[CONTRIBUTION FROM WESTERN REGIONAL RESEARCH LABORATORY[1]]

The Amino Acid Composition of Subtilin[2]

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The solids and nitrogen of subtilin are accounted for almost quantitatively by alanine 2.3, L-aspartic acid 4.1, L-glutamic acid 12.2, glycine 4.7, L-isoleucine 4.0, L-leucine 15.5, L-lysine 12.3, lanthionine 6.4, L-phenylalanine 4.8, L-proline 3.3, tryptophan 5.1, L-valine 3.3, a yet unidentified sulfur-containing amino acid 27.5 and amide nitrogen 2.2. The figures indicate the content as g. in the hydrolyzate from 100 g. of subtilin free base (16.3% N).

This paper presents the composition of subtilin,[3,4] a polypeptidic antibiotic produced by a particular strain of *Bacillus subtilis*. It is composed principally of the thirteen α-amino acids listed in Table I. Seven of the amino acids usually found in proteins (arginine, cystine (or cysteine), histidine, methionine, serine, threonine and tyrosine) were virtually absent (0.1% or less).

The sulfur of subtilin is present in part as *meso*-lanthionine[5] and in part as a yet unidentified diamino dicarboxyl thioether of the empirical formula $C_7H_{14}O_4N_2S$.[6] Subtilin thus resembles other polypeptidic antibiotics produced by various strains of *Bacillus* (*i.e.*, gramicidin, gramicidin S, tyrocidine and the polymyxins) in containing residues not commonly encountered in proteins.

The average of those estimates of minimum molecular weight (Table I) believed to be most reliable (aspartic acid, isoleucine, phenylalanine, proline and valine) is 3420. Free diffusion and ultracentrifugation measurements to be published elsewhere[7] show that at *p*H 3 subtilin has a much higher kinetic molecular weight, presumably due to association, since subtilin diffuses readily through cellophane. Certain of the data of Table I (amide N, amino N and α-amino carboxyl groups) suggest a minimum molecular weight of approximately 7000 but do not exclude the lower value since non-integral numbers might be found if subtilin were composed of a group of closely related compounds, as has been demonstrated[8] for gramicidin, gramicidin S, tyrocidine and bacitracin.

The empirical formula deduced from the analytical data is $Gly_2Ala_1Val_1Leu_4Ileu_1Pro_1Phe_1Try_1Lys_3Asp_1Glu_3Lan_1(C_7H_{12}O_3N_2S)_4(amide)_5(H_2O)_1$, corresponding to a molecular weight of 3188. This formula accounts for 38 of the 40 nitrogen equivalents determined per 3420 g., and for a somewhat lower proportion of the solids. This simple formula cannot be complete, since the numbers of free amino and carboxyl groups found experimentally are significantly fewer than those calculated from the empirical formula. Rings of residues joined solely by peptide bonds may be postulated. The number of rings is calculated to be 4 or 5 by subtracting the number of Van Slyke nitrogens per molecule (4 or 5) from the number of residues of lysine (3) plus that of diamino sulfur-containing residues (5) plus one for the principal chain.[9] On the other hand, the number of rings is calculated to be 1, 2 or 3 by subtracting the number of free carboxyl groups (2 or 3) plus the number of amide nitrogens (5 or 6) from the number of residues of glutamic acid (3) plus that of aspartic acid (1) plus that of dicarboxyl sulfur-containing residues (5) plus one for the principal chain. The discrepancy in the calculated numbers of rings (4 or 5 *vs.* 1 to 3) is not due to the presence of D-glutamic acid or D-aspartic acid, which on the contrary were shown to be absent. Non-nitrogenous organic acid was not detected by ether extraction of acidified hydrolysate prepared in a closed tube.

Experimental

Materials.—Subtilin was prepared by Fevold, *et al.*,[3] from aerated vat cultures of *Bacillus subtilis* (ATCC 6633) grown on simplified medium.[4] The material appeared to be homogeneous by electrophoresis, by fractional precipitation from aqueous sodium chloride solutions and by fractional dialysis against 1% acetic acid.[3] On the other hand, Brink, *et al.*,[10] have shown by countercurrent distribution that our most potent samples of subtilin are probably only about 90% pure on the basis of the increased bacteriostatic potency of the principal fraction. We are indebted to Dr. Karl Folkers of Merck & Co., Inc., for 215 mg. of homogeneous subtilin (lot 9R205) prepared in this way. It contained 15.7% nitrogen and 4.83% sulfur by our analyses.

Elementary analyses of two of our more potent lots of subtilin are given in Table II. All analyses in this paper are calculated for subtilin (free base), 16.3% nitrogen.

Microbiological Methods.—Except where otherwise indicated, assays were made by the methods described elsewhere[11] on samples hydrolyzed with 6 *N* hydrochloric acid at 120° for 18 hours. Seven different subtilin preparations, including that prepared by countercurrent distribution, did not show significant differences in their amino acid composition. The relative bacteriostatic potencies were as follows: 57, 69, 79, 82, 90, 90 and 100. It appears that the various potencies represent varying degrees of inactivation incurred during isolation,[3] rather than failure to remove unrelated impurities.

The microbiological tests used for isoleucine, lysine, phenylalanine, proline and valine respond only to the L-isomers. D-Alanine is equally as active as L-alanine so no inference is possible as to the isomer found in subtilin. D-Aspartic acid is 60% as active as L-aspartic acid, D-glutamic acid is 20% as active as L-glutamic acid and D-leucine is 16% as active as L-leucine, so the values reported in Table I would be in error if appreciable amounts of the D-isomers

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented in part before the Symposium on Antibiotics of the American Association for the Advancement of Science, Chicago, Ill., December, 1947, and before the Division of Biological Chemistry at the American Chemical Society Meeting, Portland, Oregon, September, 1948.

(3) H. L. Fevold, *et al.*, *Arch. Biochem.*, **18**, 27 (1948).

(4) J. A. Garibaldi and R. E. Feeney, *Ind. Eng. Chem.*, **41**, 432 (1949).

(5) G. Alderton and H. L. Fevold, THIS JOURNAL, **73**, 463 (1951).

(6) G. Alderton, unpublished data.

(7) W. H. Ward, in preparation for publication.

(8) L. C. Craig, *et al.*, in "Amino Acids and Proteins," Cold Springs Harbor Symposia on Quantitative Biology, **14**, 24 (1950).

(9) The Van Slyke nitrogen determination does not measure the free amino group of proline, so this calculation would be in error if proline were bound at a chain-end through its carbonyl group. Failure to find any dinitrophenylproline in dinitrophenylsubtilin hydrolyzed for four hours with 6 *N* hydrochloric acid at 120° suggests that this is not the case (J. F. Carson, in preparation for publication).

(10) N. G. Brink, *et al.*, THIS JOURNAL, **73**, 330 (1951).

(11) J. C. Lewis, *et al.*, *J. Biol. Chem.*, **186**, 23 (1950).

TABLE I

COMPOSITION OF SUBTILIN

Constituent	Method of determination	Found %[a]	Constituent N / % of total N	No. of residues, groups, or atoms per 3420 g.	Min. mol. wt. from amino acid analyses
Glycine	*L. mesenteroides*	4.67	5.3	2.13	3220
Alanine	*L. citrovorum*	2.3[b]	2.2	0.88	3880
Valine	*L. arabinosus*	3.27	2.4	0.95	3580
Leucine	*L. arabinosus*	15.5	10.2	4.04	3390
Isoleucine	*L. arabinosus*	3.95	2.6	1.03	3320
Proline	*L. mesenteroides*	3.3	2.5	0.98	3490
Phenylalanine	*L. mesenteroides*	4.8	2.5	0.99	3440
Tryptophan	*L. arabinosus*	4.3–6.2[n]	....	...	..
Tryptophan	Spectrophotometric	5.1, 5.2[o]	4.3	0.86	3960
Tryptophan	Colorimetric[c]	5.0[c]	4.2	0.84	..
Lysine	*L. mesenteroides*	12.3	14.4	2.88	3560
Aspartic acid	*L. mesenteroides*	4.07	2.6	1.05	3270
Glutamic acid	*L. arabinosus*	12.2[b]	7.1	2.83	3610
Lanthionine	Paper chromatography	6.4[d]	5.3	1.05	..
$C_7H_{14}O_4N_2S$	Paper chromatography	27.5[d]	21.2	4.23	..
N-Amide	Mild acid hydrolysis	2.23,[e] 2.26[f]	13.8	5.48	
N-Free amino	Van Slyke	1.86,[g] 1.83[f]	11.3	4.56	
N-Total	Micro-Kjeldahl	16.3[p]	(100)	39.8	
S-Total	Micro-Carius	4.9[p]		5.23	
		Eq. per 10^4 g.			
Acid groups	Titration curve[h]	6 to 7[f]		2.0 to 2.4	
Acid groups	Esterification at 0°[i]	7.5[f]		2.56	
Acid groups	Dye method[j]	(9.4)[k]		(3.2)[k]	
Basic groups	Dye method[j]	11.0[l]		3.76	
α-Amino carboxyl groups	Ninhydrin CO_2 titration[m]	1.2		0.41	

[a] Grams per 100 g. of subtilin containing 16.3% nitrogen, or in the hydrolysate therefrom. [b] Assays of reference proteins[11] suggest that values for alanine and in lesser degree for glutamic acid tend to run low. [c] Method and data of J. R. Spies and D. C. Chambers, *Anal. Chem.*, **21**, 1249 (1949), utilizing reaction with *p*-dimethylaminobenzaldehyde. [d] Calculated from the experimentally determined proportions of lanthionine and the $C_7H_{14}O_4N_2S$ compound, assuming all the sulfur to be present as these compounds. [e] Samples dissolved in 1.5 *N* sulfuric acid were heated in sealed tubes at 120° for 40 minutes, following which ammonia was titrated after distilling from a *p*H 7.4 phosphate buffer. [f] Data of J. F. Carson, *et al.*, THIS JOURNAL, **71**, 2318 (1949). Amide nitrogen was determined by distilling ammonia from solutions of subtilin which had been allowed to stand in 10 *N* hydrochloric acid at 35° for 200 to 220 hours. [g] Estimated by extrapolation to zero time from data obtained for 30, 60 and 120 minutes of digestion. The standard deviation of the estimate was 0.09. [h] Titration between *p*H 6.00 and *p*H 2.00 by the procedure of R. M. Herriott, *et al.*, *J. Gen. Physiol.*, **30**, 208 (1946). [i] Esterification in absolute methanol 0.03 *N* to hydrochloric acid at 0° terminated in approximately 15 days with the introduction of 7.5 equivalents per 10^4 g. of subtilin without significant loss of amide nitrogen. At 25° esterification did not terminate with the introduction of 7.5 eq. per 10^4 g. but further esterification was accompanied by marked changes in amide nitrogen and in solubility. The approximate limit of 7.5 equivalents per 10^4 g. was also noted for esterification with ethanol and with propylene glycol at 25° (see *f* above). [j] Method of H. Fraenkel-Conrat and M. Cooper, *J. Biol. Chem.*, **154**, 239 (1944). [k] The possibility that a carboxyl group was freed during equilibration with the dye at *p*H 11.5 must be considered. The antibiotic activity of subtilin is lost rapidly at this *p*H. [l] Values for the number of basic groups (11.8 and 11.4), reasonably close to that found for subtilin (11.0), were obtained with methyl esters (see *f* above) containing 5.2 and 11.3 equivalents, respectively, of methoxyl groups per 10^4 g. [m] Method of B. E. Christensen, *et al.*, *J. Biol. Chem.*, **137**, 735 (1941), modified by inclusion of 80% ethylene glycol in the reaction mixture to allow complete solution of the subtilin, reaction time, 20 minutes at 100–110°. We are indebted to Dr. K. P. Dimick for this analysis. The less specific colorimetric ninhydrin method of S. Moore and W. H. Stein, *J. Biol Chem.*, **176**, 367 (1948), gave 8.4 equivalents per 10^4 g. with standardization against leucine. The subtilin used contained less than 0.02% ammonia. [n] See Table III. [o] See Table IV. [p] See Table II.

TABLE II

ELEMENTARY ANALYSIS OF SUBTILIN

Element[a]	Lot 317[b] Not corrected, %	Lot 317[b] Corrected to 0% HCl,[d] %	Lot 215-O[c] Not corrected, %	Lot 215-O[c] Corrected to 0% HCl,[d] %
C	52.3	53.4	51.6	53.0
H	6.79	6.87	6.77	6.87
N	16.0	16.3	15.9	16.3
S	4.80	4.90	4.75	4.88
Cl	1.90	(0.00)	2.54	(0.00)
P	0.005		0.01	

[a] Carbon and hydrogen were determined by micro-combustion, sulfur and chlorine by micro-Carius, nitrogen by micro-Kjeldahl with HgO as the catalyst, and phosphorus by the colorimetric method of R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940). Analyses by courtesy of L. M. White and G. E. Secor. [b] Subtilin de-ashed by passage through a cation exchanger (see *f* of Table I) and vacuum-dried at 70° to constant weight. The antibacterial potency with *Micrococcus conglomeratus* by the method of J. C. Lewis, *et al.*, *Arch. Biochem.*, **14**, 437 (1947), was 79% of that of lot 9R205 (pure subtilin). [c] Salt-precipitated subtilin,[3] not dialyzed or de-ashed. Dried *in vacuo* at 70°. Potency 90% of lot 9R205. Spectrographic examination of a similar preparation by E. J. Eastmond disclosed 0.2% of sodium, 0.1% of silicon, and traces of aluminum, boron, calcium, copper, iron, lead, magnesium, manganese, nickel, silver and tin adding to less than 0.1%. [d] All of the chloride is assumed to be present as hydrochloric acid; complete saturation of the free amino groups requires 0.131 mole (4.78 g.) of hydrochloric acid per 100 g. of subtilin-free base.

were present in subtilin. Tests with the L-amino acid oxidase activity of *Proteus vulgaris*, discussed below, show that D-isomers of these amino acids are not present.

Bioassays with *L. arabinosus* under the conditions[12] specific for L-glutamic acid (1 mg. of DL-aspartic acid per ml. of

(12) M. N. Camien and M. S. Dunn, *J. Biol. Chem.*, **179**, 935 (1949).

TABLE III

TRYPTOPHAN ASSAYS WITH *L. arabinosus*

Hydrolysis time, hr.	Hydrolyzed with[a] 5 *N* NaOH 100°, %	5 *N* NaOH 120°, %	5 *N* $Ba(OH)_2$ 100°, %	5 *N* $Ba(OH)_2$ 120°, %
5	3.40	4.30	6.15	5.55
10	4.15	4.25	6.10	5.25
20	4.35		5.60	

[a] Hydrolysis in evacuated ampoules with 6 *N* HCl under similar conditions gave dark solutions with no tryptophan activity for *L. arabinosus*, although subtilin does not contain sulfhydryl or disulfide groups, serine or carbohydrate (*cf.* H. S. Olcott and H. Fraenkel-Conrat, *J. Biol. Chem.*, 171, 583 (1947).

TABLE IV

TRYPTOPHAN ESTIMATION FROM ABSORPTION SPECTRUM[a]

Sample	Concn., mg. per ml.	Optical density at 2865 Å.[b] Of the test solution	Of the linear interpolation between D 2780[c] and D 2890[d]	Tryptophan content, %
DL-Tryptophan	0.0496	1.092	1.331	(100)
Subtilin (#317)	.908	1.318	1.540	5.1[e]
Subtilin (#9R205)	.904	1.420	1.648	5.2

[a] In methanol–0.01 *N* hydrochloric acid; 1-cm. cell. We are indebted to G. F. Bailey for help with these determinations. [b] Absorption minimum for tryptophan and subtilin. [c] Absorption maximum for tryptophan and plateau for subtilin. [d] Absorption maximum for tryptophan and subtilin. [e] Exemplary calculation: $5.1 = \frac{(1.540 - 1.318)}{(1.331 - 1.092)} \times \frac{0.0496}{0.908} \times 100$.

single-strength Steele's medium,[13] no asparagine) and for total glutamic acid (Steele's medium without aspartic acid or asparagine) gave identical results for subtilin hydrolysate.

Tryptophan was determined in subtilin lot 317 with *L. arabinosus* and Difco tryptophan assay medium. Two-hundred mg. samples were hydrolyzed in evacuated sealed ampoules with 5 *N* sodium hydroxide or barium hydroxide under the conditions noted in Table III. Barium was removed as the sulfate. Sodium hydroxide was neutralized with hydrochloric acid, in which case the equivalent amount of sodium chloride was added to the standard solution of tryptophan. DL-Tryptophan had 50 ± 1% of the activity of L-tryptophan. Complete racemization during alkaline hydrolysis was assumed. The maximum result obtained with sodium hydroxide hydrolysis is in exact agreement with that obtained on the same lot of subtilin by Spies and Chambers (4.35% after recalculation to 16.3% nitrogen by their *p*-dimethylaminobenzaldehyde colorimetric method, *cf.* Table I). Their comprehensive investigation shows that tryptophan is destroyed under this condition of hydrolysis. By their Procedure Q, which involves hydrolysis in 5 *N* sodium hydroxide containing lead acetate in order to minimize the destructive influence of sulfur-containing compounds, they obtained 4.66% tryptophan (recalculated). By their preferred Procedure O, involving no hydrolysis, they obtained 5.01% (recalculated). This value checks the value of 5.15% obtained by calculation from the absorption spectrum of subtilin (see below). The higher values obtained microbiologically for the less severe conditions of hydrolysis with 5 *N* barium hydroxide (Table III) may represent errors due to incomplete racemization of tryptophan, although they are in best agreement with the value of 5.98% calculated on the assumption of 1 residue for a molecular weight of 3420. No inference is possible from the microbiological or other tests as to which optical isomer of tryptophan is found in subtilin.

Negative (0.1% or less) microbiological tests were obtained as follows: arginine—*Leuconostoc mesenteroides* P-60 and *Lactobacillus fermenti* 36; methionine—*L. mesenteroides* and *L. arabinosus* 17-5; threonine–*L. mesenteroides* and *Streptococcus faecalis* R; histidine, serine and tyrosine—*L. mesenteroides*. The negative response of *L. fermenti* shows that ornithine and citrulline are absent since either one of these compounds can substitute for arginine for this organism.[14] Since amino acids present in subtilin only as the D-isomers would not be detected in hydrochloric acid hydrolyzates by certain of the test organisms, barium hydroxide and sodium hydroxide hydrolysates were also tested. These gave negative results, while satisfactory recovery of 0.1% of amino acid added after hydrolysis was obtained in all cases.

Neurospora mutant 36104[15] was used to show the absence of L-cystathionine or its optical isomers in subtilin. Since only the L-isomer is active for this fungus, sodium hydroxide and barium hydroxide hydrolysates were tested as well as hydrochloric acid hydrolyzates. Moreover, the $C_7H_{14}O_4N_2S$ compound isolated by Alderton was subjected to the following racemization procedure[16]: 20 mg. of the compound was dissolved in 0.20 ml. of 1 *N* sodium hydroxide (10% excess), and 0.10 ml. of acetic anhydride was added slowly with shaking. After standing for two days at 35°, the mixture was refluxed for three hours with 6 *N* hydrochloric acid. After drying *in vacuo* to remove acid, the mixture was dissolved, neutralized, and tested by the *Neurospora* mutant. None of the preparations showed any L-cystathionine activity. Five mg. of L-cystathionine isolated from *Neurospora* culture[15] was obtained through the courtesy of Dr. Louis Jacobson of the University of California. It gave an identical growth response of mutant 36104 as an equimolecular weight of L-methionine. Subtilin thus appears not to contain D- or L-cystathionine or D- or L-*allo*-cystathionine.

Chemical Tests.—The following qualitative colorimetric tests made according to procedures outlined by Block and Bolling[17] were negative (0.1% or less): arginine (Sakaguchi), histidine (Macpherson), methionine (McCarthy–Sullivan) and cysteine or cystine. For the last, both the Sullivan–Hess–Howard and the nitroprusside (Krishnaswamy) tests were used, with and without reduction with cyanide. The nitroprusside test was also performed on unhydrolyzed subtilin. Thomas'[18] test for tyrosine was negative. Subtilin gave a negative Molisch test for carbohydrate. Acid hydrolyzates contained less than 0.1% reducing sugar by the Shaffer–Hartmann method.

Periodate oxidation by the method of Nicolet and Shinn[19] gave 0.3 equivalent of ammonia per 10^4 g. of subtilin. This shows that serine, threonine or related compounds such as hydroxylysine, are substantially absent. Less than 0.1 equivalent of Zeisel-reacting groups[20] was found per 10^4 g. of subtilin. Both 30-minute and two-hour digestion times were used.

L-Glutamic acid hydrochloride was isolated from subtilin by two techniques. Direct crystallization from a charcoal-decolorized hydrolysate by saturation with gaseous hydrochloric acid gave 144 mg. of three times crystallized product, equivalent to 3% of glutamic acid in subtilin. The nitrogen content was 7.68% (theor. 7.64%) and the specific rotation in 1.73 *N* hydrochloric acid, $[\alpha]^{24}D$ +27.8°. The other technique involved adsorption on and differential elution from an anionic resin column, and yielded 860 mg. of once-crystallized product, equivalent to 7.5% glutamic acid in subtilin. The rotation in 6.0 *N* hydrochloric acid, $c = 2.00$, $[\alpha]^{27}D$ +28.5°. Accidental manipulative loss equivalent to approximately 4.5% glutamic acid in subtilin was incurred before any fractionation between D- and L-glutamic acid would be expected. The mother liquor and washings, which might be expected to contain the more soluble DL-glutamic acid hydrochloride, contained only 30 mg. of total glutamic acid by microbiological assay. Thus the isolated glutamic acid is representative of at least 97% of the total glutamic acid of subtilin. Assuming +31.9° for the specific rotation of L-glutamic acid in 6.0 *N* hydro-

(13) B. F. Steele, *et al.*, *J. Biol. Chem.*, **177**, 533 (1949).

(14) B. E. Volcani and E. E. Snell, *ibid.*, **174**, 893 (1948).

(15) N. H. Horowitz, *ibid.*, **171**, 255 (1947). We are indebted to Dr. Horowitz for the mutant culture.

(16) V. du Vigneaud and C. E. Meyer, *ibid.*, **98**, 295 (1932).

(17) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," Chas. C. Thomas, Springfield, Ill., 1945.

(18) L. E. Thomas, *Arch. Biochem.*, **5**, 175 (1944).

(19) B. H. Nicolet and L. A. Shinn, *J. Biol. Chem.*, **142**, 139 (1942).

(20) E. P. Clark, *J. Assoc. Offic. Agr. Chem.*, **15**, 136 (1932).

chloric acid[21] and that the impurity was D-glutamic acid, the isolated product contained 95% of the L-isomer.

The following technique was used in the second isolation described above. Anion exchange resin (Permutite No. D735), 100- to 200-mesh, was adjusted to *p*H 3.0 by stirring with dilute hydrochloric acid and packed into a column 6.8 by 280 cm. The hydrolysate from 9.2 g. of subtilin, from which excess hydrochloric acid was removed by evaporation *in vacuo*, was passed into the column. Water (1800 ml.) was passed through the column followed by 0.03 *N* hydrochloric acid. Paper chromatography showed that the basic and neutral amino acids (including the sulfur-containing compounds) were removed in the first 1900 ml. of eluate. Glutamic acid appeared between 3900 and 5300 ml. and aspartic acid between 7900 and 13,600 ml. A final elution with 1 *N* sulfuric acid yielded only 32 mg. of pyrrolidonecarboxylic acid as determined by bioassay after hydrolysis. Although this resin gave a good separation in a single column,[22] it gave a marked orange color to the eluates. Control experiments showed that this soluble resin prevented the quantitative precipitation of glutamic acid by gaseous hydrochloric acid. The glutamic acid was therefore adsorbed on a small column (22.5 ml.) of Permutite S anion exchange resin, much of the color was removed by washing with water, and the glutamic acid was recovered by elution with 1 *N* hydrochloric acid, concentration, hydrolysis and precipitation with gaseous hydrochloric acid.

Spectrophotometric Estimation of Tryptophan.—The absorption spectrum of subtilin was measured over the range from 2500 to 3800 Å. with a Cary recording spectrophotometer. Lots 317 and 9R205 were measured both in water and in methanol made 0.10 *N* to hydrochloric acid (to dissolve the subtilin). The spectrum showed a steep drop in optical density from 2500 to about 3000 Å. with relatively low absorption at wave lengths greater than 3000 Å. A definite maximum was noted at 2890 Å. for subtilin in methanol–0.10 *N* hydrochloric acid and at 2870 Å. for subtilin in water, and a plateau was noted at about 2780 Å. These wave lengths correspond closely with the two major absorption maxima for tryptophan in these solvents. Tyrosine, the only other one of the familiar amino acids with an absorption maximum in this region,[23] is not present in subtilin. Although it is evident that our samples of subtilin contain other constituents whose contribution to the absorption rises with decreasing wave lengths as low as 2500 Å., the tryptophan content may be calculated from the absorption spectrum. The calculations based on data for subtilin in methanol–0.10 *N* hydrochloric acid are given in Table IV. The absorption peaks in water were less sharp, and the calculations were only about one-third as precise as those for methanol solutions; nevertheless, tryptophan contents of 5.1 and 5.3% were calculated for the two lots of subtilin. The calculation assumes a linearly-related background absorption for the three wave lengths involved in the calculation and also assumes that free tryptophan provides a suitable standard for comparison with the combined tryptophan of subtilin.[24] The close agreement of the the results with that obtained by an independent method is shown in Table I.

Paper Chromatography.—R_F values for the sulfur-containing compounds isolated from subtilin and for certain other amino acids for comparison are given in Table V. The mixture of acetone, water, acetic acid and urea gives complete resolution (in a one-dimensional chromatogram) of the sulfur-containing amino acids from all other ninhydrin-reactive constituents of hydrochloric acid hydrolysate of subtilin. To obtain complete resolution of lanthionine from the $C_7H_{14}N_2O_4S$ compound it is desirable to let the solvent flow for approximately 80 cm. This was done by the technique of Williams and Kirby,[25] the 43-cm. paper cylinder saturated with solvent being removed and dried, and then rechromatographed in the original position. Twenty-five chromatograms of simulated subtilin hydrolysates containing various ratios of *meso*-lanthionine and the $C_7H_{14}N_2O_4S$ compound and nine chromatograms of subtilin hydrolysates were compared quantitatively by extraction of the ninhydrin color of the sulfur amino acid spots with aqueous acetone and photometric estimation.[26] The results indicate the presence of one mole of lanthionine and four moles of the $C_7H_{14}N_2O_4S$ compound in 3420 g. (ratio = 0.25 ± 0.01 (standard deviation)).

TABLE V

R_F VALUES FOR THE SULFUR COMPOUNDS FOUND IN SUBTILIN

Solvent system[a]	*meso*-Lanthionine	$C_7H_{14}O_4N_2S$	Aspartic acid	Glutamic acid	Glycine	Lysine
n-Butanol, water-saturated	0.00	0.00	0.00	0.00	0.04	0.01
n-Butanol: acetic acid (9:1), water-saturated	.01	.01	.05	.09	.07	.02
Phenol (0.1% cupron), water-saturated	.19	.29	.16	.23	.39	.41
Tetrahydrofurfuryl alcohol: water (8:2)	.08	.15	.20	.27	.29	.17
Tetrahydrofuran: water (6:4)	.28	.34	.40	.46	.42	.21
Acetone:water: urea (60:40:0.5)	.17	.24	.42	.49	.43	.15
Acetone:water: acetic acid: urea (60:37.5: 2.5:0.5)	.16	.22	.40	.47	.41	.33

[a] 25 ± 1°. Changes in composition of solvents due to highly volatile components such as tetrahydrofuran or acetone affect the R_F values markedly.

The possible occurrence of a third sulfur-bearing component in the acid hydrolysate of subtilin was investigated by the use of the iodoplatinate test[27] for organically bound sulfur. One-dimensional chromatographs prepared with each of the solvents of Table V showed no evidence of a third component, except for an immobile spot observed with both the simulated and actual hydrolysates of subtilin, and with lanthionine.

Two-dimensional chromatograms of an acid hydrolysate of subtilin and of a simulated hydrolysate of subtilin showed identical spots. When water-saturated *n*-butanol, acetic acid, cupron (90:10:0.1), and water-saturated *o*-cresol, ammonia and cupron (98:2:0.1) were used as the solvent pair, spots corresponding to proline, phenylalanine, leucine–isoleucine, valine, alanine, glycine, aspartic acid, glutamic acids, lysine and lanthionine–$C_7H_{14}N_2O_4S$ were recognized. When acetone, water, acetic acid, urea and cupron (60:37.5:2.5:0.5:0.1) and water-saturated phenol, ammonia and cupron (98:2:0.1) were used as the solvent pair, spots for proline, phenylalanine–leucine–isoleucine, valine, alanine, glycine, aspartic–glutamic acids, lanthionine and the $C_7H_{14}N_2O_4S$ compound were recognized.

Optical Form by L-Amino Acid Oxidase.—The absence of significant proportions of the D-isomers of the non-sulfur-containing amino acids was tested by means of the L-amino acid oxidase of *Proteus vulgaris* (X-19).[28] Approximately 250 mg. of twice-washed moist cells grown on nutrient agar for 18 hr. at 35° were incubated in shake flasks at 35° with the hydrolysate of 400 mg. of subtilin in 20 ml. of 0.025 *M* phosphate at *p*H 7.4. Semi-quantitative paper chromatography[28] or microbiological assay where D-isomers could be measured, or both, showed that after 12 to 20 hr. only the sulfur-containing amino acids and about 5% of the original leucine–isoleucine escaped oxidation by fresh cells. Control runs with 0.5 equivalent of DL-aspartic acid, DL-glutamic acid, DL-isoleucine, DL-leucine, DL-phenylalanine, DL-proline and DL-valine per 3420 g. of subtilin, singly or in suitable combinations, showed that the D-isomers of these amino acids resisted oxidation. Suitable control runs were not obtained with alanine or lysine. Tests of oxygen uptake in the Warburg apparatus showed that neither *meso*-lanthionine nor the $C_7H_{14}N_2O_4S$ compound were oxidized at an appreciable rate.

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Acknowledgments.—We are indebted to G. Alderton for lanthionine and the $C_7H_{14}N_2O_4S$ compound and for tests for cystine, to D. K. Mecham for the periodiate oxidation test for serine and threonine, to H. Fraenkel-Conrat for colorimetric tests for acid and basic groups, tyrosine and histidine, to H. S. Olcott for the preliminary isolation of glutamic acid, to J. B. Stark for aid with ion exchange columns, and to P. A. Thompson and F. C. Marsh for technical assistance with the microbiological assays.

ALBANY, CALIF. RECEIVED MAY 31, 1951

[CONTRIBUTION FROM AMILAN DEPARTMENT, TOYO RAYON COMPANY, NAGOYA]

Studies on Synthetic Polyamides. XVIII.[1] On Hydrolysis of Polycapramide

BY KOHEI HOSHINO AND MASAMOTO WATANABE

Assuming that the molecular weight distribution of polycapramide is a Flory equilibrium distribution, the hydrolysis mechanism of polycapramide in 50% sulfuric acid was studied by viscosity measurement data using Montroll depolymerization equation. The Staudinger viscosity equation is not applicable to polycapramide, and the Sakurada-Houwink general viscosity equation holds; the following result was obtained: $[\eta] = (M_0/K)^{\frac{1}{a}} \Gamma\left(2 + \frac{1}{a}\right) \Big/ [-\ln p\ (1-\alpha)]^{\frac{1}{a}}$ and the first order velocity constant is

(a) $dB/dt = -\lambda B$

	30°	40°	50°
$\lambda(10^{-4}$ hr.$^{-1})$	4.98	8.15	29.4

(b) $\lambda t = \ln p + A/[\eta]t$

	30°	40°	50°
$\lambda(10^{-4}$ hr.$^{-1})$	4.47	11.28	36.18

Introduction

The theoretical treatment of depolymerization of the linear condensation polymer was solved previously by Montroll and Simha,[2] and in a simpler fashion, by Sakurada and Okamura.[3] Matthes[4] measured the rate of hydrolysis of polycapramide in 40% sulfuric acid at 50°. The degree of polymerization decreases in accordance with a first-order splitting of amide linkage over the entire range investigated from $\bar{x}_n = 220$ to 6. But Montroll and Simha,[2] Sakurada,[3] and Matthes[4] assumed that all bonds connecting monomeric units in the homogeneous system of the same molecular weight have the same probability of being broken regardless of their position in a given polymer in which they are found. Such a homogeneous system is difficult to prepare, so one must often be satisfied with more or less heterogeneous mixtures. Montroll[5] obtained the theoretical equation of depolymerization in a polydisperse system of long chain molecules. We used this Montroll depolymerization equation, and the hydrolysis mechanism of polycapramide in 50% sulfuric acid at 30°, 40° and 50° was studied kinetically. The aim of this paper is to establish the general law of degradation of high polymers in a polydisperse system taking polycapramide as an example.

Experimental and Results

The material for measurement was cold-drawn fiber spun from the melt of polycapramide which had been prepared by polyamidation of ε-caprolactam. Its number-average degree of polymerization was *143*. The polymer was fractionated into 6 cuts in a phenol–water, two-liquid phase system at 70°.[6] Viscosity of the fractionated materials in 50% sulfuric acid was measured in Ostwald viscometers.

Generally, number-average molecular weight $\bar{M}_n$ and intrinsic viscosity $[\eta]$ are related by the Sakurada-Houwink equation of the form

$$\bar{M}_n = K[\eta]^a \qquad (1)^7$$

where K and α are constants. Data shown in Table I are the results of the viscosity measurement in 50% sulfuric acid.

TABLE I

RESULTS OF THE VISCOSITY MEASUREMENT IN 50% SULFURIC ACID

Sample Fraction	$\bar{M}_n$	Intrinsic viscosity in 50% sulfuric acid 30°	40°	50°
1	3,793	0.222	0.307	0.275
2	4,471	.353	.343	.304
3	7,067	.392	.378	.396
4	8,165	.433	.353	.417
5	9,704	.441	.417	.421
6	10,700	.631	...	...

$\bar{M}_n$ in Table I were determined from equation (1)′ in *m*-cresol

$$\bar{M}_n = 10{,}400\ [\eta]^{1.61} \qquad (1)'^6$$

Constants K and α in equation (1) in 50% sulfuric acid were calculated by the least square method from the data shown in Table I. Its result is given in Table II.

As an example of the depolymerization reaction, hydrolysis in 50% sulfuric acid at 30°, 40° and 50° was followed by viscosity measurements. Relative viscosity was measured by Ostwald viscometers

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(7) The reciprocal of a corresponds to a' in $[\eta] = K'\bar{M}_n^{a'}$. Accordingly it is not surprising that values of a in Table I are greater than unity. An equation of the form of (1) is only generally applicable when the viscosity average molecular weight is employed, but we regard $\bar{M}_n$ equal to $\bar{M}_v$ for sharp fractions.