SYNTHESIS OF POLYMERIC PEPTIDES IN PROTEINASE-CATALYZED TRANSAMIDATION REACTIONS*

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In an earlier communication from this laboratory (1), it was reported that, at pH values near 7, beef spleen cathepsin C catalyzes the polymerization of glycyl-L-phenylalaninamide (GPA) to form an insoluble product which is, on the average, an octapeptide containing alternate glycyl and L-phenylalanyl residues. The available data justify the conclusion that this polymerization involves successive enzyme-catalyzed transamidation reactions (2). Because of the possible importance of such reactions in the biosynthesis of the peptide chains of proteins (3), and for the laboratory synthesis of long chain peptides, a study was initiated to determine the optimal conditions, the nature of the products, and the enzymatic specificity and mechanism in polymerization reactions catalyzed by cathepsin C. In the present communication, data are presented on the formation of polymeric products from GPA, from glycyl-L-tyrosinamide (GTA), and from L-alanyl-L-phenylalaninamide (APA).

Formation of Polymer from GPA by Cathepsin C—It was reported previously (1) that polymer formation was not observed at pH 5.2, near the optimal pH for the hydrolytic action of beef spleen cathepsin C (4). In order to determine more carefully the pH optimum of the polymerization reaction, a study was made of the effect of pH on the extent of transamidation when the enzyme acts on GPA at pH values near 7. The procedure employed was similar to that described previously (1), except that no buffer (other than GPA acetate) was added.

It will be seen from Table I that extensive transamidation occurred in the pH range 6.6 to 7.8, with an apparent maximum near pH 7.6. The data in Table I also show that, at pH 6.6 (as at lower pH values), the extent

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of hydrolysis exceeds that of transamidation, whereas at pH 7.6 transamidation is much more extensive than hydrolysis. At pH 6.6, no precipitate appeared within 24 hours; as the pH was increased to pH 7.6, increasing amounts of polymer were formed. The optimal pH for the preparation of the polymer is near pH 7.6, since lower yields were obtained at more alkaline pH values.

TABLE I

Catalysis of Polymerization of Glycyl-L-phenylalaninamide and of Glycyl-L-tyrosinamide by Cathepsin C

Concentration of substrate, 0.05 M; concentration of cathepsin C ($[C.\ U.]^{GPA}_{mg.\ N}$ = 18.0), 0.0073 mg. of protein N per ml. of test solution; cysteine concentration, 0.004 M; total volume, 5 ml.; time, 5 hours; temperature, 37°. The pH was adjusted by the addition of 0.1 N NaOH and checked at the start and at the end of the reaction. The initial and final pH values did not differ by more than 0.2.

Substrate*	Initial pH	Ammonia liberation	Carboxyl liberation	Extent of transamidation
		μM per ml.	*μM per ml.*	*μM per ml.*
GPA	6.6	27.1	15.4	11.7
	7.0	24.5	10.5	14.0
	7.4	24.4	8.4	16.0
	7.6	21.8	5.6	16.2
	7.8	16.4	5.1	11.3
GTA	6.6	36.9	22.4	14.5
	7.0	25.5	9.8	15.7
	7.4	26.4	6.0	20.4
	7.6	26.0	4.2	21.8
	7.6†	14.1	0.7	13.4
	7.8	23.0	4.0	19.0
	8.0	18.1	1.6	16.5

* Glycyl-L-phenylalaninamide acetate (GPA) or glycyl-L-tyrosinamide acetate (GTA).

† 0.012 M cyanide was present in place of cysteine.

In the course of these studies, it was noted that, in the pH range 6.8 to 7.6, the time interval required for the appearance of the precipitate markedly decreased with increasing pH. The appearance of initial turbidity was noted by means of a Bausch and Lomb photoelectric colorimeter with a 630 mμ filter. Under the conditions described in Table I, no decrease in light transmission was noted until 25 minutes after the start of the reaction at pH 7.6. The "lag period" observed at other pH values were 60 minutes at pH 7.0, 40 minutes at pH 7.2, and 30 minutes at pH 7.4. Once turbidity has appeared, the light transmission decreases rapidly, coincident with the formation of the flocculent precipitate. The results of a representative experiment are given in Fig. 1.

The incubation period required for the appearance of the polymeric product suggests that the formation of the long chain product requires the successive addition of glycyl-L-phenylalanyl units to a growing chain until an insoluble precipitate is formed. This hypothesis is supported by the observation that the tetrapeptide amide glycyl-L-phenylalanylglycyl-L-phenylalaninamide gives no polymer with cathepsin C at any pH tested. It would appear, therefore, that the polymerization does not involve the interaction of two tetrapeptide units, but rather that the substrate of each step of the reaction is GPA, with the addition of a dipeptide unit at the amino end of the growing chain. This mechanism is formally analo-

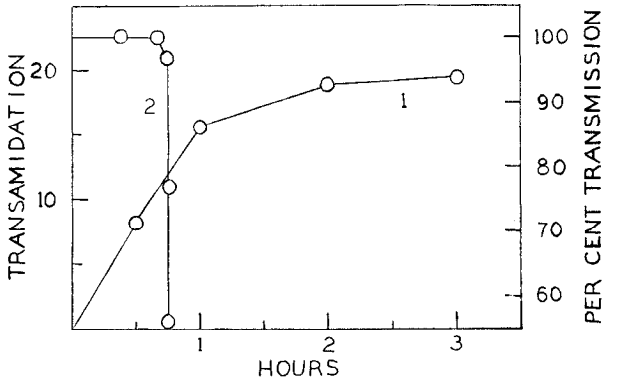

FIG. 1. Polymerization of glycyl-L-phenylalaninamide by cathepsin C at pH 7.2. The experimental conditions were similar to those given in Table I. Curve 1, rate of transamidation in micromoles per ml. (ordinate at the left); Curve 2, change in turbidity of solution as measured with Bausch and Lomb photoelectric colorimeter (ordinate at the right).

gous to the successive addition of glucose units to a growing amylose chain of a branched polysaccharide when crystalline muscle phosphorylase acts on glucose-1-phosphate (5).

Further studies on the specificity of the polymerization reactions have shown that GPA cannot be replaced by glycyl-L-phenylalanine or by glycyl-D-phenylalaninamide; this D isomer is not hydrolyzed by cathepsin C at pH 5, and does not serve as a substrate for transamidation reactions at pH 7.6. If glycyl-DL-phenylalaninamide is subjected to the action of cathepsin C at pH 7.5, the resulting insoluble polymer yields, on acid hydrolysis, phenylalanine having a specific rotation of $[\alpha]_D^{23}$ $-28.0°$ (2 per cent in H_2O). Pure L-phenylalanine has a specific rotation of $[\alpha]_D^{23}$ $-35.2°$. It follows that the enzymatic polymerization exhibits stereochemical specificity which favors the incorporation of the L isomer into the peptide chain. It cannot be stated at present whether the low rotation of the phenylalanine

obtained by hydrolysis was caused by the presence of a few D-phenylalanyl residues in the chain; as will be noted from the results of chromatographic studies to be discussed later, it appears more probable that a small quantity of glycyl-D-phenylalaninamide was adsorbed by the polymer.

From the extensive transamidation observed near pH 7 even when little or no precipitate is formed, it follows that soluble transamidation products are present in the incubation mixture. Further chromatographic studies are in progress to identify these soluble products.

In an earlier paper from this laboratory (6), it was reported that glycine is the only amino-terminal amino acid in the polymer from GPA, as judged by end-group analysis. To establish more definitely the composition and structure of the insoluble material obtained by the action of cathepsin C on GPA, 30 mg. of the material were subjected to paper chromatography on Whatman No. 1 paper with *n*-butanol-pyridine-water (5 :2 :3) as the solvent. Three ninhydrin-positive fractions were identified on marker strips and will be denoted in what follows as Fraction A (R_F 0.47; long streak), Fraction B (R_F 0.13), Fraction C (did not move). Only Fractions A and C gave a positive reaction with Durrum's reagent (7). After location of the fractions, the appropriate sections of paper were eluted with 60 per cent acetic acid. Fraction B (about 3 mg.) was identified as glycyl-L-phenylalanine by the fact that, upon methylation and hydrolysis as described previously (6), only dimethylglycine and phenylalanine were obtained. Also, an authentic sample of glycyl-L-phenylalanine showed the same chromatographic behavior as did Fraction B in butanol-pyridine and in butanol-acetic acid. An aliquot of Fraction A (about 15 mg.), on hydrolysis with 6 N hydrochloric acid at 110° for 24 hours, gave equimolar quantities of glycine and phenylalanine (as determined by the method of Moore and Stein (8)).[1] On methylation followed by hydrolysis, dimethylglycine, glycine, and phenylalanine were found in a molar ratio of approximately 1:3:3.3. The theoretical ratio for an octapeptide composed of repeating glycylphenylalanyl units is 1:3:4. Fraction C could not be eluted completely from the paper since it appears to be the least soluble component of the mixture; approximately 7 mg. were obtained. Complete acid hydrolysis gave equimolar amounts of glycine and phenylalanine; methylation and hydrolysis gave a molar ratio of dimethylglycine-glycine-phenylalanine of 1:3.9:5. The theoretical ratio for a decapeptide composed of repeating glycylphenylalanyl units is 1:4:5. For the estimation of the relative proportions of dimethylglycine, glycine, and phenylalanine formed upon hydrolysis, spots from paper chromatograms of the hydrolysates were located with the orcinol-sulfuric acid reagent (6) and were eluted. The eluates of the three spots were then analyzed by means of the copper phosphate procedure of Woiwod (9).

[1] We are indebted to Dr. Y. P. Dowmont for these determinations.

It may be concluded, therefore, that the insoluble material obtained by the enzymatic polymerization of GPA is a mixture composed largely of octa- and decapeptides, contaminated with a small amount (about 10 per cent) of glycyl-L-phenylalanine that cannot be eluted from the polymeric material with water. The capacity of the long chain peptides to adsorb peptides of lower molecular weight is also shown by the fact that the precipitate obtained by the action of cathepsin C on glycyl-DL-phenylalaninamide, on chromatographic analysis with butanol-pyridine-water, is found to have a fourth ninhydrin-reactive component (R_F 0.23) which has been identified as glycylphenylalaninamide and is probably the unreactive D isomer.

In the course of the studies on the polymerization of GPA at pH values near 7, it was found that the reaction usually stopped when the ammonia liberation corresponded to about 50 per cent of that expected upon complete deamidation of the substrate. The cause of this incomplete reaction, which was also noted with other dipeptide amides that are polymerized by cathepsin C, appears to lie in the removal of the enzyme from solution by the precipitated polymer. It will be seen from Fig. 1 that, after the appearance of the precipitate, the rate of transamidation is markedly decreased. The supernatant fluid (obtained by centrifugation of the insoluble precipitate present after an incubation period of 4 hours) did not exhibit measurable cathepsin C activity. Addition of enzyme and of cysteine to the supernatant fluid caused further ammonia liberation and polymer formation.

Formation of Polymer from GTA by Cathepsin C—It will be seen from Table I that extensive transamidation occurs when cathepsin C acts on GTA, and that the extent of this reaction is maximal near pH 7.6. This pH is also the most favorable for the formation of an insoluble polymer. A preparative experiment was conducted in a manner similar to that employed for the polymerization of GPA (1), except that the pH was adjusted with 0.1 N NaOH to pH 7.6 instead of 7.1. From 3.45 gm. of GTA acetate in 272 ml. of incubation mixture, a precipitate was obtained which was collected by centrifugation and washed four times with 50 ml. portions of water. After being dried, the product weighed 100 mg., and contained 1.0 per cent ash. On an ash-free and moisture-free basis, the product contained 13.5 per cent nitrogen (Kjeldahl) and 1.2 per cent α-amino N (Van Slyke nitrous acid method). Complete hydrolysis with 7 N hydrochloric acid for 24 hours at 110° liberated 1.2 per cent of ammonia N. After removal of the ammonia from the hydrolysate, the α-amino N was found to be 12.3 per cent. Like the polymer from GPA, the product from GTA gives a positive biuret reaction (purple color) with alkaline copper sulfate.

The analytical data given above indicate that the insoluble product is, on the average, a decapeptide amide of the structure glycyl-L-tyrosyl(gly-

cyl-L-tyrosyl)$_3$glycyl-L-tyrosinamide. It will be seen that the ratio of amino acid N (total N minus ammonia N) to α-amino N (before acid hydrolysis) equals 10.2. The fact that the α-amino N before acid hydrolysis and the ammonia N after acid hydrolysis are the same (1.2 per cent) shows that, under the conditions of the enzyme experiment, the extent of hydrolysis of GTA to glycyl-L-tyrosine was small. This is in contrast to the findings with the insoluble material obtained from GPA, and the difference may be attributed to the fact that the polymer from GPA was prepared at pH 7.1, whereas the polymer from GTA was prepared at pH 7.6. At the latter pH the extent of enzymatic hydrolysis (as measured by carboxyl liberation) is much less than at pH 7.1, and the reaction is almost exclusively one of transamidation.

Since the extent of transamidation in the polymerization of GTA at pH 7.6 amounted to 21.8 μM per ml. (Table I), it will be seen that the yield of insoluble polymer was extremely small (approximately 9 per cent of the theory), and that the major products of the reaction remained in solution. It may be assumed that these soluble products represent peptides of shorter chain length than the decapeptide amide obtained in insoluble form. Chromatographic studies designed to separate and identify these soluble peptides are in progress.

It will be noted from Table I that the substitution of cysteine by cyanide as the activator in the enzymatic transamidation of GTA causes a decrease in the rate of the reaction. The formation of polymer also occurs in the presence of cyanide, but the yields are usually lower than with cysteine as the activator.

Formation of Polymer from APA by Cathepsin C—It will be noted from Table II that APA serves as a substrate for cathepsin C at pH 7.2, and that the transamidation reaction at this pH leads to the formation of an insoluble precipitate. For the preparation of an insoluble polymer derived from APA, 0.74 gm. (0.005 mole) of APA acetate was incubated at pH 7.2 in the usual manner (1) with cathepsin C (total volume, 50 ml.). After 3 hours, the precipitate was collected by centrifugation and washed four times with 10 ml. portions of water. After being dried, the product weighed 240 mg., was ash-free, and contained 14.0 per cent nitrogen (Kjeldahl) and 2.1 per cent α-amino N (Van Slyke nitrous acid method). Complete hydrolysis with 7 N hydrochloric acid for 21 hours liberated 1.7 per cent ammonia N; the hydrolysate contained 12.6 per cent amino acid N. These analytical data indicate that the average chain length of the product is that of a hexapeptide (12.3/2.1 = 5.9). From the relative amounts of ammonia liberated on acid hydrolysis and the α-amino N before acid hydrolysis, it may be calculated that the product contains, on the average, 80 per cent of the hexapeptide amide, L-alanyl-L-phenylalanyl-

L-alanyl-L-phenylalanyl-L-alanyl-L-phenylalaninamide, and 20 per cent of the free hexapeptide.

Under the conditions of this experiment, the extent of transamidation reached approximately 50 per cent, corresponding to the conversion of 1.25 mM of APA; therefore, the theoretical yield of hexapeptide amide plus hexapeptide is approximately 0.42 mM. The amount isolated (240 mg.) corresponds to 0.36 mM, or 86 per cent of the theoretical value. This yield is significantly greater than that obtained under similar conditions from GPA (67 per cent (1)), and considerably greater than that obtained from GTA. In this connection, it is of interest to note that the yield of insolu-

TABLE II

Transamidation of L-Alanyl-L-phenylalaninamide by Cathepsin C

Concentration of L-alanyl-L-phenylalaninamide acetate, 0.05 M; concentration of cathepsin C ($[C.\ U.]^{GPA}_{mg.\ N} = 18.0$), 0.0073 mg. of protein N per ml. of test solution; cysteine concentration, 0.004 M; pH 7.2 (adjusted by the addition of 0.1 N NaOH); temperature, 37°.

Time	Ammonia liberation	Carboxyl liberation	Extent of transamidation
min.	*μM per ml.*	*μM per ml.*	*μM per ml.*
30*	7.7	2.6	5.1
60	15.0	3.5	11.5
90	21.1	4.0	17.1
120	27.5	4.5	23.0
180	31.4	6.5	24.9
240	31.0	6.5	24.5

* A gelatinous precipitate was present which increased in amount on further incubation.

ble polymer decreases as the average chain length of the product increases. It may be inferred that a decisive factor in determining the chain length and yield of the insoluble product is the solubility of intermediate peptides formed in the polymerization process. The hexapeptides derived from GPA and GTA appear to be more soluble than the hexapeptide from APA, whereas the octapeptide derived from GTA appears to be more soluble than that derived from GPA.

Further Studies on Specificity of Polymer Formation by Cathepsin C—In order to examine further the influence of structural changes in the substrate on the extent of transamidation by cathepsin C, the following dipeptide amides were tested under the experimental conditions given in Table II: L-alanyl-L-tyrosinamide (ATA), L-leucyl-L-tyrosinamide (LTA), α-L-glutamyl-L-phenylalaninamide (GluPA). Although ATA is readily hydrolyzed at pH 5 and gives a polymer at pH 7.6 (Table III), LTA and GluPA did not show extensive ammonia liberation under conditions which

gave transamidation with GPA, GTA, and APA. LTA and GluPA were hydrolyzed slowly by cathepsin C at pH 5. Interpretation of these results is made difficult by the fact that LTA and GluPA are sparingly soluble in water. It was noted previously (4) that L-phenylalanyl-L-phenylalaninamide gives an insoluble precipitate in the presence of cathepsin C at pH 5; a similar result has been noted at pH 7. Preliminary experiments have also shown that L-seryl-L-phenylalaninamide serves as a substrate for cathepsin C, and gives an insoluble precipitate at pH 7.

It was of considerable interest to find that none of the following tripeptide amides served as a substrate of cathepsin C at pH 7: glycylglycyl-L-phenylalaninamide, glycyl-L-phenylalanylglycinamide, and L-alanylglycyl-

TABLE III

Transamidation of L-*Alanyl-*L-*tyrosinamide by Cathepsin C*

Concentration of L-alanyl-L-tyrosinamide acetate, 0.05 M; concentration of cathepsin C ($[C.\ U.]^{GPA}_{mg.\ N}$ = 15.6), 0.014 mg. of protein N per ml. of test solution; cysteine concentration, 0.004 M; temperature, 37°.

pH	Time	Ammonia liberation	Carboxyl liberation	Extent of transamidation
	min.	*μM per ml.*	*μM per ml.*	*μM per ml.*
5.0	60	12.4	11.2	1.2
	120	22.3	20.3	2.0
	180	26.9	26.6	0.3
7.6	30*	10.8	0.0	10.8
	60	14.3	1.4	12.9
	120	14.7	1.4	13.3

* The solution was cloudy at 30 minutes, and a gelatinous precipitate appeared at 40 minutes.

L-phenylalaninamide. This result suggests that cathepsin C is specifically adapted to the catalysis of transamidation reactions in which a dipeptide unit such as that derived from GPA, GTA, APA, or ATA is transferred to an acceptor amino group. The fact that L-phenylalaninamide is not a substrate for cathepsin C (4), and that closely related tri- and tetrapeptide amides also are not attacked, points to the dipeptide unit as one of especial metabolic importance in the elongation of peptide chains by cathepsin C. Further studies are required to establish more clearly the rôle of dipeptides in protein synthesis. It will be of interest to examine the action of purified intracellular proteinases on derivatives of dipeptides in which the terminal carboxyl group participates in an ester or a thiol ester linkage.

Synthesis of Peptide Derivatives Tested As Substrates

*Glycyl-*L-*phenylalaninamide Acetate*—This substance was prepared as described previously (10) from carbobenzoxyglycyl-L-phenylalanine ethyl

ester, which may be obtained in good yield by the reaction of L-phenylalanine ethyl ester with carbobenzoxyglycyl chloride (10), with carbobenzoxyglycinazide, or with carbobenzoxyglycine in the presence of isovaleryl chloride and triethylamine (11).

Glycyl-D-phenylalaninamide Acetate—This substance was prepared by the coupling of carbobenzoxyglycine and D-phenylalanine ethyl ester in the presence of isovaleryl chloride and triethylamine, followed by treatment with ammonia and hydrogenolysis, as described for the L form (10).

$C_{13}H_{19}O_4N_3$ (281.2). Calculated. N 14.9, NH_2-N 5.0
Found. " 14.7, " 5.0
$[\alpha]_D^{25}$ −28.5° (5% in water)

L-Alanyl-L-phenylalaninamide Acetate—4.4 gm. of carbobenzoxy-L-alanyl-L-phenylalanine ethyl ester (12) were dissolved in 100 ml. of methanol previously saturated with ammonia at 0°. After 2 days, the solution was concentrated; the resulting product was recrystallized from methanol-water. Yield, 4.0 gm.; m.p. 210–211°.

$C_{20}H_{23}O_4N_3$ (369.4). Calculated, N 11.4; found, N 11.2

3.2 gm. of the carbobenzoxydipeptide amide were subjected to catalytic hydrogenolysis in methanol in the presence of 0.6 ml. of glacial acetic acid. After removal of the catalyst, the filtrate was concentrated *in vacuo*, yielding 2.4 gm. of crystalline product.

$C_{14}H_{21}O_4N_3$ (295.3). Calculated, N 14.2; found, N 14.3
$[\alpha]_D^{27}$ +33.4° (1.54% in water)

α-L-Glutamyl-L-phenylalaninamide—This compound was prepared as described previously (13).

Glycyl-L-tyrosinamide Acetate—This compound was prepared as described previously (10). In contrast to the report of Kaufman, Neurath, and Schwert (14), no difficulty was encountered in the hydrogenolysis of carbobenzoxyglycyl-L-tyrosinamide in the presence of acetic acid.

L-Alanyl-L-tyrosinamide Acetate—An ethyl acetate solution of carbobenzoxy-L-alaninazide (prepared in the usual manner from 4.0 gm. of the hydrazide (15)) was added to an ethyl acetate solution of L-tyrosine ethyl ester (prepared from 4.2 gm. of the hydrochloride). After 20 hours at room temperature, the solution was washed and dried in the usual manner and concentrated *in vacuo* to yield an oil which was dissolved in 100 ml. of methanol previously saturated with ammonia. After 2 days, the solution was concentrated *in vacuo*, yielding a crystalline product. After recrystallization from methanol-water, the substance melted at 216°; yield, 3.0 gm.

$C_{20}H_{23}O_5N_3$ (383.4). Calculated, N 10.9; found, N 11.2

Hydrogenolysis of 2.5 gm. of the carbobenzoxydipeptide amide in the presence of 0.5 ml. of glacial acetic acid gave 1.9 gm. of the dipeptide amide acetate.

$C_{14}H_{21}O_5N_3$ (311.3). Calculated. N 13.5, NH_2-N 4.5
Found. " 13.2, " 4.4
$[\alpha]_D^{27}$ +30.2° (1.2% in water)

L-*Leucyl*-L-*tyrosinamide Acetate*—This substance was prepared from 1.7 gm. of carbobenzoxy-L-leucinhydrazide and 1.4 gm. of L-tyrosine ethyl ester hydrochloride in a manner similar to that described for L-alanyl-L-tyrosinamide. A crystalline carbobenzoxy dipeptide amide was not obtained; hydrogenolysis of the syrup gave a crystalline dipeptide amide acetate. The product was recrystallized from methanol-ethyl acetate.

$C_{17}H_{27}O_5N_3$ (353.4). Calculated. N 11.9, NH_2-N 4.0
Found. " 12.0, " 4.0
$[\alpha]_D^{27}$ +6.2° (2.1% in pyridine)

Glycylglycyl-L-*phenylalaninamide*—A chloroform solution of carbobenzoxyglycylglycinazide (from 5.0 gm. of the hydrazide) was added to an ethyl acetate solution of L-phenylalanine ethyl ester (prepared from 4.1 gm. of the hydrochloride). After 16 hours at room temperature, the solution was washed and dried in the usual manner and concentrated *in vacuo* to yield 4 gm. of carbobenzoxytripeptide ester; m.p., 73–74°. Hydrogenolysis of 2.0 gm. of this product in the presence of 0.27 ml. of glacial acetic acid gave 1.5 gm. of the tripeptide ester acetate. The substance was recrystallized from methanol-ether; m.p., 121–122°.

$C_{17}H_{25}O_6N_3$ (367.3). Calculated, N 11.4; found, 11.7

Treatment of 2.6 gm. of the carbobenzoxytripeptide ester with ammonia in methanol gave the corresponding amide. After recrystallization from methanol-water, the yield was 2.1 gm.; m.p., 196–197°. Hydrogenolysis in methanol solution gave the tripeptide amide, which was recrystallized from methanol-ethyl acetate.

$C_{13}H_{18}O_3N_4$ (278.3). Calculated, N 20.1; found, N 20.0

Glycyl-L-*phenylalanylglycinamide*—1 gm. of the corresponding carbobenzoxy compound (16) was subjected to hydrogenolysis in the usual manner to yield a syrupy hygroscopic product which was dissolved in water. The aqueous solution was analyzed for its nitrogen content, and aliquots were used for the enzyme experiments.

L-*Alanylglycyl*-L-*phenylalaninamide*—An ethyl acetate solution of carbobenzoxy-L-alanylglycinazide (prepared in the usual manner from 4.9 gm. of the hydrazide (17)) was added to an ethyl acetate solution of L-phenyl-

alanine ethyl ester (prepared from 4.2 gm. of the hydrochloride). After 16 hours at room temperature, the solution was washed and dried in the usual manner and concentrated *in vacuo*, yielding a crystalline residue which was recrystallized from ethyl acetate-petroleum ether. Yield, 5.2 gm.; m.p., 123–124°.

$C_{24}H_{29}O_6N_3$ (455.4). Calculated, N 9.2; found, N 9.4

Upon treatment of 1.2 gm. of the carbobenzoxytripeptide ester with ammonia in the usual manner, the corresponding amide was obtained. After recrystallization from methanol-ethyl acetate, the substance melted at 224–225°; yield, 0.8 gm.

$C_{22}H_{26}O_5N_4$ (426.4). Calculated, N 13.1; found, N 13.2

0.8 gm. of the carbobenzoxytripeptide amide was subjected to catalytic hydrogenolysis in methanol. After removal of the solvent, the resulting product was recrystallized from methanol-ethyl acetate. Yield, 0.4 gm.

$C_{14}H_{20}O_3N_4$ (292.3). Calculated, N 19.2; found, N 19.1

*Glycyl-*L*-phenylalanylglycyl-*L*-phenylalaninamide*—Carbobenzoxyglycyl-L-phenylalaninhydrazide was prepared by the treatment of the corresponding ester (18) with hydrazine hydrate in the usual manner. After recrystallization from hot water, the hydrazide melted at 85–86°. An ethyl acetate solution of the corresponding azide (prepared in the usual manner from 2.5 gm. of the hydrazide) was added to an ethyl acetate solution of glycyl-L-phenylalaninamide. After 16 hours at room temperature, the reaction mixture was washed and concentrated *in vacuo*, to yield 2.5 gm. of the desired product; m.p., 192–193°.

$C_{30}H_{33}O_6N_5$ (559.5). Calculated, N 12.5; found, N 12.65

Upon hydrogenolysis of 1.2 gm. of the carbobenzoxytetrapeptide amide, 0.9 gm. of a crystalline product was obtained. It was recrystallized twice from ethanol-petroleum ether.

$C_{22}H_{27}O_4N_5$ (425.4). Calculated. N 16.4, NH_2-N 3.3
Found. " 16.5, " 3.2
$[\alpha]_D^{27}$ +17.6° (2% in pyridine)

In the hydrogenolysis of carbobenzoxy tri- and tetrapeptide amides such as those described above, it is preferable to conduct the reaction in the absence of acetic acid.

SUMMARY

Studies on the polymerization of glycyl-L-phenylalaninamide by cathepsin C have shown that this reaction has a pH optimum near 7.6, and leads

to the formation of an insoluble product that contains long chain peptides, probably octa- and decapeptides. The formation of the polymer appears to proceed by the stepwise addition, in successive transamidation reactions, of glycyl-L-phenylalanyl units to the amino end of a growing peptide chain.

Similar polymerization reactions have been described with other dipeptide amides (glycyl-L-tyrosinamide, L-alanyl-L-phenylalaninamide, L-alanyl-L-tyrosinamide) that are substrates of cathepsin C. On the other hand, experiments with amino acid amides, tri-, or tetrapeptide amides showed that, under the conditions of these studies, the amide group of none of the compounds tested was attacked measurably by cathepsin C.

BIBLIOGRAPHY

1. Jones, M. E., Hearn, W. R., Fried, M., and Fruton, J. S., *J. Biol. Chem.*, **195,** 645 (1952).
2. Fruton, J. S., *Yale J. Biol. and Med.*, **22,** 263 (1950).
3. Fruton, J. S., 2e Congrès international de Biochimie, Symposium sur la biogénèse des protéines, Paris, 1 (1952).
4. Tallan, H. H., Jones, M. E., and Fruton, J. S., *J. Biol. Chem.*, **194,** 793 (1952).
5. Cori, C. F., Cori, G. T., and Green, A. A., *J. Biol. Chem.*, **151,** 39 (1943).
6. Ingram, V. M., *J. Biol. Chem.*, **202,** 193 (1953).
7. Durrum, E. L., *J. Am. Chem. Soc.*, **72,** 2943 (1950).
8. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192,** 663 (1951).
9. Woiwod, A. J., *Biochem. J.*, **45,** 412 (1949).
10. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145,** 253 (1942).
11. Vaughan, J. R., Jr., and Osato, R. L., *J. Am. Chem. Soc.*, **73,** 5553 (1951).
12. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **145,** 247 (1942).
13. Fruton, J. S., Johnston, R. B., and Fried, M., *J. Biol. Chem.*, **190,** 39 (1951).
14. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177,** 793 (1949).
15. Harris, J. I., and Fruton, J. S., *J. Biol. Chem.*, **191,** 143 (1951).
16. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118,** 405 (1937).
17. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **117,** 189 (1937).
18. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134,** 225 (1940).