[CONTRIBUTION NO. 1676 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The α-Chymotrypsin Catalyzed Synthesis of the Phenylhydrazides of Certain Acylated α-Amino Acids[1]

BY WALTER HARRY SCHULLER AND CARL NIEMANN[2]

RECEIVED APRIL 14, 1952

It has been found that the optimum *p*H for the α-chymotrypsin catalyzed synthesis of benzoyl-L-tryptophanyl-, benzoyl-L-tyrosyl- and benzoyl-L-phenylalanylphenylhydrazide from the corresponding acylated α-amino acids and phenylhydrazine appears to be determined in part by the nature of the α-amino acid side chain present in the anionic substrate and in part by the nature of the anionic component of the buffer system. For the above three anionic substrates and for 0.5 *M* acetate and 0.5 *M* citrate buffers the optimum *p*H is in the region between *p*H 5.5 and 6.5. A number of essentially qualitative experiments which indicate the scope of the above reaction are also described.

The α-chymotrypsin catalyzed synthesis of a peptide, or peptide-like, bond was first reported by Bergmann and Fruton[3] who noted that when an aqueous solution of sodium benzoyl-L-tyrosinate and glycinanilide acetate of *p*H 7.5 was incubated with α-chymotrypsin at 37.5°, benzoyl-L-tyrosylglycinanilide precipitated from the reaction mixture in an amount equivalent to a yield of 31%. In a subsequent communication,[4] in which no experimental details were given, a similar synthesis, *i.e.*, of benzoyl-L-tyrosyl-L-leucinanilide, from benzoyl-L-tyrosine and L-leucinanilide was described. In contrast to the above, it was stated[3] that no benzoyl-L-tyrosinanilide was precipitated when a mixture of benzoyl-L-tyrosine and aniline was subjected to presumably the same treatment as that accorded the mixture of benzoyl-L-tyrosine and glycinanilide.

In 1949 it was noted in these laboratories[5] that benzoyl-L-tyrosylphenylhydrazide was precipitated in low yields when an aqueous solution of sodium benzoyl-L-tyrosinate was treated with phenylhydrazine acetate in the presence of α-chymotrypsin, and a study was undertaken to explore more fully the characteristics of this reaction. Shortly thereafter, Tauber[6] reported that an insoluble protein-like substance was formed when an aqueous solution of Witte peptone was treated with α-chymotrypsin at *p*H 7.0, and subsequently this investigator made similar observations with respect to the α-chymotrypsin catalyzed synthesis of protein-like products from the peptic digests of a number of proteins.[7] During the same period, Brenner, *et al.*,[8-10] and Fruton, *et al.*,[11-13] clearly demonstrated the existence of several α-chymotrypsin catalyzed transacylation and transamidation reactions, which not only confirmed an earlier speculation on an alternative mode of peptide bond synthesis,[14,15] but which was also of considerable interest to the immediate problem under investigation. The most recent observation made in this area is that of Tauber,[16] who has noted that the α-chymotrypsin catalyzed synthesis of L-phenylalanyl-L-phenylalanine ethyl ester from L-phenylalanine ethyl ester appears to give a maximum yield of the above product at about *p*H 8.6 at 37°.

Reports that the optimum *p*H for the papain catalyzed synthesis of acylated α-amino acid anilides appeared to vary with the nature of the anionic substrate[17-20] encouraged us to investigate in detail this aspect of the α-chymotrypsin catalyzed synthesis of acylated α-amino acid phenylhydrazides. Since it was known that the optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of several acylated-L-phenylalaninamides, in a 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, was 7.9 ± 0.1 at 25°,[21] six acylated L-phenylalanines, *i.e.*, acetyl-, benzoyl-, carboethoxy-, carbobenzoxy-, benzenesulfonyl- and phenylcarbamyl-L-phenylalanine, were allowed to react, at 35° and *p*H 7.8 ± 0.1, with either phenylhydrazine or *p*-toluidine in the presence of α-chymotrypsin and the above buffer system. In no case was the formation of a phenylhydrazide or *p*-toluide observed. From these negative results and the earlier experience with benzoyl-L-tyrosine and phenylhydrazine[5] it was concluded that phenylhydrazide formation was more likely to be observed at lower *p*H values. Therefore three benzylated α-amino acids, *i.e.*, benzoyl-L-tryptophan, benzoyl-L-tyrosine and benzoyl-L-phenylalanine were selected for study and the extent of phenylhydrazide formation with each of these anionic substrates was determined using a variety of buffers which individually or collectively allowed observations to be made from *p*H 4 to 7. The results of this investigation are summarized in Figs. 1 to 3.

Due to the fortunate circumstance that a sodium citrate–citric acid buffer can be used over a rela-

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **124**, 321 (1938).

(4) M. Bergmann and J. S. Fruton, *Ann. N. Y. Acad. Sci.*, **45**, 409 (1944).

(5) Unpublished observation of Dr. R. V. MacAllister.

(6) H. Tauber, THIS JOURNAL, **71**, 2952 (1949).

(7) H. Tauber, *ibid.*, **73**, 1288, 4965 (1951).

(8) M. Brenner, H. R. Muller and R. W. Pfister, *Helv. Chim. Acta*, **33**, 568 (1950).

(9) M. Brenner and R. W. Pfister, *ibid.*, **34**, 2085 (1951).

(10) M. Brenner, E. Sailer and K. Rufenacht, *ibid.*, **34**, 2096 (1951).

(11) J. S. Fruton, *Yale J. Biol. Med.*, **22**, 263 (1950).

(12) R. B. Johnson, M. J. Mycek and J. S. Fruton, *J. Biol. Chem.*, **187**, 205 (1950).

(13) J. S. Fruton, R. B. Johnson and M. Fried, *ibid.*, **190**, 39 (1951).

(14) M. Bergmann and H. Fraenkel-Conrat, *ibid.*, **119**, 707 (1937).

(15) M. Bergmann and C. Niemann, *Science*, **86**, 187 (1937).

(16) H. Tauber, THIS JOURNAL, **74**, 847 (1952).

(17) S. W. Fox and C. W. Pettinga, *Arch. Biochem.*, **25**, 13 (1950).

(18) S. W. Fox, C. W. Pettinga, J. S. Halverson and H. Wax, *ibid.*, **25**, 21 (1950).

(19) S. W. Fox and H. Wax, THIS JOURNAL, **72**, 5087 (1950).

(20) N. F. Albertson, *ibid.*, **73**, 452 (1951).

(21) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).

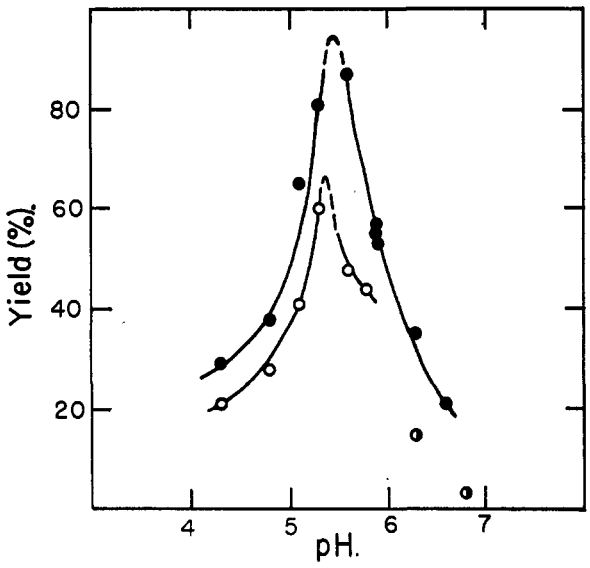

Fig. 1.—Yield of benzoyl-L-tryptophanylphenylhydrazide: ●, 0.5 *M* sodium citrate buffer; ○, 0.5 *M* sodium acetate buffer; ◑, 0.5 *M* sodium phosphate buffer.

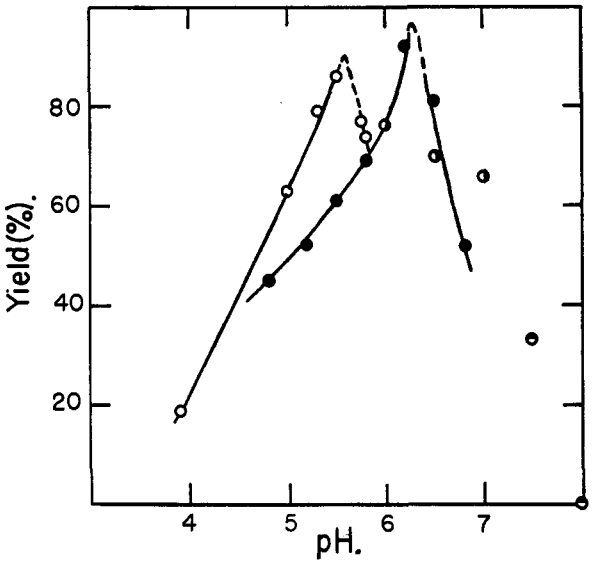

Fig. 2.—Yield of benzoyl-L-tyrosylphenylhydrazide: ●, 0.5 *M* sodium citrate buffer; ○, 0.5 *M* sodium acetate buffer; ◑, 0.5 *M* sodium phosphate buffer; ◓, 0.1 *M* sodium tetraborate–0.2 *M* potassium dihydrogen phosphate buffer.

tively wide *p*H range, it was possible to determine the apparent optimum *p*H for the α-chymotrypsin catalyzed synthesis of the phenylhydrazides of all three of the above anionic substrates in a single buffer system, *i.e.*, a uni-polyvalent 0.5 *M* citrate buffer. In this buffer system the optimum *p*H for the α-chymotrypsin catalyzed synthesis of benzoyl-L-tyrosylphenylhydrazide, *i.e.*, 6.3 ± 0.1, is identical, within the limits of experimental error, with the optimum *p*H for the α-chymotrypsin catalyzed synthesis of benzoyl-L-phenylalanylphenylhydrazide, *i.e.*, 6.2 ± 0.1. However, the optimum *p*H for the α-chymotrypsin catalyzed synthesis of benzoyl-L-tryptophanylphenylhydrazide, *i.e.*, 5.5 ± 0.1, is clearly in a more acid region. Thus in this buffer system it is seen that the optimum *p*H for phenylhydrazide formation may vary with the nature of the α-amino acid side chain present in the anionic substrate.

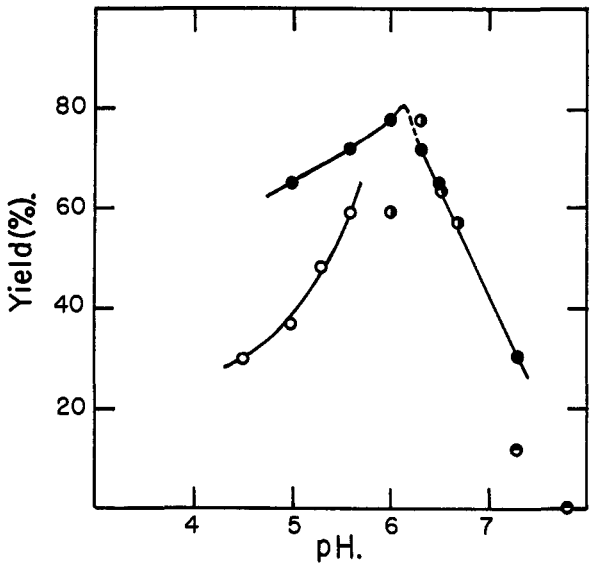

Fig. 3.—Yield of benzoyl L-phenylalanylphenylhydrazide: ●, 0.5 *M* sodium citrate buffer; ○, 0.5 *M* sodium acetate buffer; ◑, 0.5 *M* sodium phosphate buffer; ◓, 0.1 *M* sodium tetraborate–0.2 *M* potassium dihydrogen phosphate buffer.

In a 0.5 *M* uni-univalent acetate buffer the optimum *p*H for the α-chymotrypsin catalyzed synthesis of benzoyl-L-tryptophanylphenylhydrazide, *i.e.*, 5.4 ± 0.1, is identical, within the limits of experimental error, with that observed in the 0.5 *M* citrate buffer, *i.e.*, 5.5 ± 0.1. However, when the same comparison is made with respect to the α-chymotrypsin catalyzed synthesis of benzoyl-L-tyrosylphenylhydrazide it is seen that in this case the optimum *p*H in the 0.5 *M* acetate buffer, *i.e.*, 5.6 ± 0.1, is approximately 0.7 of a *p*H unit lower than the optimum *p*H in the 0.5 *M* citrate buffer. While it is not possible to specify the optimum *p*H for the α-chymotrypsin catalyzed synthesis of benzoyl-L-phenylalanylphenylhydrazide in the 0.5 *M* acetate buffer because of the lack of sufficient data it appears that in this case the optimum *p*H may also be lower than that observed in the 0.5 *M* citrate buffer. Thus it appears that in the α-chymotrypsin catalyzed synthesis of acylated α-amino acid phenylhydrazides from the corresponding acylated α-amino acids and phenylhydrazine the optimum *p*H may in part be determined by the nature of the α-amino acid side chain of the anionic substrate and in part by the nature of the anionic component of the buffer system. It is not obvious whether this latter phenomenon is to be associated with a specific ion effect or to a difference in ionic strengths, or is due to the concerted action of both of these factors. Although there is an indication that a change from the uni-univalent acetate buffer to the uni-polyvalent citrate buffer has the effect of shifting the optimum *p*H to higher *p*H values the generality of this conclusion cannot be regarded as being established.

It is of interest to note that in the two cases where a comparison is possible, *i.e.*, with benzoyl-

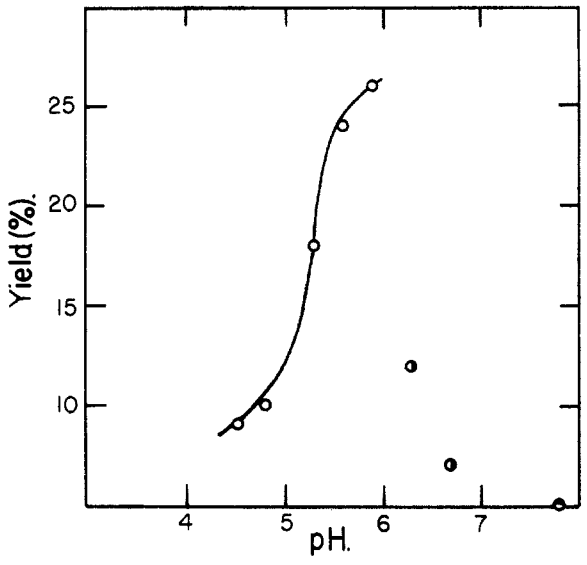

Fig. 4.—Yield of carbobenzoxy-L-phenylalanylphenylhydrazide: ○, 0.5 *M* sodium acetate buffer; ◑, 0.5 *M* sodium phosphate buffer; ◓, 0.1 *M* sodium tetraborate —0.02 *M* potassium dihydrogen phosphate buffer.

L-tryptophanylphenylhydrazide and benzoyl-L-tyrosylphenylhydrazide, a change from the 0.5 *M* acetate buffer to the 0.5 *M* citrate buffer, with each system adjusted to the optimum *p*H for the buffer and the anionic substrate, has the effect of markedly increasing the yield of the former compound whereas with the latter the increase in yield is hardly significant.

While 11 experiments were conducted with a 0.5 *M* phosphate buffer and five with a mixed borate–phosphate buffer the information obtained from these experiments is not sufficiently extensive to afford any conclusion other than the inference that the optimum *p*H appears to lie in the same general region as that observed with the acetate and citrate buffers.

The fact that the optimum *p*H for achieving a maximum yield of all three acylated α-amino acid phenylhydrazides appears to lie in the region between *p*H 5.5 and 6.5, for all buffer systems investigated, is somewhat surprising for in the α-chymotrypsin catalyzed hydrolysis of a number of acylated α-amino acid amides the optimum *p*H is in every case within the region between *p*H 7.0 and 8.5.[21-26] Whether this phenomenon is simply due to a difference in the behavior of amides and phenylhydrazides in both the synthetic and hydrolytic reactions, or whether it is to be ascribed to a fundamental difference in the mode of action of α-chymotrypsin in hydrolytic and synthetic reactions cannot be answered at present.

In a separate series of experiments an attempt was made to determine the optimum *p*H for the α-chymotrypsin catalyzed synthesis of carbobenzoxy-L-phenylalanylphenylhydrazide in a 0.5 *M* acetate buffer. As with benzoyl-L-phenylalanylphenylhydrazide sufficient data were not collected to permit the construction of a satisfactory yield *versus* *p*H curve. However it may be inferred from the data given in Figs. 3 and 4 that the optimum *p*H for the system in question is not very far from *p*H 6.0, *i.e.*, still within the region observed for the other synthetic reactions reported in this communication.

The second part of this investigation was principally qualitative in character and had for its purpose a limited exploration of the scope of the α-chymotrypsin catalyzed synthesis of acylated α-amino acid phenylhydrazides from the corresponding acylated α-amino acids and phenylhydrazine. In the first series of experiments six acylated-L-phenylalanines and three acylated-D-phenylalanines were allowed to react with phenylhydrazine at 35° in the presence of α-chymotrypsin and a 0.5 *M* citrate buffer adjusted to *p*H 6.0, *cf.* Tables I and II. While the α-chymotrypsin catalyzed synthesis of acetyl-, benzoyl-, carboethoxy- and carbobenzoxy-L-phenylalanylphenylhydrazide was expected on the basis of previous hydrolytic studies with this enzyme[21-26] the formation of benzenesulfonyl- and phenylcarbamyl-L-phenylalanylphenylhydrazide clearly indicates that the nature of the acyl moiety of the anionic substrate can be varied over rather wide limits in both the hydrolytic and synthetic reactions. The negative results obtained with benzoyl-, carbobenzoxy- and phenylcarbamyl-D-phenylalanine, *cf.* Table I, are particularly interesting because with the corresponding L-acids the phenylhydrazides were obtained in yields in excess of 79%. The fact that two of the above D-acids, *i.e.*, benzoyl- and carbobenzoxy-D-phenylalanine, were converted into the corresponding phenylhydrazides in the papain catalyzed reaction[27] is important in that it serves to emphasize an additional point of distinction between the mode of action of α-chymotrypsin and that of cysteine activated papain.

TABLE I

α-CHYMOTRYPSIN CATALYZED SYNTHESIS OF VARIOUS ACYLATED PHENYLALANYLPHENYLHYDRAZIDES AT 35° AND *p*H 6.0[a]

Anionic substrate			Base[d]	Time,	Yield,[e]
Phenylalanine	Concn.[b]	Amount[c]	concn.[b]	hr.	%
Acetyl-L-	100	0.5	400	132	6.7
Benzenesulfonyl-L-	5.6	.5	22.4	153	2.0[g]
Carboethoxy-L-	58.8	.5	235	130	6.7
Phenylcarbamyl-L-	8.3	.5	33.2	108	103
Phenylcarbamyl-D-	8.3	.5	33.2	360	0[h]
Benzoyl-L-	14.3	.25	28.6	105	79
Benzoyl-D-	14.3	1.0	28.6	360	0
Carbobenzoxy-L-[f]	2.0	0.25	4.0	119	26
Carbobenzoxy-L-	2.9	.25	11.6	119	82
Carbobenzoxy-D-	2.9	.75	11.6	360	0

[a] In a sodium citrate buffer 0.5 *M* in citrate unless otherwise noted. [b] In millimole per liter. [c] Actual amount in millimole. [d] Phenylhydrazine. [e] Of acylated phenylalanylphenylhydrazide, *cf.* Table II for properties. [f] In a sodium acetate buffer 0.5 *M* acetate. [g] Yield calculated on basis of weight of once recrystallized product. [h] *Cf.* Exptl. section.

(22) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(23) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(24) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

(25) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).

(26) H. J. Shine and C. Niemann, *ibid.*, **74**, 97 (1952).

(27) W. H. Schuller and C. Niemann, *ibid.*, **73**, 1611 (1951).

TABLE II

PROPERTIES OF ACYLATED α-AMINO ACID PHENYLHYDRAZIDES OBTAINED FROM ENZYME EXPERIMENTS

Phenylhydrazide of	Melting point,[d] °C. Found	Lit.[e]	Mixed	$[\alpha]_D$[d]
Benzoyl-L-phenylalanine	215–216	215–216	215–216	
Carbobenzoxy-L-phenylalanine	175–175.5	175–176	175–175.5	
Acetyl-L-phenylalanine	204–205	205–206	205–206	
Benzenesulfonyl-L-phenylalanine	162–163	163–164	162–163	
Carboethoxy-L-phenylalanine	159.5–160	159.5–160	159.5–160	
Phenylcarbamyl-L-phenylalanine[a]	223.5–224.5	.....	.....	−23.8[f]
Benzoyl-L-tyrosine[b]	249.5–251	.....	.....	−60.2[g]
Benzoyl-L-tryptophan[c]	213–214.5	.....	.....	−32.1[h]

[a] *Anal.* Calcd. for $C_{22}H_{22}O_2N_4$: C, 70.6; H, 5.9; N, 15.0. Found: C, 70.6; H, 6.0; N, 14.9. [b] *Anal.* Calcd. for $C_{22}H_{21}O_3N_3$: C, 70.4; H, 5.6; N, 11.2 Found: C, 70.3; H, 5.8.; N, 11.1. [c] *Anal.* Calcd. for $C_{24}H_{22}O_2N_4$: C, 72.3; H, 5.6; N, 14.1. Found: C, 72.4; H, 5.5; N, 14.0. [d] Of product recrystallized from aqueous ethanol. [e] *Cf.* ref. 27. [f] *c* 5.92 in pyridine, *t* 23°. [g] *c* 7.1 in pyridine, *t* 24°. [h] *c* 2.74 in pyridine, *t* 25°.

The papain catalyzed synthesis of a *sym*-bisacylhydrazine was first demonstrated by Niemann and Nichols[28] and subsequently by two other groups of investigators.[29,30] In contrast to the above, when benzoyl-L-phenylalanine was allowed to react with hydrazine in the presence of α-chymotrypsin, and a 0.5 *M* citrate buffer adjusted to *p*H 6.0, no *sym*-bis-(benzoyl-L-phenylalanyl)-hydrazine was obtained. Negative results were also obtained when benzoyl-DL-alanine was allowed to react with phenylhydrazine in the presence of α-chymotrypsin and a series of 0.5 *M* citrate buffers adjusted to *p*H 3.5, 4.1, 4.5, 5.0, 5.5, 6.0 and 6.5, respectively. In a single experiment conducted in a 0.5 *M* citrate buffer of *p*H 6.0 the α-chymotrypsin catalyzed reaction of benzoyl-L-phenylalanine with *p*-toluidine gave a 16% yield of the corresponding toluide.

Experimental[31,32]

Anionic Substrates.—The preparation and properties of all of the acylated phenylalanines listed in Table I have been described previously.[27] Benzoyl-L-tryptophan was prepared from the amino acid *via* the conventional Schotten–Baumann procedure, and the crude product recrystallized from hot water to give benzoyl-L-tryptophan, m.p. 105–106° with preliminary softening at 98–99°, $[\alpha]^{26}D$ −37.6° (*c* 1.0 in acetone), $[\alpha]^{25}D$ −12.5° (*c* 3.8 in absolute ethanol); lit.[33] m.p. 104–105° with preliminary softening at 95°, $[\alpha]^{20}D$ −37° (*c* 1.0 in acetone). The same procedure gave benzoyl-L-tyrosine, m.p. 163–164°, $[\alpha]^{29}D$ 17.0° (*c* 5.2 in 0.2 *N* aqueous potassium hydroxide); lit.[34] m.p. 165–166°, $[\alpha]^{20}D$ 18.24° (*c* 5.2 in 0.2 *N* aqueous potassium hydroxide) and benzoyl-DL-alanine, m.p. 165–166°, lit.[35] m.p. 165–166°.

Enzyme Experiments.—For the experiments summarized in Tables I and III the acylated α-amino acids and recrystallized phenylhydrazine hydrochloride were weighed into stoppered erlenmeyer flasks and sufficient buffer added to dissolve these reactants. When solution was effected the *p*H of the solutions were adjusted by the addition of 10 *N* sodium hydroxide, or the acid component of the buffers, and the solutions made up to volume by the further addition of buffer. In every case care was taken to select flasks which minimized exposure of the solutions to the atmosphere. α-Chymotrypsin, Armour preparation, lot no. 90402, was then added in amount equal to 1 mg. per ml. of solution, and the resulting solutions incubated at 35° for the times indicated in Tables I and III. At the end of these periods the precipitates were collected on sintered-glass filters of medium porosity, the filters and their contents dried *in vacuo* over phosphorus pentoxide, and then weighed. The *p*H of the filtrates were determined and in no case were the values found to differ from the initial values by more than 0.1 of a *p*H unit. The precipitates remaining on the glass filters were extracted with hot ethanol until no significant amount of ethanol soluble material remained, whereupon the filters were again dried and weighed. Unless specified to the contrary the loss in weight upon ethanol extraction was taken as the yield of acylated amino acid phenylhydrazide. The ethanolic filtrates were freed of solvent and the crystalline residues were recrystallized from aqueous ethanol to give the phenylhydrazides listed in Table II. For those experiments where the incubation periods were greater than 150 hours it was found necessary to base yields on the amount of once recrystallized product actually isolated because in these cases substantial amounts of water-insoluble, ethanol-soluble oxidation products of phenylhydrazine were also formed. This was particularly true in the control experiments in which benzoyl-L-tryptophan, benzoyl-L-tyrosine and benzoyl-L-phenylalanine were incubated with phenylhydrazine in a 0.5 *M* citrate buffer adjusted to *p*H 6.0 under the same conditions specified in Table III except that no enzyme was added. After 200 hours at 35° a small amount of tarry material was obtained in all three cases but in no case could a crystalline product be obtained from the ethanolic solutions.

The attempted reaction of phenylcarbamyl-D-phenylalanine with phenylhydrazine in the presence of α-chymotrypsin, *cf.* Table I, resulted in the isolation of a water-insoluble,

TABLE III

*p*H *versus* YIELD EXPERIMENTS[a]

Buffer	Anionic substrate Nature	Concn.[b]	Amount[c]	Base[d] concn.[b]	Time,[e] hr.
Acetate[f]	Bz-L-Tr[i]	5.0	0.25	10.0	99
Citrate[g]	Bz-L-Tr[i]	5.0	.50	10.0	99
Phosphate[h]	Bz-L-Tr[i]	5.0	.25	10.0	99
Acetate[f]	Bz-L-Ty[k]	31.3	.25	62.6	96
Citrate[g]	Bz-L-Ty[k]	31.3	.50	62.6	96
Phosphate[h]	Bz-L-Ty[k]	31.3	.25	62.6	96
Borate[i]	Bz-L-Ty[k]	31.3	.25	62.6	96
Acetate[f]	Bz-L-Ph[l]	14.3	.50	28.6	105
Citrate[g]	Bz-L-Ph[l]	14.3	.25	28.6	105
Phosphate[h]	Bz-L-Ph[l]	14.3	.50	28.6	105
Borate[i]	Bz-L-Ph[l]	14.3	.50	28.6	105
Acetate[f]	Cbz-L-Ph[m]	2.0	.25	4.0	119
Phosphate[h]	Cbz-L-Ph[m]	2.0	.25	4.0	119

[a] Experimental conditions for experiments summarized in Figs. 1–4. [b] In millimole per liter. [c] Actual amount in millimole. [d] Phenylhydrazine. [e] At 35°. [f] Sodium acetate–acetic acid buffer 0.5 *M* in acetate. [g] Sodium citrate–citric acid buffer 0.5 *M* in citrate. [h] Sodium phosphate–phosphoric acid buffer 0.5 *M* in phosphate. [i] Borate–phosphate buffer 0.1 *M* in sodium tetraborate and 0.2 *M* in monopotassium phosphate. [j] Benzoyl-L-tryptophanate. [k] Benzoyl-L-tyrosinate. [l] Benzoyl-L-phenylalaninate. [m] Carbobenzoyl-L-phenylalaninate.

(28) C. Niemann and P. L. Nichols, Jr., *J. Biol. Chem.*, **143**, 191 (1942).

(29) F. W. Holley, J. J. Cahill, Jr., and K. Folkers, THIS JOURNAL, **73**, 2944 (1951).

(30) N. F. Albertson, *ibid.*, **73**, 5438 (1951).

(31) All melting points are corrected.

(32) The authors are indebted to Dr. A. Elek for all microanalyses reported in this communication.

(33) J. R. Spies, THIS JOURNAL, **70**, 3717 (1948).

(34) E. Fischer, *Ber.*, **32**, 3638 (1899).

(35) E. Fischer, *ibid.*, **32**, 2451 (1899).

ethanol-soluble product which weighed 37 mg. after one recrystallization from aqueous ethanol and which melted at 150–152° after three additional recrystallizations from the same solvent. The m.p. of phenylcarbamyl-D-phenylalanine is 171°.[27] However, it was noted that when an earlier preparation of this acid which melted at 167–168° was recrystallized from hot water a product was obtained which melted at 150–152° and whose m.p. was unchanged upon further recrystallization from the same solvent.

Anal. Calcd. for $C_{11}H_{11}O_2N$ (189): C, 69.9; H, 5.9; N, 7.4. Found: C, 69.6; H, 5.9; N, 7.5.

Since the above analysis clearly indicated that the product, m.p. 150–152°, could arise from one of the starting materials it was concluded that there was no evidence for the formation of phenylcarbamyl-D-phenylalanylphenylhydrazide.

Miscellaneous Enzyme Experiments.—In the attempted synthesis of *sym*-bis-(benzoyl-L-phenylalanyl)-hydrazine, benzoyl-L-phenylalanine (0.5 millimole, 12.5 millimoles per liter), was incubated at 35°, with hydrazine (100 millimoles per liter) in the presence of α-chymotrypsin (1 mg. per ml. of reaction mixture) and a 0.5 *M* citrate buffer adjusted to *p*H 6.0. No precipitation was observed even after 15 days.

In the attempted synthesis of benzoyl-L-alanylphenylhydrazide, benzoyl-DL-alanine (1.0 millimole, 33.3 millimoles per liter) was incubated at 35° with phenylhydrazine (66.6 millimoles per liter) in the presence of the usual quantity of α-chymotrypsin and a series of 0.5 *M* citrate buffers adjusted to *p*H 3.5, 4.1, 4.5, 5.0, 5.5, 6.0 and 6.5, respectively. After 10 days only a small amount of a black tar was formed in all cases.

From the incubation of benzoyl-L-phenylalanine (1.0 millimole, 8.7 millimoles per liter) with *p*-toluidine (17.4 millimoles per liter) at 35° in the presence of the usual amount of α-chymotrypsin and a 0.5 *M* citrate buffer adjusted to *p*H 6.0, there was obtained, after 114 hours, 16.5% of benzoyl-L-phenylalanin-*p*-toluide, m.p. 219.5–220° after two recrystallizations from aqueous ethanol, $[\alpha]^{25}D$ +30.3° (*c* 1.06 in pyridine).

Anal. Calcd. for $C_{23}H_{22}N_2O_2$ (233): C, 77.1; H, 6.2; N, 7.8. Found: C, 77.2; H, 6.2; N, 7.7.

PASADENA 4, CALIFORNIA

[CONTRIBUTION NO. 1688 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate in Aqueous Solutions at 25° and *p*H 7.9[1]

BY H. T. HUANG AND CARL NIEMANN[2]

RECEIVED APRIL 28, 1952

It has been found that the α-chymotrypsin catalyzed hydrolysis of methyl hippurate in aqueous media proceeds at a sufficiently rapid rate at 25° and *p*H 7.9 to allow a study to be made of the kinetics of this reaction. From inhibition experiments with the above specific substrate and several trifunctional competitive inhibitors whose enzyme–inhibitor dissociation constants had been evaluated previously it appears that the catalytically active site involved in the hydrolysis of methyl hippurate is the same as that responsible for the hydrolysis of a number of acylated amides derived from L-tryptophan, L-tyrosine and L-phenylalanine. It has also been found that trypsin will catalyze the hydrolysis of methyl hippurate but at a much lower rate than is observed with α-chymotrypsin.

It has been suggested in a previous communication[3] that specific substrates and competitive inhibitors of α-chymotrypsin, derived from α-amino acids and described by the general formula R_1CHR_2-R_3,[4] may combine with the enzyme through interaction of the groups R_1, R_2 and R_3 with their respective complementary centers ρ_1, ρ_2 and ρ_3 which are assumed to be a characteristic feature of the catalytically active site of the enzyme. The well defined antipodal specificity of α-chymotrypsin which distinguishes the behavior of an L-specific substrate from that of the corresponding enantiomorphic D-competitive inhibitor, may then be interpreted in terms of steric limitations which the configuration about the asymmetric α-carbon atom must necessarily impose upon the three major interactions, *i.e.*, R_1–ρ_1, R_2–ρ_2 and R_3–ρ_3, assumed to be responsible for the formation of the intermediate complexes ES or EI.[5] Thus, in the case where the configuration about the asymmetric α-carbon atom is L it is possible that the formation of ES results in a strain which is centered in the R_3 group and which facilitates the transformation of ES into enzyme and reaction products either directly or by subsequent attack of ES by other reactants present in the reaction medium. Formally these two routes would correspond to the unimolecular and bimolecular mechanisms which have been proposed for the acid or base catalyzed solvolysis of esters.[6,7] In contrast, when the configuration about the asymmetric α-carbon atom is D two factors may be operative in contributing to the stability of R_3, one, the formation of EI by the interaction of all three R groups with their respective ρ centers with little or no strain, and two, an unfavorable orientation of the R_3 group in EI with respect to subsequent attack.

It is important, in a consideration of the mode of action of α-chymotrypsin, to inquire to what extent bifunctional compounds of the type $R_1CH_2R_3$ and $R_2CH_2R_3$, in which the asymmetry of the α-carbon atom is eliminated but where the optimum distance between R_3 and the remaining R group is

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

(4) Where R_1 = an amino or acylamino group, R_2 = the side chain of the α-amino acid, and R_3 = the carboxyl group or a functional derivative thereof.

(5) For definition of symbols, *cf.*, H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(6) *Cf.*, E. A. Braude, *Annual Reports*, **46**, 119 (1950).

(7) I. B. Wilson and D. Nachmansohn, in F. F. Nord, *Advances in Enzymology*, **12**, 302 (1951), have proposed a detailed unimolecular mechanism of hydrolysis for the system acetylcholine–acetylcholine esterase which appears to accommodate the available data on the effect of *p*H on the activity of this enzyme toward a number of its specific substrates and competitive inhibitors. For the hydroperoxidases B. Chance, *cf.*, Sumner and Myrbäck, "The Enzymes," Vol. II, Part I, Academic Press, New York, N. Y., 1951, p. 428, has definitely established the bimolecular nature of the reaction between the enzyme–substrate complexes and the donor molecules. See also C. G. Swain, *Record Chem. Prog.*, **12**, 21 (1951).