isovaleryl chloride as the condensation reagent. L-Butyrine ethyl ester and L-norvaline ethyl ester hydrochlorides [$[\alpha]^{25}D$ +6.1° and +9.5° (*c* 2, 5 *N* HCl), respectively] were used in the synthesis of the four dipeptides. The over-all yields varied from 65 to 80% of the theoretical. Data on the intermediates are as follows.

Ethyl carbobenzoxy-L-alanyl-L-butyrinate: m.p. 77°; calcd. N, 8.3; found N, 8.3. Ethyl carbobenzoxy-D-alanyl-L-butyrinate: m.p. 93°, calcd. N, 8.3; found N, 8.8. Carbobenzoxy-L-alanyl-L-butyrine: m.p. 154°; calcd. N, 9.1; found N, 9.3. Carbobenzoxy-D-alanyl-L-butyrine: m.p. 141°; calcd. N, 9.1; found N, 9.2.

Ethyl carbobenzoxy-L-alanyl-L-norvalinate: m.p. 108°; calcd. N, 8.0; found N, 8.0. Ethyl carbobenzoxy-D-alanyl-L-norvalinate: m.p. 90°; calcd. N, 8.0; found N, 8.1. Carbobenzoxy-L-alanyl-L-norvaline: m.p. 145°; calcd. N, 8.7; found N, 8.7. Carbobenzoxy-D-alanyl-L-norvaline: m.p. 151°; calcd. N, 8.7; found N, 8.7.

The carbobenzoxy peptides were hydrogenated as usual in the presence of palladium black, and the free peptides recrystallized from ethanol–H_2O. The analytical data are collected in Table V. Carbobenzoxy-L-alanyl-L-phenylalanine and carbobenzoxy-D-alanyl-L-phenylalanine were prepared by the interaction of the respective carbobenzoxyalanine with L-phenylalanine ethyl ester in the presence of

TABLE V

PHYSICAL CONSTANTS OF L- AND D-ALANYL-L-AMINO ACIDS

Compound	$[\alpha]^{25}D$[a]	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
D-Alanyl-L-alanine	−71.1	45.0	7.6	17.5	44.7	7.4	17.3
L-Alanyl-L-butyrine	− 8.0	48.3	8.1	16.1	48.4	8.4	16.2
D-Alanyl-L-butyrine	−52.8	48.3	8.1	16.1	48.2	8.2	16.2
L-Alanyl-L-norvaline	− 5.0	51.0	8.6	14.9	51.1	8.4	14.8
D-Alanyl-L-norvaline	−47.4	51.0	8.6	14.9	50.9	8.4	14.8

[a] In degrees, *c* 2, H_2O.

isovaleryl chloride and triethylamine, and the resulting coupling product subsequently saponified and acidified. The compounds were recrystallized first from ethyl acetate and petroleum ether and then from acetone and petroleum ether; m.p. for the former compound was 122°, for the latter compound 74°. Bergmann and Fruton reported a m.p. of 56–58° for the former compound.[24]

(24) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **145**, 247 (1942).

Anal. Calcd. for $C_{20}H_{22}O_5N_2$: C, 64.9; H, 6.0; N, 7.6. Found (for carbobenzoxy-L-alanyl-L-phenylalanine): C, 64.8; H, 5.8; N, 7.6. Found (for carbobenzoxy-D-alanyl-L-phenylalanine): C, 64.6; H, 6.1; N, 7.5.

Enzymatic Studies.—The hydrolysis of all substrates studied was followed by the manometric ninhydrin–CO_2 procedure. A single lyophilized preparation of renal acylase I was employed.[1] The enzyme solution in 0.01 *M* phosphate buffer at *p*H 7.0 was prepared fresh daily. Digests consisted of 1 ml. of 0.1 *M* phosphate buffer at *p*H 7.0, 1 ml. of 0.025 *M* neutralized substrate, and 1 ml. of appropriately diluted enzyme solution. The period of incubation, which was at 37°, varied up to 120 minutes. As with all substrates studied with acylase I under these conditions, zero-order kinetics were obtained. Rates were calculated from the initial linear portion of the time curves, and were expressed as micromoles of substrate hydrolyzed per hour per mg. of protein N. All rate values given, both with acylase I and with pancreatic carboxypeptidase, were the average of from 4 to 8 separate determinations with an average variation of less than 10%.

Studies with pancreatic carboxypeptidase were conducted with a crystalline Armour preparation. The enzyme was suspended in chilled 5% LiCl solution and filtered clear after 18 hours of standing at 5°. Digests consisted of 1 ml. of veronal buffer at *p*H 7.45, 1 ml. of 0.025 *M* neutralized substrate and 1 ml. of appropriately diluted enzyme solution. Rates were expressed in terms of the first-order velocity constant per mg. of protein N per ml. of digest. The experimental conditions were different from those frequently employed which include an initial substrate concentration of 0.05 *M* and an incubation temperature of 25°.[14,24] The present results are therefore difficult to compare with those in the literature. Thus, Bergmann and Fruton report a rate value of 7.3 for the action of carboxypeptidase on carbobenzoxy-L-alanyl-L-phenylalanine,[24] a value subsequently altered to 11.3 by Neurath and Schwert.[14] The present value of 71.0 (Table III) obtained for this compound with an initial substrate concentration of 0.008 *M*, divided by the ratio of 0.05 to 0.008 or close to 6, would yield a value of 11.8. Inasmuch as the temperature at which the present and the cited experiments were performed was quite different (by 12°), and because there is no reason to expect that the proteolytic coefficients would be linear in the substrate concentration over so wide a range of concentration, the comparison made is quite approximate and serves only to reveal a similar order of magnitude.

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE VIRUS LABORATORY AND THE HORMONE RESEARCH LABORATORY, DEPARTMENT OF BIOCHEMISTRY UNIVERSITY OF CALIFORNIA, BERKELEY]

A General Micromethod for the Stepwise Degradation of Peptides

BY H. FRAENKEL-CONRAT[1] AND J. IEUAN HARRIS

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The phenyl isothiocyanate method for the stepwise degradation of peptides from the amino end has been adapted to a microscale and modified in such a manner as to be applicable to peptides containing all natural amino acids, with the possible exception of cystine. This is achieved by following with the spectrophotometer the formation of the phenylthiohydantoins in strong aqueous acid and thus avoiding unnecessary exposure to acid.

The application of the phenyl isothiocyanate (PTC) method[2] to the stepwise degradation of peptides and proteins was greatly facilitated when it was shown that the phenylthiohydantoin (PTH) derivative of the N-terminal amino acid could be released in aqueous acid solution.[3] Several different conditions have been used for the aqueous acid cleavage of phenylthiohydantoins from the corresponding phenylthiocarbamyl (PTC-) peptides. Thus Ottesen and Wollenberger[4] employed 0.1 *N* HCl at 75° for the successive removal of N-terminal amino acids from the hexapeptide (ala·gly·val·asp·ala·ala) released during the transformation of ovalbumin to plakalbumin. Under these conditions, however, the very labile bond between aspartic acid and alanine was also hydrolyzed during the course of the second step in the degradation. Christensen[5] found extensive non-specific splitting

(1) Aided by a grant from the National Foundation for Infantile Paralysis.

(2) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(3) H. Fraenkel-Conrat and J. Fraenkel-Conrat, *ibid.*, **5**, 1409 (1951).

(4) M. Ottesen and A. Wollenberger, *Compt. rend. trav. lab. Carlsberg, ser. chim.*, **28**, 463 (1953).

(5) H. N. Christensen, *ibid.*, **28**, 265 (1953).

of peptide bonds when he degraded insulin in guanidine hydrochloride at 75° with 0.1 N acid. This was in contrast to the earlier study employing 0.6–1.2 N acid at 36° in which the PTH derivatives of the N-terminal and next two amino acids of insulin were released without significant hydrolysis of other peptide bonds, as indicated by the same amount of PTH being formed in three consecutive steps (3 equivalents per 12,000 molecular weight).[3] Dahlerup-Petersen, *et al.*,[6] also investigated the use of milder conditions for the ring closure and concomitant cleavage of PTH's from peptides. Preliminary results showed that the PTH's of alanine and glycine could be formed in quantitative yield from certain di- and tri-peptides at 60–70° in 0.05 M aqueous citrate buffer at *p*H 4.5–5.0; in order to avoid unnecessary exposure to the hot buffer the released PTH's were removed from solution by continuous extraction with benzene.

In the course of these and other investigations[7] it became apparent that the various conditions described above were not in general applicable to peptides containing polyfunctional amino acids. The main limitations appeared to be the instability of the PTH's, particularly those of serine, threonine and cystine in dilute aqueous acid, and the insolubility of the PTH's of arginine and histidine in organic solvents. A technique has now been developed which appears to overcome these difficulties and is shown to be applicable on a micro scale to a large variety of different peptides. The transformation of the PTC-peptide to the phenylthiohydantoin is followed spectrophotometrically by making use of the different absorption maxima of the two classes of compounds (240 or lower *vs.* 265–270).[6] In this way the minimum time necessary to obtain the maximum yield of PTH at a given acid strength and temperature can be ascertained and hence excessive exposure to acid of the released PTH is prevented.

Experimental

1. **Material and Reagents.**—Dioxane and diethyl ether were purified to remove peroxides; phenyl isothiocyanate and ethyl acetate were redistilled while other reagents were of the best commercial grades. The peptides containing L-serine were synthesized by one of the authors (J. I. H.) and have in part been described,[8] while the arginine peptides were generously donated by Dr. F. H. Carpenter.[9] Other peptides were obtained from the Emil Fischer collection. Serine PTH, as well as many other PTH's, were synthesized and kindly placed at our disposal by Dr. A. L. Levy.

2. **Procedure. a. Reaction with Phenyl Isothiocyanate.**—The peptide (0.2–3 mg.) dissolved in 50% aqueous dioxane (4 ml.) was brought to *p*H 8.7–9.0 with 0.01 N sodium hydroxide and stirred for 1.5 hours at 40° with phenyl isothiocyanate (0.1 ml.)[7,10] The reaction mixture was extracted seven times with benzene and the aqueous solution concentrated to dryness *in vacuo* over NaOH.

b. Formation of Phenylthiohydantoin.—The residual sodium salt of the PTC-peptide was redissolved in water (2–10 ml.); aliquots corresponding to 0.2–1.0 micromole were then made 3 N with respect to hydrochloric acid—in some cases 1 N or 0.24 N (see Table I)—and 0.2–1.0 $\times$ 10^{-4} M with respect to peptide, by the addition of the necessary amounts of water and redistilled constant boiling hydrochloric acid. The absorption spectra of the acidified solutions were read in a model DU Beckman spectrophotometer over the range of 235–280 mμ and subsequently re-examined at intervals during the next two hours. In this way the rate of release of the PTH derivatives of the N-terminal amino acid from the PTC-peptide could be ascertained from the rate of change of the absorption maximum of the reaction solution from 240 or lower to 265–270 mμ. If the transformation was found to proceed slowly for a given peptide at a given concentration of acid, the temperature of the reaction mixture could be raised to 40–45° and/or the acid concentration could be increased. The absorption spectrum was noted at intervals until the absorption peak characteristic of PTH's (about 265 mμ) attained a maximum value. This value could be used to calculate the approximate yield of PTH released, assuming an average molecular extinction coefficent of 16,000 for PTH's.

c. Extraction of Released Phenylthiohydantoins.—The PTH derivatives of all the N-terminal amino acids, excepting those of arginine and histidine, were extracted into ethyl acetate from the aqueous acid solutions (3 extractions), pooled and washed twice with small amounts of water; the PTH derivatives of alanine, valine and leucine were readily extracted with ether.

The phenylthiohydantoin derivatives of histidine and arginine could not be extracted into ethyl acetate from acid solution (as ascertained spectrophotometrically); in such cases solutions were concentrated to dryness *in vacuo* over NaOH, redissolved in water, neutralized with sodium hydroxide, and again extracted with ethyl acetate.[5] Histidine PTH (but not arginine PTH) was extracted in this way. Arginine PTH resisted extraction even from alkaline solution, beginning to decompose at *p*H 9.0. Its presence was revealed when a neutral aqueous solution, after extraction with ethyl acetate, still retained an absorption maximum at 268 mμ, and was then confirmed by direct chromatography of an aliquot of the aqueous solution (see section e). Although it was not found possible to separate arginine PTH from the remaining amino acid or peptide, the PTH was found to disappear (either by decomposition or by extraction with the dioxane–benzene mixture) during the sequence of reactions leading to the cleavage of the next amino acid as its PTH, and thus did not interfere with further sequence elucidation.

d. Recovery of Peptide for Second Cycle of Reactions.—After extraction of the PTH, the aqueous acid solution (plus washings) was transferred to a 10-ml. beaker and again concentrated to dryness *in vacuo* over NaOH and concentrated H_2SO_4. The residue was then redissolved in 50% aqueous dioxane (4 ml.), reacted a second time with phenyl isothiocyanate, and again submitted to the whole cycle of operations leading to the release of the PTH derivative of the next amino acid along the chain. This process was continued until the peptide under investigation was completely degraded.

e. Identification of Phenylthiohydantoins.—The ethyl acetate or ether extracts containing the PTH's were evaporated *in vacuo*, redissolved in small volumes of acetone, and subjected to descending one-dimensional paper chromatography according to the procedures of Sjöquist.[11] Chromatograms were run on starch-impregnated Whatman No. 1 paper and the phenylthiohydantoins revealed as white spots on a purple background by spraying with the iodine–azide reagent. In most cases only a single spot was obtained, but sometimes smaller amounts of immobile spots or of diffuse and uncharacteristic spots were obtained. Solutions of standard PTH's were always run as controls, and the R_f values of the experimental PTH's were in all cases in good agreement with those of the standards. The expected amino acids were clearly identified at each step during the degradation of a peptide, usually with little or no evidence of contamination with adjacent amino acids. The C-terminal amino acid was generally identified by direct chromatography (in butanol–acetic acid–water (4:1:5)) of the aqueous solution after evaporation of the HCl. In a few cases the residual free amino acid was also converted to its corresponding PTH as would be the procedure with a peptide of unknown length; both procedures led to the unequivocal identification of the C-terminal amino acid. The identification

(6) B. Dahlerup-Peterson, K. Linderstrøm-Lang and M. Ottesen, *Acta Chem. Scand.*, **6**, 1135 (1952).

(7) H. Fraenkel-Conrat and B. Singer, THIS JOURNAL, **76**, 180 (1954).

(8) J. I. Harris and J. S. Fruton, *J. Biol. Chem.*, **191**, 143 (1951).

(9) D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 5872 (1953).

(10) H. Fraenkel-Conrat, in "The Chemical Structure of Proteins," J. and A. Churchill Ltd., London, 1953, p. 102.

(11) J. Sjöquist, *Acta Chem. Scand*, **7**, 447 (1953).

TABLE I

EFFECT OF EXPERIMENTAL CONDITIONS ON FORMATION AND DECOMPOSITION OF PHENYLTHIOHYDANTOINS

	3 *N* Acid (HCl)			*N* Acid (R.T.)[a]		0.24 *N* Acid		
3 *N* Acid (HCl)	Time, hr.	Temp., °C.	Yield,[b] %	Time, hr.	Yield,[b] %	Time, hr.	Temp., °C.	Yield,[b] %
PTC-leu-gly-ala	1	R.T.[a]	80					
	4.3	R.T.	83	4.5	71			
	13	R.T.	80	13	77	13	R.T.	65[c]
	36	R.T.	70(24)[d]	36	75	36	R.T.	70
						50	R.T.	68
	2	0	85					
	0.5	40	85					
	2.3	40	81			4	40	74
	20	40	50[d]			10	40	73
	44	40	44(69)[d]					
PTC-leu-ala-gly	1	R.T.	87					
	4.3	R.T.	91	4.5	80			
	13	R.T.	89	13	85	13	R.T.	72[c]
	36	R.T.	78(24)[d]	36	82	36	R.T.	76
						50	R.T.	75
	2	0	92					
	0.5	40	91			4	40	79
	24	40	45[d]			10	40	79
	54	40	44(64)[d]			22	40	75
PTC-ser-ala	1	R.T.	76					
	4.3	R.T.	85	4.5	66			
	13	R.T.	81	13	69	13	R.T.	62[c]
	36	R.T.	74(27)[e]	36	62[e]	36	R.T.	59[e]
				50	50[e]	50	R.T.	49[e]
				84	49(54)[e]	84	R.T.	44[e]
						125	R.T.	42(62)[e]
	1	40	71			4	40	60[e]
	2.3	40	68			9	40	50[e]
	20	40	59(25)[e]			22	40	39(64)[e]

[a] Room temperature (near 24°). [b] Calculated from optical density at maximum (265–267 mμ), assuming 16,000 as the molar extinction coefficient of all PTH's. For brevity's sake not all readings were included in the table. As an example of the rate of formation of the PTH of leucine from PTC-leu-ala-gly in 3 *N* HCl at R.T., the following data are listed: 8 min., no PTH maximum; 20 min., 67%; 30 min., 74% (max. 262.5 mμ); 40 min., 82%; 60 min., 87%; 80 min., 90%; 120 min., 91% yield. [c] Absorption maximum at 260 mμ, typical PTH maximum not yet reached. [d] Second maximum appears at 325 mμ; figures in parentheses indicate the highest percentage of the total (sum of the two peaks) reached by the new component (325 mμ). All absorptions decrease with longer time. [e] Second maximum appears at 315 mμ; for significance of figures in parentheses see *d*.

of the PTH's was usually confirmed by hydrolyzing them in sealed evacuated tubes with 6 *N* HCl for 16 hours at 150° and chromatographing the regenerated amino acids.[10,12]

Results

A considerable number of experiments, exemplified by a few typical ones listed in Table I, have demonstrated the following facts: (1) The rate of formation of all PTH's is a function of the acid strength. (2) The rate of decomposition of the PTH's in aqueous acid appears to be almost independent of the acid strength. Therefore, the highest yield of PTH is obtained by use of the strongest acid for the shortest possible time. (3) Secondary transformation which occurs with the PTH's of serine, threonine and cystine, and yields products with absorption maxima near 315 mμ, is slow and greatly favored by weak acid. (4) In strong acid (3 *N*) leucine PTH, as well as certain others, shows the gradual appearance of a new maximum above 320 mμ while other PTH's seem to decompose to products showing no characteristic absorption.

The latter points will be discussed in more detail below. On the basis of these observations, the PTC- derivatives of the peptides listed in Table II were treated with 3 *N* acid for the shortest possible time to obtain the highest possible yield of PTH at each step. The rate of formation of the different PTH's was found to vary appreciably; glycine was generally the slowest, while alanine was found to be among those most rapidly formed (*cf.* also 4). Depending on the nature of the peptide under investigation, from 1.5 to 24 hours at room temperature or from 0.5 to 7 hours at 40° were required, in 3 *N* acid, for the maximal formation of the PTH's of the various N-terminal amino acids listed in Table II.

The amounts of PTH released, as measured spectrophotometrically, varied from 70–95% of the expected theoretical values. In part, the loss is undoubtedly due to experimental handling, such as the repeated extraction with benzene, but the instability of the various PTH's in the acid reaction mixture is also an important factor for which corrections could be applied. It must be noted that, for practical reasons, the PTH's were usually extracted only after the absorption had reached the descending part of the curve. Thus the yields were no lower than one might expect. What is important is that both the reaction with phenyl isothiocyanate and

(12) Unpublished results of A. L. Levy.

TABLE II

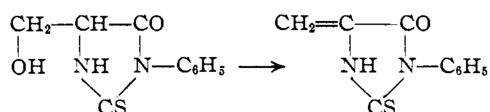
STEPWISE DEGRADATION OF PEPTIDES

Peptide	Step I Yield,[a] %	Step I Amino acid	Step II Yield,[a] %	Step II Amino acid	Step III Yield,[a] %	Step III Amino acid
Ala-gly-gly	86	Ala	81	Gly		Gly
Ala-aspNH$_2$	80	Ala	98	AspNH$_2$(Asp)[b]		
Ala-ala	82	Ala		Ala		
Ala-gly	93	Ala		Gly		
Gly-aspNH$_2$-leu[c]	(90)	Gly	84	AspNH$_2$(Asp)		Leu
Gly-tyr-gly	81	Gly	77	Tyr		Gly
Gly-asp-gly-gly	100	Gly	65	Asp	55	Gly (Gly)[d]
Gly-gly	61	Gly		Gly		
Ser-gly	74	Ser		Gly		
Ser-ala	85	Ser	62	Ala		
Ser-ala-glu	73	Ser	85	Ala		Glu
Ser-tyr	100	Ser		Tyr		
Tyr-ser	79	Tyr		Ser		
Arg-leu	94	Arg	93	Leu		
Arg-glu	74	Arg	80	Glu		
Leu-gly-ala	85	Leu	67	Gly		Ala
Leu-ala-gly	93	Leu	79	Ala		Gly

[a] The yields, all based on the original peptide, were those obtained under the most favorable conditions (usually 3 *N* acid). [b] Only asparagine was found when the first step was carried out in 0.3 *N* acid at 40° (5 hours); 3 *N* acid caused the appearance of small amounts of aspartic acid (about 10%). [c] An unweighable amount of this peptide (sticking to walls of empty tube) only was available. Thus the yield in the first step was assumed to be 90%. This step was performed with the entire sample in 3 *N* acid which probably accounts for the appearance of about 25% aspartic acid PTH in the asparagine PTH fraction in all aliquots even though different techniques were used in evaporating the acid and performing step II. [d] Step IV.

the formation and cleavage of the PTH derivative of the N-terminal amino acid appear to have occurred in essentially quantitative manner, since chromatograms at each step showed no evidence of contamination with adjacent amino acids. It has thus been possible to apply the described technique with success to the stepwise degradation of about 20 di-, tri- and tetrapeptides, containing representatives of most classes of amino acids (Table II).

Peptides Containing N-Terminal Serine.—One of the main objects of the present investigation was to ascertain the optimal conditions for the quantitative cleavage of serine PTH from peptide chains. Under the conditions generally employed, *i.e.*, in the presence of dilute acid, serine PTH appears to be readily decomposed to give a new compound showing an ultraviolet absorption maximum at 315 mμ and presumably formed through loss of one molecule of water to give an unsaturated PTH



derivative, which may subsequently polymerize through its reactive methylene group.

This transformation which is characteristic of the PTH's of serine, threonine and cystine[13] occurs in dilute acid at a rate which is comparable to the rate of release of the PTH's from peptide chains under similar conditions (*cf.* Table I).

In contrast, serine PTH could be rapidly and almost quantitatively released from PTC seryl peptides in 3 *N* HCl at 40° or at room temperature; only after excessively long exposure to acid was a small amount of the dehydro-form observed (Table I). It has thus emerged that the PTC procedure can be used for N-terminal serine if strong acid is used for the acid-catalyzed cyclization step.

Effect of 3 *N* Acid on Leucine PTH.—Leucine PTH (whether prepared from synthetic DL-leucine or released from PTC-L-leucyl peptides) was almost completely transformed to a derivative showing a sharp absorption peak at 325 mμ after 10 days in 3 *N* HCl solution at room temperature. This product (x) was isolated after treating leucine PTH (30 mg.) in about 20% alcoholic solution with 3 *N* HCl at 40° for several days. Its properties are summarized and compared with those of leucine PTH in Table III.

TABLE III

COMPARISON OF LEUCINE PTH WITH ITS ACID DECOMPOSITION PRODUCT

Properties	Leucine PTH	Compound X
M.p., °C.	178	212
Elementary analysis	C 62.8; H 6.45; N 11.3; S 12.9	C 63.9; H 6.19; N 14.0; S 15.35
Empirical formula	$C_{13}H_{16}O_2S$	$C_{11}H_{13}N_2S$
Mol. wt. (Rast method)	248	202
Spectrophotometric data, mol. extinction coefficient and λ_{max}		
In ethanol	16,300 (268 mμ)	20,000 (325 mμ)
In 0.1 *N* HCl	15,500 (264 mμ)	19,000 (322 mμ)
In *p*H 9.2 borate	Decomposes	12,000 (332 mμ)
In 0.1 *N* NaOH	Decomposes	11,500 (337 mμ)
Ratio min./max. in ethanol	245/270 mμ = 0.36	255/325 mμ = 0.17
R_f (Sjöquist solvent A)	0.63	0.70
Hydrolysis 6 *N* HCl 150°, 16 hr.	Leucine	Much ammonia, some leucine, glycine, other amino compounds

Similar products (abs. max. ≃ 325 mμ) were obtained more slowly and in poorer yields from isoleucine and valine PTH's, while aspartic acid PTH gave a product with an absorption maximum at 350 mμ under the influence of 3 *N* HCl at room temperature. Further work will be needed to elucidate the nature of these transformations.

Stepwise Degradation of Various Peptides.—Strong hydrochloric acid at room temperature cannot *a priori* be advocated for routine use with unknown peptides since the possibility of hydrolytic fission of peptide bonds along the chain under these conditions cannot be excluded. However, none of the peptides which were used in the present investigations was appreciably affected by 3 *N* acid nor by the subsequent evaporation of the acid in an evacuated desiccator containing NaOH and concentrated H_2SO_4 at room temperature. This was indicated by the direct chromatographic analysis of the peptides remaining in the reaction solution after the extraction of the first PTH, and also by the appearance of a clean single spot when the next amino acid was in turn isolated and chromatographed as its phenylthiohydantoin. Some splitting of the amide bond of asparagine was found to occur but this usually amounted to only a

(13) We are indebted to A. L. Levy for communicating to us his original observation that serine and threonine PTH are readily transformed to products absorbing maximally at 315–320 mμ. Cystine PTH is unstable even during storage in the crystalline form; its maximum absorption gradually shifts to 315–320 mμ, and insoluble polymer is formed. Serine PTH decomposes and becomes insoluble upon storage in alcoholic solution at 3°.

small proportion of the total asparagine present. As shown in Table II, the 3 *N* acid procedure has been successfully applied to a variety of tripeptides. However, with an unknown and precious peptide it would be advisable to try *N* HCl at room temperature at each step, and to raise the temperature or increase the concentration of acid only if the appearance of the PTH maximum at 265 mμ is slow under the initial conditions.

Two peptides were found to give results not in accord with expectation. Glycylaspartyldiglycine gave a low yield of the PTH of glycine in the third step and little free glycine remained in the aqueous acid solution.[14] This may be due to the known tendency of diglycine to form diketopiperazine.[15] Progressively lower yields of glycine PTH were also obtained from the polyglycine chain of leucyl-triglycyl-leucyl-octaglycyl-glycine, but our inability to find any but the N-terminal leucine even after 12 cycles of reaction remains unexplained.[16]

Application to Naturally Occurring Peptides.—In recent experiments which are still in progress the described method has been applied to oxytocin, and to the A and B chains of oxidized insulin,[17] which are naturally occurring peptides containing 8, 20 and 29 amino acid residues, respectively.[18-20] Oxytocin and the A chain of insulin presented no technical difficulties, but the B chain of insulin resembled a protein in that its PTC derivative precipitated upon acidification.

In the case of oxytocin no PTH could be extracted in the first step (the N-terminal cystine remains attached to the peptide through its disulfide linkage) but the expected amino acids (tyrosine, isoleucine, glutamic acid and aspartic acid) were unequivocally identified during the course of the next four steps. These results are in accord with the structure of oxytocin as elucidated, in part through use of the PTC- method, by du Vigneaud and co-workers.[18] The A chain of insulin yielded the first 5 amino acids (glycine, isoleucine, valine, glutamic acid and glutamic acid) in a similarly unequivocal fashion, although the chromatograms showed small amounts of other amino acid PTH's after the third step. The amide groups were apparently hydrolyzed off in both oxytocin and the insulin chains, in the course of the repeated treatments with strong acid.[21] These results indicate the upper limit of complexity of the peptides which can be degraded by the present technique. A technique which has recently been described for the stepwise degradation of proteins[22] can also be used to advantage for these large natural peptides since it does not cause appreciable hydrolysis of amide bonds; it cannot, however, be applied to the smaller peptides.

ADDENDUM IN PROOF.—In a recent publication (G. Schramm and J. W. Schneider, *Z. f. Naturforschung.*, **9b**, 209 (1954)) the claim is made that appreciable amounts of glycine and alanine may be split out of the middle of simple peptides. Much of this work, while referring to the Edman method, is performed at the quite unusually high *p*H of 11.5. One commercial peptide of unknown purity is used to indicate a similar behavior at *p*H 8.5. As indicated in Table II, and in the text, we have found no other than the terminal amino acid in all our seven peptides containing glycine and/or alanine in the chain.

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(14) This behavior became more marked the longer the neutral solution of the PTC-peptide was stored, indicating an instability on the part of this derivative. Other PTC-peptides were also found to give diminished yields of PTH after prolonged storage prior to treatment with acid.

(15) R. Hirohita, V. Kanda, M. Nakamura, N. Izumiya, A. Nagamatsu, T. Ono, S. Jufii and M. Kimitsuki, *Z. physiol. Chem.*, **295**, 368 (1953).

(16) The formation of glycine PTH in the later stages was obviously incomplete as shown by the broadness, or non-existence of maxima in the region of 260–270 mμ, even after prolonged exposure to acid. Yet, chromatography of the ethyl acetate extracts revealed at each step glycine and no leucine.

(17) The authors are greatly indebted to Drs. V. du Vigneaud and F. Sanger for samples of oxytocin and of the B chain of insulin.

(18) V. du Vigneaud, C. Ressler and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953).

(19) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(20) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353, 366 (1953).

(21) No further PTH's could be split from oxytocin, while from the insulin A chain similar amounts of PTH were obtained during further steps of degradation, but were found to be heterogeneous in composition. It appears that the disulfide-linked half-cysteine, and the cysteic acid occurring in position six of the two materials, respectively, interfere with the reaction. Experimental attempts to overcome this difficulty through reduction and alkylation of the disulfide bonds are in progress.

(22) H. Fraenkel-Conrat, THIS JOURNAL, **76**, 3606 (1954).