evolution and their rate constants determined (Tables II and III). The possibility of controlled peptide synthesis in aqueous solution from the Leuchs anhydrides is discussed and experiments are reported showing the limitations of such a procedure.

Introduction

The anhydrides (I) of N-carboxy-α-amino-acids, first prepared by Leuchs,[2] have been investigated



I

II

chiefly in inert solvents where with controlled amounts of active-hydrogen compounds as initiators they lead to polypeptides[3] of moderate or high molecular weight while at very low temperatures the decarboxylation of the initial carbamic acid (II) can be repressed sufficiently by salt formation[4] to afford good yields of simple peptides. Even more attractive possibilities of control of this reaction would present themselves if it were possible to operate in aqueous solutions; with this in mind, we have studied the nature and rates of the reactions of some simple N-carboxy-anhydrides in water.

Reactions of N-Carboxy-anhydrides with Water.—The N-carboxy-anhydrides of glycine, *dl*-alanine and α-amino-isobutyric acid, on solution in water at 0°, evolve carbon dioxide at a rate convenient for measurement. The reactions were followed by observing the increase in pressure in a closed, initially evacuated system; with the first and last of these anhydrides a rate measurement by following volume increase at constant pressure agreed with the results of the pressure method. Except for a short initial period (see below) the reactions, as followed in this manner, were of the first order, yielding rate constants as shown in Table I. Reactions of anhydro-N-carboxy-α-aminoisobutyric acid in 0.1 *M* barium chloride, 0.1 *M* hydrochloric acid and 1.0 *M* sulfuric acid yielded the same average rate constant, 2.4×10^{-3} sec.$^{-1}$, as the reaction in pure water, with a mean deviation of $\pm 0.07 \times 10^{-3}$. The effect of acid catalysis upon the hydrolysis of the anhydride is therefore negligible.

TABLE I

RATE CONSTANTS FOR UNCATALYZED HYDROLYSIS AT 0°

N-Carboxy-anhydride of	No. of runs	Concentration range	k_1, sec.$^{-1}$ $\times 10^3$	Mean deviation
Glycine	6	0.01– 0.028	4.1	0.23
dl-Alanine	3	.022– .028	6.4	.23
α-Aminoisobutyric acid	5	.019– .028	2.4	.12

Carbon dioxide is not evolved when a carboxy-anhydride reacts with two or more equivalents of sodium or barium hydroxide. An attempt was made to measure the rate of the reaction of anhydro-N-carboxyglycine with hydroxyl ion by following the conductivity of a solution of barium hydroxide to which the anhydride was added. There was a very rapid reaction which produced 74% of the expected change in conductivity within the first 40 seconds, followed by a slower process to which a second-order rate constant of 0.30 l./mole sec. could be assigned. The latter process is far too slow to be the reaction of anhydride with hydroxyl ion, as a later experiment proves. According to Stadie and O'Brien,[5] carbamates such as the Siegfried salts come rapidly to equilibrium with carbon dioxide and amino acid anion, but the carbon dioxide is much more slowly equilibrated with bicarbonate and carbonate. The calculated equilibrium concentration of carbonate ion during the preparation of a Siegfried barium salt is more than sufficient to precipitate barium carbonate; the non-appearance of such a precipitate is evidently the result of slow attainment of the carbonate equilibrium. The slow reaction observed in the conductivity experiments may well be the conversion of hydroxyl ion into carbonate.

Hydrolysis of N-Carboxy-anhydrides in Buffer Solutions.—During this work it became apparent that the amino acid formed by hydrolysis of the

(1) This work was supported by the Office of Naval Research under Contract No. N5ori-07653 with Harvard University, 1952–1953.

(2) H. Leuchs, *Ber.*, **39**, 857 (1906).

(3) For a review see E. Katchalski, "Advances in Protein Chemistry," Vol. VI, Academic Press, Inc., New York, N. Y., 1951, pp. 123–185. More recent work: D. G. H. Ballard and C. H. Bamford, "Symposium on Peptide Chemistry," Special Publication No. 2, Chemical Society, London, 1955, pp. 25–48; E. R. Blout and R. H. Karlson, THIS JOURNAL, **78**, 941 (1956); P. Doty and R. D. Lundberg, *ibid.*, **78**, 4810 (1956); E. R. Blout and M. Idelson, *ibid.*, **78**, 3857 (1956).

(4) J. Leggett Bailey, *J. Chem. Soc.*, 3461 (1950).

(5) W. C. Stadie and H. O'Brien, *J. Biol. Chem.*, **103**, 521 (1933); **112**, 723 (1935).

anhydride was itself competing appreciably with water, especially in the case of glycine, for reaction with the anhydride after the reaction was under way. The effect of this competition could be reduced by operating either in excess base to prevent decarboxylation of N-carboxy-glycine or in a buffer solution sufficiently acid to keep the amino group of the amino acid overwhelmingly in the ammonium ion form. An acetate buffer was adopted containing 1.00 *M* sodium acetate and 0.875 *M* acetic acid and having a *p*H of 4.76. The experiments described above indicated that the hydrolysis of an N-carboxy-anhydride is subject to basic but not to acid catalysis. In view of the possibility of general basic catalysis (*e.g.*, catalysis by acetate ions as well as by hydroxyl ions), a series of rate determinations were made in which anhydro-N-carboxyglycine was hydrolyzed at 0° at concentrations from 0.023 to 0.026 *M* in this acetate buffer at various buffer dilutions up to tenfold, the ionic strength being maintained constant at 1.00 by means of potassium chloride. The results fit the equation

$$k = 0.00028 + 0.00049[OAc^-] \text{ (time in sec.)}$$

closely. That this is catalysis by the acetate ion and not by acetic acid is already indicated by the complete insensitivity of the rate to strong acids in the case of anhydro-N-carboxy-α-aminoisobutyric acid. From this result it appears that about 30% of the rate measured in pure water was due to the overlaying of the hydrolysis by a reaction with amino acid formed during the hydrolysis and existing at a varying *p*H during the reaction, which did not produce a clear deviation from first-order kinetics. Later work verified this indication that the carboxy-anhydrides do indeed react preferentially with amino groups even in aqueous solution.

Kinetic Salt Effect.—The salt effect on the hydrolysis of anhydro-N-carboxyglycine is negative, as shown by the first-order rate constants in buffer solutions consisting of 0.1 *M* sodium acetate and 0.0875 *M* acetic acid alone (*k* 0.00043 sec.$^{-1}$). No study was made to apportion this between "primary" and "secondary" kinetic salt effect.

The "Induction Period."—Most of the manometric runs had an initial period of ten minutes during which the reaction appeared to be very slow, before the uniform first-order rate of gas evolution was attained. Since the carbon dioxide is the product of the second of two successive reactions

$$I \longrightarrow \underset{\underset{NHCOOH}{|}}{RCHCOOH} \longrightarrow \underset{\underset{NH_2}{|}}{RCHCOOH} + CO_2$$

we considered the possibility that the decarboxylation of the carbamic acid was a slow enough reaction to account for this effect. This was tested by taking advantage of the fact that a stable salt of glycine N-carboxylic acid can be prepared[6] by saturating a solution of barium glycinate with carbon dioxide. At 0°, in the absence of excess barium hydroxide, this saturation was accomplished without any precipitation of barium carbonate. The decarboxylation of N-carboxyglycine was now observed by the rapid addition to the stable Siegfried salt of enough acetic acid to make an acetate buffer of *p*H 4.76, or in other experiments to give an unbuffered solution of *p*H 0–2. The evolution of carbon dioxide in these experiments followed an approximately first-order course with k_1 varying from 0.023 to 0.042 sec.$^{-1}$. However, when a solution of sodium carbonate was acidified in the same manner, similar results were obtained, k_1 being again from 0.023 to 0.042 sec.$^{-1}$. That the measurable reaction was just the rate of evolution of carbon dioxide from aqueous solution was verified in a series of experiments in which the rate constants varied from 0.005 to 0.030 sec.$^{-1}$ as the speed of the magnetic stirrer in the apparatus was varied over an approximately fivefold range. We conclude that the slow approach to linearity in the kinetic curves is due to the appreciable time required for the establishment of liquid-vapor equilibrium, a steady state being approached as the carbon dioxide evolved in a period of a few minutes becomes a smaller fraction of the total present.

The rate of equilibration of carbonic acid with water and carbon dioxide, an important factor in physiological processes, is greater than the rate of gas evolution here measured. The rate constant is reported[7] as 1.97 sec.$^{-1}$ by Roughton and coworkers and 1.4 sec.$^{-1}$ by Stadie and O'Brien at 0°. The rate constant for decarboxylation of unsubstituted carbamic acid at this temperature is reported[8] to be even greater, 80 sec.$^{-1}$. It appears that whatever the position of the equilibrium in the carbonation of glycine, this equilibrium is attained rapidly in comparison with the reactions whose rates are being measured here.

Reaction of N-Carboxy-anhydrides with Amino Acids.—With the information at hand it was possible to measure the rates of reaction of the N-carboxyanhydrides with various amino acids in aqueous systems. For this purpose it was not possible to suppress the hydrolysis of the anhydride entirely, but the rate constant of this process could be accurately evaluated and subtracted from the over-all pseudo-unimolecular rate constant observed in the presence of an excess of amino acid in the same buffer solution. The reactivity of an amino acid toward an N-carboxyanhydride resides entirely in the free amino group, and the fraction of the amino acid molecules possessing such a free amino group is controlled by the buffer solution. By knowing the second acid dissociation constant of an amino acid and the *p*H of the buffer, it should therefore be possible to determine the rate constant for reaction of an N-carboxyanhydride with the free-base form of any amino acid. In the 0.1 *M* acetate buffer of *p*H 4.74 the pseudo-unimolecular rate constant k_{obs} was used as follows to determine the second-order rate constant k_{AA} for the reaction of anhydro-N-carboxyglycine with amino acid

$$k_{obs} = 0.00043 + k_{AA} \text{ (amino acid)}$$

and for anhydro-N-carboxyalanine

$$k_{obs} = 0.00071 + k_{AA} \text{ (amino acid)}$$

(6) M. Siegfried, *Z. physiol. Chem.*, **44**, 85 (1905).

(7) R. Brinkman, R. Maragria and F. J. W. Roughton, *Phil. Trans. Roy. Soc.*, **A232**, 65 (1933); W. C. Stadie and H. O'Brien, *J. Biol. Chem.*, **103**, 521 (1933).

(8) F. J. W. Roughton, THIS JOURNAL, **63**, 2834 (1941).

In the latter case the correction to be made for hydrolysis rate amounted to as much as 80% of the quantity being measured; in most cases the relative rates were more favorable than this. An idea of the degree of reliability of the results may be had from the data in Table II, which were determined in order to learn how great the selectivity might be between the optical isomers of alanine in reaction with *d*- or *l*-anhydro-N-carboxyalanine.

TABLE II

OPTICALLY ACTIVE ANHYDRO-N-CARBOXY-ALANINES AND OPTICALLY ACTIVE ALANINES IN ACETATE BUFFER AT 0°
0.100 *M* NaOAc, 0.0875 *M* HOAc

Anhydride concentration	Alanine concentration	k_1, sec.$^{-1}$ × 10^3	$\frac{k_1 - 0.00071}{\text{(alanine)}}$ l./mole sec. × 10^3
	L-Alanine anhydride alone		
0.0396	0	0.70	
.0426	0	.71	
.0465	0	.70	
	Average	0.70	
	D-Alanine anhydride alone		
0.0454	0	0.71	
.0423	0	.72	
.0446	0	.72	
	Average	0.72	
	L-Alanine anhydride + L-alanine		
0.0408	0.225	1.02	1.29
.0416	.449	1.31	1.33
.0442	.449	1.31	1.33
	Average		1.32
	D-Alanine anhydride + D-alanine		
0.0454	0.337	1.15	1.30
0.0439	0.449	1.31	1.33
	Average		1.32
	D-Alanine anhydride + L-alanine		
0.0456	0.225	0.96	1.12
.4038	.337	1.07	1.06
.0422	.449	1.04	1.10
	Average		1.09
	L-Alanine + D-alanine		
0.0420	0.225	0.96	1.13
.0438	.449	1.16	1.00
.0411	.449	1.19	1.07
.0449	.337	1.07	1.07
	Average		1.10

The four possible pairs were run in triplicate, with mean deviations of 1–3% in the second-order rate constants determined. There was agreement between the values for *d*-anhydride with *d*-alanine and for *l*-anhydride with *l*-alanine and also between the constant for *d*-anhydride with *l*-alanine and that for *l*-anhydride with *d*-alanine. In each case the reaction between the configurationally similar reactants was about 20% faster than that between the configurationally opposite reactants. It is not surprising that the difference is small, since neither reacting center is itself asymmetric.

Table III summarizes the second-order rate constants for reaction of anhydro-N-carboxyglycine and alanine with several amino acids and peptides. These measurements are made under conditions where only a minute fraction of the amino acid exists with its amino group in the free-

TABLE III

ANHYDRO-N-CARBOXYGLYCINE AND AMINO ACIDS AT 0° IN ACETATE BUFFER

0.1 *M* sodium acetate, 0.0875 *M* acetic acid

Amino acid	k_{AA}, l./mole sec. × 10^3	pK_2^0	$(k_{AA})^-$ corr.
Glycine	3.8 ± 0.25	10.53[a]	2330
dl-Alanine	1.10 ± .12	10.62[a]	830
α-Aminoisobutyric acid	0.265 ± .045	10.57[a]	179
l-Leucine	1.85 ± .13	10.73[a]	1800
Glycylglycine	23.7 ± .17	8.86[b]	311
Triglycine	29.0 ± .17	(8.58)[c]	200
Anhydro-N-carboxy-*d*-alanine			
dl-Alanine	1.2	10.62[a]	917
dl-Alanylalanine	4.4 ± 0.7	(9.17)[c]	118
l-Leucine	1.11	10.73[a]	1100

[a] From an equation of H. S. Harned and R. A. Robinson, *Trans. Faraday Soc.*, **36**, 973 (1940), and data selected by J. T. Edsall in Cohn and Edsall, "Amino Acids, Peptides and Proteins," Reinhold Publ. Corp., New York, N. Y., 1943, p. 79. [b] J. Greenstein, *J. Biol. Chem.*, 101, 603 (1933). [c] Estimated assuming the same temperature coefficient as for the amino acids, from data in Cohn and Edsall, p. 84.

base form, yet even under these conditions the amino acid competes successfully with the water for reaction with the N-carboxyanhydride. It is of interest to calculate how fast the active form of the amino acid (having its amino group free) is reacting with the anhydride. For this purpose there are two molecular species to be considered, the anion $H_2NCHRCOO^-$ and the unfavored uncharged form $H_2NCHRCOOH$. The fraction of the former species present in our buffer solution is $10^{(4.74 - pK_2)}$, and the fraction of the latter species is 10^{pK_z} where K_2 is the second ionization constant of the amino acid, and K_z is the equilibrium constant between the dipolar ion and the uncharged amino acid.[9] The latter quantity has been evaluated by Edsall and Blanchard for most of the amino acids with which we are concerned. If for glycine we extrapolate their value of pK_z from 25 to 0° by means of their value of ΔH = 11,500 cal., we find that pK_z under our conditions is −5.79, and this leads to an estimate of the fraction of neutral glycine present at *p*H 4.74 as 1/1,500,000. From the values of pK_2 at 0° given in Table III, the fraction of anion has been estimated; for glycine it is 1/620,000. Thus about two-thirds of the free amino group which is responsible for the reaction with N-carboxyanhydride at *p*H 4.74 is situated on the glycine anion, and the other third is on neutral glycine. These are by no means accurate figures in view of the assumptions underlying the estimate of K_z, but they suggest that we should not be too far wrong in calculating the bimolecular rate constant for reaction between N-carboxyanhydride and glycine anion if we were to attribute all the reaction ob-

(9) J. T. Edsall and M. H. Blanchard, THIS JOURNAL, **55**, 2344 (1933).

served at pH 4.74 to this species. In favor of this approximation is the expectation that a negative charge on the carboxyl group might enhance the nucleophilic reactivity of the amino group, so that the anion would be inherently more reactive than the neutral form. Against this is the possibility that an undissociated carboxyl group in the amino acid might be strategically located to assist catalytically in the reaction of its amino group with the carbonyl group in the anhydride, which might enhance the importance of the rare neutral species in contributing to the observed rate. In a series of experiments in acetate buffers at constant acetate concentration and ionic strength but varying acetic acid concentration and pH, the rate of reaction was inversely proportional to the acetic acid concentration.

This provides evidence that the amino acid anion is the dominant species in the reaction with anhydro-N-carboxyglycine, rather than the neutral form which is present at constant concentration in this series of buffers.

Accordingly, in Table III the corrected bimolecular rate constant for reaction of anhydride with each amino acid anion has been computed by multiplying k_{AA} by the factor $10^{(pK_2 - 4.74)}$. It will be seen that these rate constants are very large, indicating a surprising ability of the amino group to compete with solvent water. More will be said of this in a later section.

The rate constants for reaction of anhydro-N-carboxyglycine with glycine, glycylglycine and glycylglycylglycine are in the ratio of 1:6:7.5. This means experimentally that in the acetate buffer there is a strong tendency toward the formation of higher peptides through competition of the glycylglycine already formed during a run with the glycine added and so on for the higher peptides. As the last two columns show, *this is not due to an inherently higher reactivity of the peptide anions* toward the anhydride in comparison with glycine, but rather to the fact that the second ionization of di- and triglycine occurs at less basic pH values and there is more anion in proportion present at pH 4.74. The *corrected rate constants*, referred to the anions, are in the *reverse order:* 12:1.6:1. Likewise, anhydro-N-carboxyalanine reacts more than three times as fast with alanylalanine at pH 4.74 as with alanine, although the anion of the latter is the more reactive by a factor of almost eight.

A regularity is discernible in the anion rate constants when a "Brønsted plot" is made of the logarithms of the rate constants *referred to pure anion* against the logarithms of the second ionization constants, the measure of the thermodynamic basicity of the amino groups. The table contains data for three compounds with amino groups on primary carbon atoms reacting with anhydro-N-carboxyglycine and for three compounds with amino groups on secondary carbon atoms reacting with anhydro-N-carboxyalanine. Each class of amino compound determines a straight line (Fig. 1), the rates for the secondary amino acids and peptides being slower than for comparably basic primary compounds. In the case of the one amino acid examined which has an amino group on a tertiary carbon atom, the point if plotted would lie below either line. Toward alanine and leucine the reactivities of anhydro-N-carboxyglycine and the corresponding alanine derivative do not appear to be very different. Also plotted in Fig. 1 are the rate constants for three reactants in the "secondary" class, from Part II (see following paper).

The Possibility of Controlled Peptide Syntheses in Aqueous Solution.—The selective reaction of anhydro-N-carboxyglycine with amino acids in aqueous solution raises the question whether it would be possible to control this process with enough precision to synthesize a peptide step by step by the successive addition of a series of N-carboxyanhydrides to a growing peptide in solution in water without isolating the peptide at each step. Obviously this would require very high yields in each step in order to be feasible for chains of any length. Our measurements show that there are two competing reactions of importance to interfere with the clean production of a desired peptide. One of these is the reaction of the anhydride with hydroxyl ion, the other is the reaction of the product peptide with anhydride to give the peptide of one more amino acid unit. The rate of uncatalyzed or buffer-catalyzed hydrolysis can be negligible in the pH range above 7.

As far as the reaction with hydroxyl ion is concerned, we did not succeed in measuring its rate. We might attempt to estimate it by taking advantage of the relationship, shown in Fig. 1, between the strength of the basic amino group of an amino acid or peptide and its rate of attack on anhydro-N-carboxyglycine, extrapolating the glycine line to the right to an abscissa of 15.74 corresponding to the basic strength of the hydroxyl ion. This is an unjustified procedure, since the Brønsted relation holds only among acids or bases of the same chemical type, and there is reason to think that for a given basic strength a primary amine should be a more powerful reagent in attack on an anhydride carbonyl group than a corresponding oxygen reagent. The rate constant estimated in this way, 1.6×10^6 l./mole sec., is probably a generous upper limit for purposes of predicting the favorable conditions for producing dipeptide with a minimum of hydrolysis.

Since we know the rate constants for uncatalyzed hydrolysis and for reaction of the anhydride with glycine as a function of pH, we can make a hypothetical calculation to the effect that

$$\text{rate of hydrolysis} = [A]0.00041 + k_{OH}10^{pH-15.74}$$

$$\text{rate of diglycine formation} = \frac{2530\,[A]\,[G]}{1 + 10^{10.53-pH}}$$

By differentiating to make the ratio of the second rate to the first a maximum, we find that the pH should be most favorable around 8, at which point diglycine formation should be favored over hydrolysis by a factor of 3.3 times the glycine concentration. This factor is not very sensitive to pH in this region, being adversely affected by the constant water hydrolysis in the more acid media and by the fact that the hydroxyl ion concentration continues rising beyond pH 10–11 where the concentration of the reactive form of the amino acid ceases to in-

crease. The results reported below (Table IV) show that hydrolysis by hydroxyl ion cannot be as extensive as this, so that $K_{OH} < 1.6 \times 10^6$.

TABLE IV

ATTEMPTS AT SYNTHESIS OF DIPEPTIDES FROM ANHYDRO-N-CARBOXYGLYCINE AND GLYCINE AT 0°

Solution	pH[a]	% polymer with anhydride alone	G = final concn. glycine,[b] $M \times 10^2$	100 (1–50 G) % dipeptide (side reaction → triglycine)[d]	100 (1–12.5 G) % dipeptide (side reaction → glycine)[d]
0.174 N $Ba(OH)_2$	(13.2)	11	5.5	0	31
0.50 M Na_2CO_3	11.71	18	5.4	0	33
1.0 M NaH_2BO_3 + 0.2 M H_3BO_3	10.89	31	0.65	68	91
0.5 M NaH_2BO_3	10.61	50	0.35	83	96
0.5 M Na_2CO_3 + 0.5 M $NaHCO_3$	(10.3)	10	>5.5	0	31
Catalytic amount $Ba(OH)_2$	(9.5)	98	3.0[c]	0	63

[a] pH values are those of the solution before the addition of anhydride or amino acids. Values in parentheses are calculated. Others were measured at 28° using a Beckman pH meter with glass electrode for solutions of pH 10. [b] Initial concentrations of all solutions: 4.0×10^{-2} M glycine and 4×10^{-2} M N-carboxyglycine anhydride. [c] Calculated from a reaction where two portions of glycine anhydride were used in an attempt to synthesize triglycine. [d] If anhydride (A) reacts exclusively with either glycine (G) to yield diglycine (GG) or diglycine to yield triglycine (GGG) and zero subscripts denote concentrations at $t = 0$, then at the end of the reaction: (G) + 2(GG) + 3(GGG) = $(A)_0 + (G)_0 = 0.08$. Since $(A)_0 = (G)_0$ in all cases, (G) = (GGG), and 4(G) + 2(GG) = 0.08, so that (GG) = 0.04 − 2(G) and $(GG)/(A)_0 = (0.04 - 2(G))/0.04 = 1 - 50(G)$. In similar fashion, if A reacts only with hydroxyl ion to yield G or with G to yield GG, then (G) + 2(GG) = 0.08, (GG) = 0.04 − (G)/2 and $(GG)/(A)_0 = 1 - 12.5(G)$.

The competition between glycine and the desired product, glycylglycine, for reaction with the N-carboxyanhydride can be estimated more reliably and is more serious. We have observed that because of the lower basicity of glycylglycine (pK_2 8.86) in comparison to glycine (pK_2 10.53), the dipeptide appears to be the stronger competitor toward the anhydride when examined in the buffer of pH 4.75. This situation should be reversed at pH 10–11, where both amino compounds are largely in their reactive forms, but the ratio between the rates of formation of diglycine and triglycine can never exceed 2330/311 (= 7.5) times the ratio of glycine and diglycine concentrations present (Table III). For this reaction to be useful, it would be necessary that we start with equivalent amounts of glycine (or other desired starting material) and N-carboxyanhydride. The concentration of diglycine in this example would start at zero and would approach a concentration whose ratio to that of glycine was equal to k_G/k_{GG} the ratio of the two rate constants. At the time of total consumption of the anhydride, an amount of tripeptide would have been formed which was equal to that of the glycine remaining unattacked, in this case 12% of the total hydrolysis being neglected. For higher peptides, where the differences between members of the series become less, the outlook is correspondingly less encouraging.

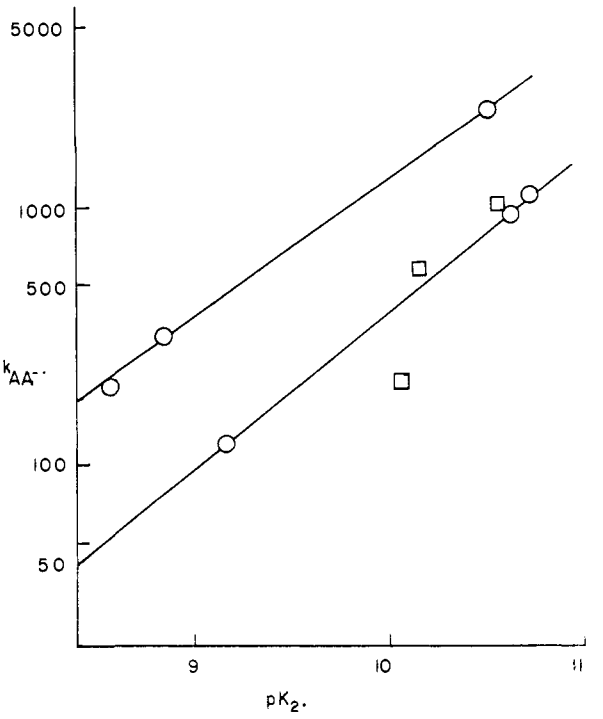


Fig. 1.—Logarithmic relationship between k_{AA^-}, the second-order rate constant for reaction in water at 0° between an anhydro-N-carboxy-amino-acid and an amino acid or peptide anion, and pK_2, the second ionization constant of the amino acid: upper line, anhydro-N-carboxyglycine with triglycine, glycylglycine and glycine anions; lower line, anhydro-N-carboxyalanine with alanylalanine, alanine and leucine (see Table III); square points, data from Part II, THIS JOURNAL, 79, 2159 (1957): phenylalanine, methionine, aspartic acid.

The experiments on peptide synthesis in water bear out these conclusions in general. Quantitative experiments were made in which, in each of a series of solutions of different pH, equimolar amounts of glycine and anhydro-N-carboxyglycine were introduced and the reaction was allowed to run to completion. The remaining glycine was then determined by the Van Slyke ninhydrin technique, by which amino acid can be estimated in the presence of peptides which do not evolve carbon dioxide on treatment with the reagent. Table IV lists the results obtained.

In a reaction yielding only hydrolysis and dipeptide formation there will be two molecules of amino acid remaining in excess for each anhydride molecule hydrolyzed, for this reaction not only wastes anhydride but adds to the store of amino acid. In a reaction yielding only di- and tripeptide, the amount of tripeptide will be equal to the amount of amino acid remaining unattacked. In Table IV the yield of the desired dipeptide has been computed on both assumptions and recorded in the last two columns.

Two solutions in which the pH was controlled by borate buffers at pH's of 10.89 and 10.61 gave results in the expected range. The above considerations having shown that competition from tr peptide formation is more important in this p

range than loss by hydrolysis, the figures of 68 and 83% are the most likely ones for the yields of dipeptide. Solutions where similar *p*H's were maintained by means of carbonate buffers showed large amounts of glycine remaining at the end of the run. The significant difference may well be the presence of carbon dioxide at sufficient concentration to convert an appreciable fraction of the glycine into the related N-carboxylate. This would lower the over-all ability of the glycine to compete for the anhydride, relative to hydroxyl ion. We do not know the position of the carbonation equilibrium for glycylglycine, but in accord with its lower basicity it is possible that this dipeptide should be less carbonated and hence the carbonate buffer might well affect the relative rates of formation of di- and tripeptide as well as the relative rates of reaction with glycine and hydroxyl ion. This explanation is borne out by some experiments in which anhydro-N-carboxyglycine was allowed to react directly in the buffer solutions without added glycine. Column 3 of Table IV shows that the ability of the glycine formed by hydrolysis to react further with the anhydride is much reduced in the carbonate buffers relative to the buffers containing borate.

The two experiments using barium hydroxide, in catalytic amounts and in excess, gave results as expected from the above kinetic discussion. With the small amount of base, the *p*H began at 9.5 and dropped. Polymerization was so important that only 2% of amino acid remained in the control experiment without added glycine. Obviously here the formation of tri- and higher peptides is the important side reaction and the yield of dipeptide from the 1:1 reaction mixture is zero, as indicated in column 5. In 0.174 *N* barium hydroxide, anhydride alone yields 89% of glycine. Hydrolysis is therefore rapid, as expected, and the results from the 1:1 experiment mean a 31% yield of diglycine. The upshot of these experiments is that the conditions for selective formation of glycylglycine are rather critical, and the selectivity is not sufficient to make this a useful general method for the stepwise synthesis of polypeptides.

Experimental

Reagents.—Various methods were used for preparing the Leuchs anhydrides but by far the most satisfactory was the method of Ben-Ishai and Katchalski[10] using the N-carbobenzoxy-amino acids and phosphorus tribromide. The purity of the anhydrides was best determined by the quantitative measurement of the carbon dioxide liberated. This was done during each kinetic run and consistent values were obtained from the various samples of anhydrides used. The anhydrides were stored in a desiccator over phosphorus pentoxide, being stable under these conditions for several weeks. In practice, however, the anhydrides were used within three days of the final recrystallization.

Benzyl chloroformate was obtained from three sources. It was prepared[11] by the method of reference 11, some was purchased from the Mann Research Laboratories and some was kindly donated by Dr. E. R. Blout.

N-Carbobenzoxyglycine was prepared from benzyl chloroformate and glycine by the method of reference 11.

N-Carbomethoxyglycine was prepared by the method described[12] by Schramm.

N-Carbethoxyglycine was prepared from ethyl chloroformate and glycine in a procedure similar to that used for N-carbomethoxyglycine; the melting point of the crude material was 74–78°.

Anhydro-N-carboxyglycine was prepared by the method of Katchalski. The crude product was recrystallized four or five times from dry ethyl acetate. Good yields of the anhydride were also obtained with 20.9 g. of N-carbobenzoxyglycine and 25 g. of phosphorus pentachloride dissolved in 150 ml. of dry ethyl acetate at 0°. The mixture was shaken every two or three minutes and the temperature maintained at 0° for 0.5 hr. The solution was allowed to warm to room temperature and was filtered to remove excess phosphorus pentachloride. The ethyl acetate was removed under vacuum at temperatures up to 60°. After the removal of most of the ethyl acetate, the residue was recrystallized from ethyl acetate.

N-Carbomethoxy-α-aminoisobutyric acid was prepared by the method of Schramm.

Anhydro-N-carboxy-α-aminoisobutyric acid was made from N-carbomethoxy-α-aminoisobutyric acid and thionyl chloride, according to the method of Schramm. The product was recrystallized six times from a benzene–cyclohexane mixture.

N-Carbomethoxy-*dl*-alanine, m.p. 75–77°, was prepared according to the method of Schramm.

N-Carbobenzoxy-*dl*-alanine, N-carbobenzoxy-*d*-alanine and N-carbobenzoxy-*l*-alanine were prepared in 90% yield by the "Organic Syntheses" procedure, described for N-carbobenzoxyglycine. The product was recrystallized by dissolving in chloroform and adding petroleum ether. The melting points were 111–115°, 85.2–86.0° and 85.0–85.5°, respectively.

Anhydro-N-carboxy-*dl*-alanine was prepared from N-carbobenzoxy-*dl*-alanine and phosphorus pentachloride, following Schramm's directions for the preparation using N-carbomethoxy-*dl*-alanine. The product was recrystallized several times from dry benzene, although it was necessary to seed each time. Several attempted preparations from the N-carbomethoxy-amino acid were unsuccessful, as was the attempted use of thionyl chloride or phosphorus tribromide on the N-carbobenzoxy-*dl*-alanine.

Anhydro-N-carboxy-*d*-alanine and anhydro-N-carboxy-*l*-alanine were prepared easily by the method of Katchalski from the corresponding N-carbobenzoxyalanine and phosphorus tribromide. The product was recrystallized three or more times from dry benzene. It was occasionally necessary to add dry petroleum ether to cause crystallization of the anhydrides.

Benzene, ether and petroleum ether (30–60°) were reagent grade dried over sodium wire.

Cyclohexane was Eastman Kodak Co. technical grade distilled and dried over sodium wire.

Reagent quality ethyl acetate was dried over calcium hydride and distilled.

Glycine and *dl*-alanine were obtained from the Eastman Kodak Co. and were twice recrystallized from ethyl alcohol–water solution, before being used in kinetic runs. These and all the other amino acids and peptides were dried at 110° before use.

α-Aminoisobutyric acid and *l*-leucine were obtained from Eastman Kodak Co. and were recrystallized once from an ethyl alcohol–water solution before being used in kinetic runs.

d-Alanine was obtained from Schwartz Laboratories and was used without further purification; $\alpha^{25}D$ −14.8 (c = 2 in 2 *N* hydrochloric acid).

l-Alanine was obtained from Schwartz Laboratories, $\alpha^{25}D$ +14.3° (c = 2 in 2 *N* hydrochloric acid).

Glycylglycine was obtained from the Nutritional Biochemical Corporation and was recrystallized from 50% ethyl alcohol before use.

Glycylglycylglycine was obtained from the Nutritional Biochemical Corporation and was recrystallized from water.

dl-Alanyl-*dl*-alanine was obtained from the Nutritional Biochemical Corporation and was used without further purification.

Kinetic Procedure.—The reaction vessel was a 250-ml. erlenmeyer flask equipped with a side arm to which a mercury manometer could be attached through a 10/30 joint. The mercury manometer was made of 1-mm. capillary tubing with a reservoir made of 20-mm. tubing, so that the change in height in the reservoir would be negligible during

(10) E. Ben-Ishai and E. Katchalski, THIS JOURNAL, **74**, 3688 (1952).

(11) *Org. Syntheses*, **23**, 13 (1943).

(12) C. H. Schramm, Thesis, Harvard University, 1947.

the course of a run. Evacuation was accomplished through a stopcock attached to the arm between the flask and the manometer. The erlenmeyer flask was closed with a 24/40 cap equipped with a glass hook from which a glass bucket containing the anhydro-N-carboxy-amino acid could be suspended by a platinum wire.

First the weighed amino acid or peptide, when any was used, was added to the erlenmeyer flask, followed by the water or buffered solution (50 ml. for the runs with optically active anhydro-N-carboxy-alanine and 100 ml. in all other cases). The system was assembled using silicone stopcock grease, and the reaction flask was immersed in an ice-bath for 1 hr. before the start of the run. The ice-bath, stirred by air and surrounded by two concentric beakers, was so arranged as to permit magnetic stirring through the bottom by means of an Arthur H. Thomas Co. magnetic stirrer and a plastic coated magnet within the reaction flask.

After the attainment of thermal equilibrium, a weighed amount of the anhydride was suspended from the cap and the system was evacuated. After three or four minutes, the anhydride was dropped into the solution by agitation of the reaction vessel and the run was begun. The manometer was always tapped immediately before reading and its response characteristics were determined before use. The pressure at infinite time was that observed after at least ten times the half-life.

In studying the decomposition of the Siegfried salts, a solution of amino acid in barium hydroxide was placed in the reaction flask and carbon dioxide was bubbled through the solution until added phenolphthalein turned colorless. In this instance, acetic acid or sulfuric acid was suspended in the glass bucket and was dropped into the basic solution at zero time to start the reaction.

For the kinetic experiments using the volume method, the manometer was replaced by a connection to a 50-ml. gas buret and a butyl phthalate manometer. The connection was made almost completely from glass with two short joints of Tygon tubing.

Product Studies.—Exactly 5 ml. of 0.400 molar glycine and 50 ml, of an appropriate buffer were placed in a stoppered 250 ml. erlenmeyer flask and cooled to 0°. Exactly 0.202 g. (0.002 mol.) of anhydro-N-carboxy-glycine was added, and the mixture was rapidly stirred with a magnetic stirrer. After several hours the solution was neutralized to *p*H 7.0 (brom thymol blue) with dilute hydrochloric acid and quantitatively transferred to a 250 ml. volumetric flask. The solution was acidified with 50 ml. of *p*H 4.5 acetate buffer (1 *M*) and diluted with water to 250 ml. A sample of the solution (5 ml.) was analyzed by the Van Slyke ninhydrin method. Acetate buffer was used instead of citrate buffers since all the kinetic runs that were analyzed were already buffered with acetate. The procedure used did not give quantitative yields of carbon dioxide but was helpful as a qualitative measure of the amino acid concentration. Thus standard alanine solutions yielded 98 ± 1% of the calculated carbon dioxide, whereas standard glycine solutions yielded 92 ± 3%. Glycylglycylglycine, when analyzed by this method, gave 0.074 mole of glycine per mole of glycylglycylglycine.

CAMBRIDGE, MASSACHUSETTS

[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

A Kinetic Study of the Leuchs Anhydrides in Aqueous Solution. II[1]

BY PAUL D. BARTLETT AND DONALD C. DITTMER

RECEIVED SEPTEMBER 7, 1956

Rate constants are reported for the reaction in water at 0° between anhydro-N-carboxy-DL-alanine and DL-phenylalanine, DL-methionine, DL-aspartic acid, L-histidine, L-cysteine and glutathione. Cysteine reacts 45 times as fast as methionine. In the reaction of cysteine with anhydro-N-carboxy-DL-phenylalanine, the concentration of free SH groups shows an initial decline followed by a return to its original value. This is interpreted as an initial reaction of the anhydride with the thiol group followed by an intramolecular transfer of the phenylalanyl group from sulfur to nitrogen. Glutathione shows a similar rapid reaction, but the thiol titer drops much more and is slow to return, as would be expected from the less favorable location of groups for an intramolecular acylation. The effect of conditions upon the reaction of alanine with both anhydro-N-carboxy-DL-alanine and anhydro-N-carboxy-DL-phenylalanine in aqueous solution has been studied.

It was shown in Part I[2] that the anhydro-N-carboxy derivatives of certain amino acids reacted in aqueous solution with amino groups so rapidly as to raise the hope that this might be a useful method for controlled, stepwise peptide synthesis. In a further exploration of systems of this kind, we have measured the rate constants listed in Table I between anhydro-N-carboxy-DL-alanine and amino acids of different types. The rate constants were determined as described in Part I.[2] An attempt has been made in Table I, as in the cases previously reported, to refer the rate constants to the active anion of the amino acid by means of the published second ionization constants of the amino acids in question.

Table I shows that cysteine and glutathione react substantially faster with anhydro-N-carboxy-alanine than does the closely comparable methionine which lacks the sulfhydryl group. Some experiments were carried out in which the sulfhydryl group was titrated with iodine during the reaction between cysteine and anhydro-N-carboxy-DL-phenylalanine. The results are shown in Fig. 1, together with the results of a similar run on the tripeptide glutathione. In both cases there is an initial attack of the carboxy-anhydride on the sulfhydryl group. In the case of cysteine, the sulf-

TABLE I

REACTIONS OF ANHYDRO-N-CARBOXY-DL-ALANINE AT 0° IN ACETATE BUFFER (0.102 *M* NaOAc, 0.0875 *M* HOAc) AT *p*H 4.75

Added compound	k_1, sec.$^{-1}$ × 10^3	k_{AA} l./mole sec. × 10^3	pK_2[c]	k_{AA^-}, l./mole sec.
None	0.715			
DL-Phenylalanine	.772	1.0	10.06	200[d]
DL-Methionine	.988	2.3	10.14	565[d]
DL-Aspartic acid	.837	1.4	10.53	840[d]
L-Cysteine	14.8[a]	103	9.11	1770
L-Histidine	6.25	61[b]	10.10	1370
Glutathione	2.83	68	9.59	4640

[a] Did not show clean first-order kinetics. [b] *p*H increased during run. [c] E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 84 ff., assuming same temperature coefficient as for glycine and alanine. [d] Plotted in Fig. 1, Part I, THIS JOURNAL, 79, 2153 (1957).

(1) This work was supported by the Office of Naval Research under Contract No. N5ori-07653, Task 53, with Harvard University, 1953–1954.

(2) P. D. Bartlett and R. H. Jones, THIS JOURNAL, 79, 2153 (1957).