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]

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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).

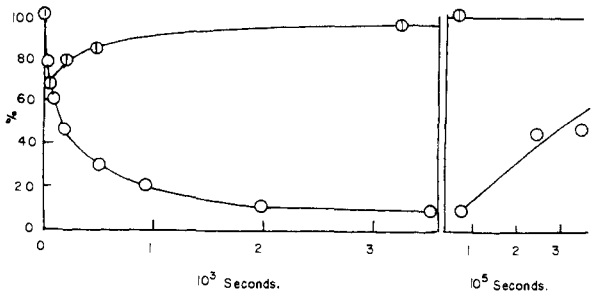

Fig. 1.—Decline and return of sulfhydryl titer during reaction of anhydro-N-carboxy-*dl*-phenylalanine with cysteine (dashed circles) and with glutathione (open circles). Note break in time scale.

hydryl titer had dropped at the end of one minute to 67% of that initially present but had already begun to rise again. The rapid formation of thioester evidently is followed up immediately by a transfer of the acyl group from sulfur to nitrogen with the formation of a normal peptide. Such transfer reactions have been observed before,[3] being a consequence of the reactivity of thioesters and the fact that such a transfer can take place here by way of a six-membered cyclic transition state.

In the case of glutathione the sulfhydryl titer also drops but does not return nearly so rapidly. At the end of an hour the sulfhydryl titer has reached 9% of that initially present and appears to be still dropping. After 20 hr. the titer is again 9%; after three days it has risen again to 45%. The difference between the cases of cysteine and glutathione would appear to be largely the less favorable situation in the latter case for the transfer of an alanyl group from sulfur to nitrogen internally.

The attempts reported in Part I to achieve high yields in the formation of specific peptides were extended to the use of anhydro-N-carboxy-DL-alanine and anhydro-N-carboxy-DL-phenylalanine. As judged by the amount of amino acid remaining after reaction, the yields obtained from these anhydrides and alanine were never as high as the best yields of glycylglycine under comparable conditions. Experiments were conducted at *p*H 9, 10, 11 and 12, the *p*H being generally controlled by the use of a Beckman auto-titrator. With anhydro-N-carboxy alanine as with anhydro-N-carboxyglycine, the highest yields of dipeptide were obtained at *p*H 10, but these yields never reached 90%. The presence or absence of a borate or phosphate buffer and the use of lithium, sodium or thallium hydroxide as the titrating base produced no marked effect upon the course of the reaction. In the case of anhydro-N-carboxy-*dl*-phenylalanine the yields were consistently below 80% and the sensitivity to *p*H appeared to be somewhat less. These results are all consistent with the view that the aqueous reactions of the anhydro-N-carboxyamino acids are determined by a set of protonation and carboxylation equilibria which are established rapidly compared to the speed of the attack of the water or amino acids upon the anhydride.

(3) T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang and H. Lau, *Ann.*, **583**, 129 (1953).

Experimental

Materials.—DL-Alanine, DL-methionine and DL-aspartic acid were Eastman Kodak Co. White Label grades. DL-Phenylalanine was the C.P. grade obtained from H. M. Co., Ltd. L-Cysteine, glutathione and L-histidine were from the Nutritional Biochemicals Corporation. Benzyl chloroformate was from Mann Biochemicals. N-Carbobenzoxy-DL-alanine and N-carbobenzoxy-DL-phenylalanine were prepared by the method of reference 4. Anhydro-N-carboxy-DL-alanine and anhydro-N-carboxy-DL-phenylalanine were prepared from the corresponding N-carbobenzoxy derivatives and phosphorus tribromide by the method of Ben-Ishai and Katchalski.[5] They were recrystallized from ethyl acetate and petroleum ether. Ethyl acetate was Merck reagent grade dried over calcium hydride.

The manometric kinetic determinations were carried out as described in Part I.

The reactions of L-cysteine and glutathione with anhydro-N-carboxy-DL-phenylalanine were done in an acetic acid-sodium acetate buffer (*p*H 4.66) at 0°. The anhydride was added in 10 ml. of dimethylformamide. Aliquots were taken at various times, quenched in 2 *N* hydrochloric acid and cooled in an ice-bath. A measured amount of standard iodine solution was added to react with the excess SH groups and the excess of iodine was back titrated with standard sodium thiosulfate solution using starch as an indicator. There was no reaction between cysteine and dimethylformamide under the reaction conditions employed. The accuracy of the glutathione reactions was impaired by precipitation early in the reaction.

The solution of the product of reaction between L-cysteine and anhydro-N-carboxy-DL-phenylalanine was oxidized with hydrogen peroxide. The resulting material was submitted to paper chromatography with butanol–acetic acid[6] resulting in three ninhydrin-positive spots. Two of these readily were identified as DL-phenylalanine and cysteic acid. From a large number of paper chromatograms carried out on small aliquots of the solution, the area of the unknown spot was cut out and eluted. The solution of the unknown was evaporated and the α-amino group of the peptide was removed with nitrosyl chloride.[7] The product was then hydrolyzed and chromatographed on paper. One ninhydrin-positive spot appeared which had the same R_f value as cysteic acid. The original dipeptide was accordingly probably DL-phenylalanylcysteine.

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(5) E. Ben-Ishai and E. Katchalski, THIS JOURNAL, **74**, 688 (1952).

(6) K. Slotta and J. Primosigh, *Nature*, **168**, 696 (1951).

(7) R. Consden, A. Gordon and A. Martin, *Biochem. J.*, **41**, 590 (1947); F. Sanger and H. Tuppy, *ibid.*, **49**, 463 (1951).