Polymerization of Dipeptide Amides by Cathepsin C*

HANNELORE WÜRZ, ATSUSHI TANAKA,† AND JOSEPH S. FRUTON‡

From the Department of Biochemistry, Yale University, New Haven, Connecticut

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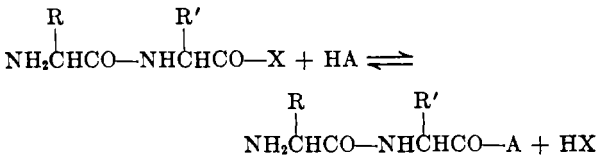
A study of the polymerization, by successive transamidation reactions, of glycyl-L-tyrosinamide to a decapeptide amide by cathepsin C near pH 7.5 has shown that the tetrapeptide amide glycyl-L-tyrosylglycyl-L-tyrosinamide is present in the reaction mixture. Addition of synthetic tetrapeptide amide does not increase the initial rate of deamidation of glycyl-L-tyrosinamide. When C^{14}-labeled glycyl-L-tyrosinamide is incubated with cathepsin C in the presence of added unlabeled tetrapeptide amide, there is extensive incorporation of unlabeled glycyltyrosyl units into the polymer. The data at hand are consistent with the conclusion that, in contrast to enzyme-bound tetrapeptide amide formed during the polymerization, the added tetrapeptide amide serves largely as a donor of glycyltyrosyl units by enzymic cleavage, rather than as a "primer" in the polymerization. It is suggested, as a working hypothesis, that the polymerization of the dipeptide amide proceeds by a single-chain mechanism whereby the growing peptide chain is not released from specific binding sites on the enzyme. When glycyl-DL-tryptophanamide is subjected to the action of cathepsin C at pH 7.5, an insoluble polymer is rapidly formed. This product has been characterized as an octapeptide amide, presumably containing alternating glycyl and L-tryptophyl residues. At pH 7.5, no hydrolysis of the dipeptide amide is observed, in contrast to the situation at pH 5, where hydrolysis is the sole reaction. Upon the action of cathepsin C on a mixture of glycyl-DL-tryptophanamide (0.05 M) and glycyl-L-tyrosinamide (0.025 M) at pH 7.5, an insoluble product is obtained whose ratio of tryptophan to tyrosine to amide-N is 3:1:1.

Cathepsin C, a proteolytic enzyme extensively purified from beef spleen (Tallan *et al.*, 1952; de la Haba *et al.*, 1959), has been shown to catalyze specifically reactions of the type:



where R and R′ are the side chains of L-α-amino acids, X is either NH_2 (as in an amide) or OC_2H_5 (as in an ester), and HA is either water or a suitable amine (NH_2OH, peptides, proteins). Near pH 5, the predominant reaction is one of hydrolysis; at more alkaline pH values, as the pK′ value of the substrate (*e.g.* glycyl-L-tyrosinamide) or of an added amine (*e.g.* NH_2OH, L-argininamide) is approached, the predominant reaction becomes one of transamidation (Jones *et al.*, 1952). Of special interest to the present study was the observation that, when cathepsin C is allowed to act near pH 7.5 on one of a group of dipeptide amides (glycyl-L-phenylalaninamide, glycyl-L-tyrosinamide, L-alanyl-L-phenylalaninamide, L-alanyl-L-tyrosinamide, L-selry-L-tyrosinamide), in each case an insoluble product is formed. These products were shown (Jones *et al.*, 1952; Fruton *et al.*, 1953) to be polymeric peptides of different chain length, but in each case to have as a repeating unitt he dipeptide residue present in the original substrate. Thus, the insoluble product obtained from glycyl-L-tyrosinamide was estimated to be, on the average, a decapeptide amide, whereas the product from L-alanyl-L-phenylalaninamide was found to be a hexapeptide amide. Also, under comparable conditions, there was considerable difference in the time of appearance and the yield of the insoluble polymeric precipitate. The suggestion was made (Fruton *et al.*, 1953; Fruton, 1957) that the polymeric peptides were formed in a sequence of successive transamidation reactions in which a dipeptide residue (*e.g.* glycyl-L-tyrosyl) was added to the amino end of the growing peptide chain until a chain length was reached that rendered the product insoluble.

One of the principal objectives of the present study was to examine more closely the validity of the above hypothesis about the mechanism of the polymerization reaction, by seeking to identify the nature and role of intermediates in the process. For this purpose, the polymerization of glycyl-L-tyrosinamide was selected for closer study because extensive deamidation of this substrate occurs before an insoluble precipitate appears, and it seemed likely that intermediates might be present in the reaction mixture in readily detectable amounts. During the course of recent investigations on polymerization reactions catalyzed by cathepsin C, it was found that glycyl-DL-tryptophanamide is an exceptionally favorable substrate, and a report of initial experiments with this compound is included in this communication.

RESULTS

Studies with Glycyl-L-tyrosinamide.—When glycyl-L-tyrosinamide (0.05 M) was subjected to the action of cysteine-activated cathepsin C at pH 7.6,

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† Alexander Brown Coxe Fellow of the Yale School of Medicine.

‡ To whom inquiries should be sent.

and samples of the reaction mixture were analyzed by paper electrophoresis (for details, see experimental section), it was found that the ninhydrin-reactive spot (at 35 cm) corresponding to unchanged substrate became less intense, denoting its gradual disappearance. It should be added, however, that even after prolonged incubation (5–24 hours at 37°), glycyl-L-tyrosinamide was still present in the incubation mixture, and the deamidation did not reach 100% of the theory. Insoluble polymer appeared at about 80 minutes, and it is probable that the inhibition is caused partly by removal of the enzyme from solution by the precipitate, since earlier work (Fruton *et al.*, 1953) had indicated that addition of fresh enzyme and cysteine (but not cysteine alone) results in the resumption of deamidation.

During the early stages of the incubation (1–2 hours), the principal new ninhydrin-reactive components that appear upon paper electrophoresis are at 12 cm and at 25 cm. The first of these has a mobility corresponding to that of glycyl-L-tyrosine, which is formed by hydrolysis of the dipeptide amide. Earlier work (Fruton *et al.*, 1953) had shown that, even at pH 7.6, an appreciable fraction of glycyl-L-tyrosinamide undergoes enzymic hydrolysis, in contrast to the behavior of L-alanyl-L-tyrosinamide or, as will be mentioned later in this paper, of glycyl-L-tryptophanamide.

The other major new ninhydrin-reactive component (at 25 cm) has a mobility identical to that exhibited by an authentic sample of the tetrapeptide amide glycyl-L-tyrosylglycyl-L-tyrosinamide, whose synthesis is described in the experimental section. The amount of tetrapeptide amide (in μmoles per ml of incubation mixture) was estimated to be: at 60 minutes, 2.0; at 140 minutes, 5.0; at 300 minutes, 2.3. Because of the limitations in the precision of the electrophoretic and spectrophotometric procedures employed in the analyses, these amounts can be considered only approximate, and no inferences based on the differences among them appear warranted. They appear to justify the conclusion, however, that the tetrapeptide amide is formed during the polymerization process, and that under the conditions of this experiment, there was relatively little change in the amount of this intermediate between 1 and 5 hours. Additional evidence for the presence of the tetrapeptide amide in the incubation mixture was provided by paper chromatography with 1-butanol–pyridine–water as the solvent; the R_F values of glycyl-L-tyrosinamide, glycyl-L-tyrosine, and the tetrapeptide amide are 0.66, 0.28, and 0.79 respectively.

In several experiments there appeared, upon paper electrophoresis of samples taken from the incubation mixture, a discrete but weak ninhydrin-reactive component of slightly lower mobility (21 cm) than that of the tetrapeptide amide. From the effect of chain length on the relative mobilities of the dipeptide amide and the tetrapeptide amide, it may be inferred tentatively that this additional component corresponds to a longer peptide amide, possibly the hexapeptide amide. The synthesis of glycyl-L-tyrosylglycyl-L-tyrosylglycyl-L-tyrosinamide was attempted by the coupling of carbobenzoxyglycyl-L-tyrosine with glycyl-L-tyrosylglycyl-L-tyrosine ethyl ester in the presence of dicyclohexylcarbodiimide, followed by ammonolysis and hydrogenolysis. The amount of homogeneous product obtained was insufficient for elementary analysis, but its electrophoretic mobility was the same as that of the presumed hexapeptide amide from the enzyme experiment.

Before concluding this summary of results obtained with glycyl-L-tyrosinamide as the sole substrate, it should be mentioned that, upon paper electrophoresis of samples taken after prolonged incubation (5–24 hours) of this compound with cathepsin C, a ninhydrin-reactive component appears at 38 cm, corresponding to L-tyrosinamide. The formation of this product may be attributed to the presence, in the enzyme preparation, of an aminopeptidase-like enzyme that slowly cleaves glycyl-L-tyrosinamide to L-tyrosinamide and glycine (whose mobility is similar to that of glycyl-L-tyrosine). The occurrence of this side-reaction may partly explain the cessation of enzyme action before complete deamidation of glycyl-L-tyrosinamide, since L-tyrosinamide has been found to be a competitive inhibitor of cathepsin C (Fruton and Mycek, 1956). Tyrosinamide is resistant to hydrolysis by the cathepsin C preparation under the conditions of the present experiments.

Earlier studies had shown that cathepsin C catalyzes reactions involving dipeptide esters (Wiggans *et al.*, 1954), and an experiment analogous to that described above with glycyl-L-tyrosinamide was also performed with glycyl-L-tyrosine ethyl ester. Upon paper electrophoresis of a sample of the incubation mixture, a ninhydrin-reactive component was observed at 26 cm, corresponding to the mobility of an authentic sample of glycyl-L-tyrosylglycyl-L-tyrosine ethyl ester. and a somewhat slower component at 20.5 cm may tentatively be suggested as being the corresponding hexapeptide ester. Glycyl-L-tyrosine and the unchanged dipeptide ester were identified at 10 cm and 35 cm respectively.

Action of Cathepsin C on Glycyl-L-tyrosylglycyl-L-tyrosinamide.—When cathepsin C (0.2 units per ml) is allowed to act on the synthetic tetrapeptide amide (0.05 M) at pH 7.5 and 37°, in the presence of 0.004 M cysteine, the extent of ammonia liberation (in μmoles per ml of incubation mixture) during the first 2 hours is very small (60 minutes, 0; 120 minutes, 2.0). However, during this period, appreciable amounts of glycyl-L-tyrosine are formed, as estimated by elution of the appropriate region (11 cm) of paper electropherograms, and measurement of the absorbance of eluates at 280 mμ. From these determinations, it may be estimated that the amounts of glycyl-L-tyrosine formed from the tetrapeptide amide (in μmoles per ml) in the above experiment were 4.7 in 60 minutes, and 15.6 in 120 minutes. In addition to glycyl-L-tyrosine, a strong new ninhydrin-reactive component was observed at 35 cm (assigned to glycyl-L-tyrosinamide), and a weak component was noted at 21 cm (possibly the hexapeptide amide).

These observations are consistent with the known specificity of cathepsin C in its preferential action

at CO–NH bonds separated from a free α-amino group by two α-amino acid residues (Izumiya and Fruton, 1956). Presumably, the tetrapeptide amide is first hydrolyzed to glycyl-L-tyrosine and glycyl-L-tyrosinamide; the latter product then may be expected to undergo deamidation with the liberation of the small amount of ammonia measured in 2 hours, and to participate in the polymerization reaction (an insoluble precipitate was observed at 4 hours in the above experiment). The conclusion that the liberated ammonia is derived from the secondary reactions of the dipeptide amide, rather than direct hydrolytic deamidation of the tetrapeptide amide, is supported by the failure to observe the appearance of a ninhydrin-reactive component corresponding to glycyl-L-tyrosylglycyl-L-tyrosine upon paper electrophoresis of the incubation mixture.

Although the presence of glycyl-L-tyrosylglycyl-L-tyrosinamide can be demonstrated in a system in which cathepsin C catalyzes the polymerization of glycyl-L-tyrosinamide, and cathepsin C is capable of subjecting the tetrapeptide amide to hydrolysis in a manner consistent with the known specificity of the enzyme, free tetrapeptide amide does not appear to function as a "primer" in the polymerization of glycyl-L-tyrosinamide. If free tetrapeptide amide could be used directly in the formation of the polymer from the dipeptide amide, it might have been expected that addition of tetrapeptide amide would increase both the rate of deamidation of the dipeptide amide and the rate of appearance of insoluble polymer, in analogy to the effect of oligoglucosides on the action of starch phosphorylase on glucose-1-phosphate (Whelan and Bailey, 1954). This expectation was not met by the data obtained in an experiment in which various amounts of synthetic tetrapeptide amide were added to cysteine-activated cathepsin C and glycyl-L-tyrosinamide at pH 7.6. As may be seen from Table I, the addition

TABLE I

EFFECT OF TETRAPEPTIDE AMIDE ON DEAMIDATION OF GLYCYL-L-TYROSINAMIDE BY CATHEPSIN C

Concentration of glycyl-L-tyrosinamide acetate, 0.05 M; concentration of glycyl-L-tyrosylglycyl-L-tyrosinamide acetate (GTGTA) indicated below; cysteine, 0.004 M; pH adjusted to 7.6 with 0.1 N NaOH; 0.2 units of cathepsin C per ml.

	Ammonia Liberation			
Time (min.)	No GTGTA[a] (μmoles per ml)	0.01 M GTGTA[b] (μmoles per ml)	0.025 M GTGTA[c] (μmoles per ml)	0.05 M GTGTA[d] (μmoles per ml)
30	3.3	3.2	3.3	3.6
60	6.1	6.5	7.3	7.9
90	8.5	9.7	11.1	10.0
120	10.3	10.8	13.6	12.7
240	10.6	11.1	13.0	14.0

[a] Precipitate appeared in 75 minutes. [b] Precipitate appeared in 90 minutes. [c] Precipitate appeared in 110 minutes. [d] Precipitate appeared in 125 min.

of increasing amounts of the tetrapeptide amide caused only slight increases in the extent of ammonia liberation, the differences from the control being attributable largely to the cleavage of the tetrapeptide amide, and subsequent deamidation of the resulting dipeptide amide. Moreover, the time of appearance of the insoluble polymer, instead of being shortened by the addition of the tetrapeptide amide, was lengthened. It should be added that the addition of glycyl(diglycyl)glycine (0.01 M, 0.03 M, or 0.05 M) to a reaction mixture in which cathepsin C acted on glycyl-L-tyrosinamide under the conditions given in Table I (except for the use of 0.01 M β-mercaptoethylamine as activator) gave results similar to those reported in the Table. No significant change in the rate of deamidation was noted, and the time for the appearance of polymer was lengthened with increased concentration of added tetrapeptide. With 0.01 M tetrapeptide, the increase in time over the control was 14 minutes; with 0.03 M tetrapeptide, 36 minutes; with 0.05 M tetrapeptide, 41 minutes. The amino acid composition of the resulting insoluble polymers has not yet been determined.

To obtain additional information about the role of glycyl-L-tyrosylglycyl-L-tyrosinamide in the polymerization reaction, C^{14}-labeled glycyl-L-tyrosinamide (0.05 M, 4,560 cpm per μmole) was incubated with cathepsin C in the absence and the presence of unlabeled tetrapeptide amide (0.01 M or 0.025 M). These concentrations of tetrapeptide amide are larger than that found (about 0.003 M) in the reaction mixture when cathepsin C acts upon glycyl-L-tyrosinamide alone. The polymer isolated from the reaction mixture without added tetrapeptide amide had a specific radioactivity of 4,650 cpm per μmole tyrosine; the polymer obtained in the presence of 0.01 M tetrapeptide amide had 3,120 cpm per μmole tyrosine; the polymer obtained in the presence of 0.025 M tetrapeptide amide had 1,840 cpm per μmole tyrosine. These values were unchanged (within the precision of the counting procedure) upon two reprecipitations of the polymer preparations, and a control experiment showed that the method employed for washing the polymer was effective in removing unlabeled tetrapeptide amide. It must be concluded, therefore, that tyrosyl residues (in the form of glycyltyrosyl units) from the added tetrapeptide amide had become incorporated in the polymer. Although the time of appearance of polymer was lengthened by the addition of tetrapeptide amide (as in the experiment reported in Table I), the yield of insoluble polymer was markedly increased; with 0.025 M tetrapeptide amide, about three times as much polymer was obtained as in the experiment without added tetrapeptide amide.

Studies with Glycyl-DL-tryptophanamide.—When cathepsin C acts on glycyl-DL-tryptophanamide at pH 7.6, the most striking result is the appearance of a gelatinous precipitate within a few minutes of the start of the reaction, and the continued liberation of ammonia until approximately 40–50% of the theoretical amount (based on the DL-compound) is released. It will be seen from the data in Table II that, at this pH value, there is no measurable hydrolytic liberation of carboxyl groups, as measured by the Grassmann-Heyde (1929) titration, and the entire ammonia liberation may therefore be attributed to a transamidation reaction. At pH 5.1, however, the extent of transamidation is negligible, the ammonia release being attributable almost entirely to hydrolysis, and no insoluble precipitate is noted.

TABLE II

ACTION OF CATHEPSIN C ON GLYCYL-DL-TRYPTOPHANAMIDE

Substrate concentration, 0.05 M; enzyme concentration, 0.2 units per ml; cysteine concentration, 0.01 M. In the absence of enzyme, no ammonia liberation was noted.

pH	Time (min.)	Ammonia Liberation (μmoles per ml)	Carboxyl Liberation (μmoles per ml)	Extent of Transamidation (μmoles per ml)
5.1[a]	20	2.1	2.5	−0.4
	40	5.3	4.3	1.0
	120	13.0	13.2	−0.2
6.1[b]	20	3.0	2.1	0.9
	40	6.1	4.1	2.0
	80	10.3	5.7	4.6
	120	14.0	7.7	6.3
7.1[c]	20	6.4	0	6.4
	40	10.9	0	10.9
	80	16.7	0	16.7
	120	19.7	0.5	19.2
7.6[d]	20	9.7	0	9.7
	40	14.6	0	14.6
	80	21.0	0	21.0

[a] No precipitate observed within 120 minutes. [b] Turbidity observed at 70 minutes. [c] Precipitate observed at 20 minutes. [d] Precipitate observed at 7 minutes.

At pH 6.1, the extent of transamidation is roughly similar to that of hydrolysis. It will be noted that the rate of deamidation (sum of transamidation and hydrolysis) markedly increases from pH 5.1 to pH 7.6, suggesting that the transamidation reaction leading to polymer formation is favored over the hydrolytic process.

Paper electrophoresis of an incubation mixture at pH 5.1 gave ninhydrin-positive spots corresponding in mobility to that of glycyl-L-tryptophan (15 cm) and of glycyltryptophanamide (32 cm). On the other hand, the incubation mixture at pH 7.6 failed to give a distinctive spot of glycyltryptophan, but instead there appeared a new ninhydrin-reactive component at 20 cm. By analogy with the results from the study of the enzymic polymerization of glycyl-L-tyrosinamide, this component may be the tetrapeptide amide.

Examination of the chemical nature of the insoluble precipitate suggests, by analogy with the results on other polymers formed by cathepsin C, that it is an octapeptide amide containing alternating glycyl and tryptophyl residues. This is indicated by the result of alkaline hydrolysis, and the finding that the molar ratio of tryptophan (estimated spectrophotometrically) to amide-NH_3 is approximately 4; the reliability of this analytical procedure was tested by subjecting glycyl-DL-tryptophanamide to the same operations. The spectrophotometric estimation of the molecular weight of the 2,4-dinitrophenyl derivative of the polymer gave a value of 1185, in fair agreement with the value of 1156 for the derivative of the presumed octapeptide amide. The analysis of the polymer for C, H, and N (Dumas) also gave values in fair accord with the theory, and the ratio of total N to amide-N was 12.6 (theory, 13).

In view of the known stereochemical specificity of cathepsin C in its preferential action on dipeptide amides in which the sensitive carbonyl group belongs to an L-amino acid residue (Fruton *et al.*, 1953; Izumiya and Fruton, 1956), it appears reasonable to assume that the tryptophyl residues in the polymer are largely in the L-configuration, although the possibility exists that the carboxyl-terminal amino acid residue of some of the peptide chains may be a D-tryptophyl residue. This possibility is a consequence of the uncertainty in regard to the stereochemical specificity of cathepsin in its utilization of glycyl-D-tryptophanamide as the amine acceptor in a transamidation reaction. The question of the configuration of the tryptophyl residues in the polymer has presented some experimental difficulties, because acid hydrolysis is excluded, because alkaline hydrolysis causes racemization, and because the insolubility of the polymer in water renders enzymic hydrolysis (*e.g.* by chymotrypsin) uncertain. The only evidence that can be offered at present in favor of the view that the tryptophyl residues of the polymer are largely (or entirely) of the L-configuration is the fact that the polymer is optically active and that the filtrate after the removal of the insoluble octapeptide amide is levorotatory. β-Mercaptoethylamine was used as activator of cathepsin C (Fruton and Mycek, 1956) so as to avoid interference by L-cysteine or L-cystine in the measurement of optical activity, and the result was consistent with the conclusion that the filtrate contained glycyl-D-tryptophanamide. Since neither of the enantiomorphs of glycyltryptophanamide is available in pure form at present, the expectation that glycyl-D-tryptophanamide is levorotatory at pH 7.5 is based only on analogy with the similarity of the sign of optical rotation (in water) for glycyl-L-tryptophan, glycyl-L-tyrosine, and glycyl-L-tyrosinamide, all of which are dextrorotatory (specific rotations, +25° to +50°).

For reasons that are unclear at present, it has proved to be difficult to prepare analytically pure samples of glycyl-L-tryptophanamide acetate (or hydrochloride) by hydrogenolysis of the carbobenzoxy derivative (Smith, 1948). Studies are in progress in the hope of resolving this problem, since the availability of pure samples of the L-substrate would permit unequivocal experiments on its polymerization by cathepsin C.[1]

As a further approach to the problem of the specificity of the polymerization reactions catalyzed by cathepsin C, an experiment was performed in which a mixture of 0.05 M glycyl-DL-tryptophanamide and 0.025 M glycyl-L-tyrosinamide was subjected to the action of the enzyme at pH 7.5. It will be noted from the data in Table III that the extent of deamidation in the mixture is less than the sum of that observed when the two substrates were incubated separately; this is consistent with the approximate values of K_m (pH 7.5) of 0.003 M for glycyltryptophanamide and of 0.008 M for glycyl-L-tyrosinamide. It is of interest that the time of appearance of the insoluble polymer was later with the mixture of the two substrates than with glycyl-DL-tryptophanamide alone. Examination of the insoluble product (for details, see experimental section) suggests that it may be an octapeptide amide in which the ratio of tryptophyl residues to tyrosyl residues to amide-N is 3:1:1

[1] ADDED IN PROOF: Since the submission of this paper, Dr. D. M. Theodoropoulos of this laboratory has prepared pure glycyl-L-tryptophanamide as the *p*-toluenesulfonate.

TABLE III

ACTION OF CATHEPSIN C ON MIXTURE OF GLYCYL-DL-TRYPTOPHANAMIDE AND GLYCYL-L-TYROSINAMIDE

Concentration of glycyl-DL-tryptophanamide, 0.05 M; concentration of glycyl-L-tyrosinamide, 0.025 M; cysteine, 0.01 M; pH adjusted to 7.5 with 0.1 N NaOH; enzyme concentration, 0.2 units per ml.

Substrate	Time (min.)	Ammonia Liberation (μmoles per ml)
Glycyl-DL-tryptophanamide[a]	15	9.4
	30	10.3
	45	16.9
Glycyl-L-tyrosinamide[b]	15	4.6
	30	7.1
	60	9.8
	90	13.9
Glycyl-DL-tryptophanamide + Glycyl-L-tyrosinamide[c]	15	8.1
	30	12.5
	60	20.3
	90	25.7

[a] Precipitate appeared 6 minutes after start. [b] No precipitate observed during 90-minute period. [c] Precipitate appeared 20 minutes after start.

DISCUSSION

The results presented in this communication are consistent with the working hypothesis that cathepsin C catalyzes the polymerization of dipeptide amides such as glycyl-L-tyrosinamide by a sequence of transamidation reactions, the first of which involves the formation of a tetrapeptide amide (*e.g.*, glycyl-L-tyrosylglycyl-L-tyrosinamide). The data do not appear to support the idea that free tetrapeptide amide can serve as a "primer" in polymer formation, although further work is needed to establish this conclusion more definitely. If, in the experiment with C^{14}-labeled glycyl-L-tyrosinamide, the main role of the added unlabeled tetrapeptide amide were to serve as acceptor for C^{14}-labeled glycyltyrosyl units, the insoluble decapeptide amide obtained in this experiment should have had a specific radioactivity of 2,740 cpm per μmole tyrosine (4560 × 0.6), a value higher than that found in the presence of added 0.025 M tetrapeptide amide. As a primer, the tetrapeptide amide might have been expected, at a concentration of 0.025 M, to lead to more rapid polymer formation than at 0.01 M, but not to cause more extensive dilution of C^{14} in the polymer. The results actually obtained are contrary to these expectations, and a major role of the added tetrapeptide amide as a primer seems unlikely under the experimental conditions of this study.

A more satisfactory interpretation of the data obtained in the isotope experiment is that the tetrapeptide amide is a better donor of its amino-terminal glycyltyrosyl unit than is glycyl-L-tyrosinamide. It has been shown in this paper that the tetrapeptide amide is rapidly cleaved by cathepsin C to glycyl-L-tyrosine and glycyl-L-tyrosinamide when no acceptor such as the dipeptide amide is initially present in high concentration. In the presence of 0.05 M glycyl-L-tyrosinamide, however, the dipeptide amide may be expected to compete effectively with water for reaction with the "activated" glycyltyrosyl units from the tetrapeptide amide. The view that the amino-terminal glycyltyrosyl unit of the tetrapeptide amide is used more effectively for polymer formation (in the presence of glycyl-L-tyrosinamide) is supported by the increased dilution of C^{14} in the polymer with increased concentration of added unlabeled tetrapeptide amide and by the fact that the yield of polymer obtained with 0.025 M added tetrapeptide amide (0.05 M glycyltyrosyl units) is more than twice that with 0.05 M glycyl-L-tyrosinamide alone. Experiments are now in progress to determine the relative extent of incorporation of C^{14} into the decapeptide amide when cathepsin C is incubated with unlabeled glycyl-L-tyrosinamide and with tetrapeptide amide labeled either in the amino-terminal or the carboxyl-terminal glycyltyrosyl unit.

The available data suggest therefore that, although glycyl-L-tyrosylglycyl-L-tyrosinamide is an intermediate in the polymerization of glycyl-L-tyrosinamide by cathepsin C, the tetrapeptide amide formed on the enzyme may be handled differently from tetrapeptide amide either added to the solution or released from the active region of the enzyme. The release of a portion of the tetrapeptide amide formed from the dipeptide amide is suggested by the finding of about 3 μmoles per ml of tetrapeptide amide by electrophoretic separation of the components of an incubation mixture (initial concentration of dipeptide amide, 50 μmoles per ml). Although the accuracy of this figure must be taken with reserve, in view of the uncertainties of the analytical method used, it would seem that the amount of tetrapeptide amide detected in the incubation mixture is much greater than could be accounted for solely on the basis of enzyme-bound intermediate. In the present experiments, the incubation mixture usually contained about 0.04 mg of protein per ml, and the particle weight of cathepsin C has been estimated to be about 235,000 (de la Haba *et al.*, 1959). In the absence of data on the specific activity of a homogeneous preparation of cathepsin C, or on the number of catalytic sites per unit of particle weight, a calculation of the absolute enzyme concentration cannot be made, but it appears safe to assume that it is much lower than 3 μmoles per ml. If, as seems likely, a portion of the tetrapeptide amide formed by transamidation is released from the enzyme, the results presented above suggest that the free tetrapeptide amide is preferentially cleaved instead of recombining with the enzyme in a manner that permits it to serve as a primer in the polymerization reaction. It may be suggested, therefore, that in the enzymic polymerization of glycyl-L-tyrosinamide (and of other suitable dipeptide derivatives), only that portion of the intermediate tetrapeptide amide which remains bound to the enzyme participates in the next step of the polymerization reaction leading to the formation of the corresponding hexapeptide amide.

By analogy with the behavior of other proteinases that act at both amide and ester bonds, it is reasonable to assume that, in the action of cathepsin C, acyl-enzyme intermediates (*e.g.*, glycyltyrosyl-enzyme) are formed. The efficiency of the polymerization, as expressed in yield of polymer, would then depend not only on the net rate of formation of acyl-enzyme, but also on the extent to which the acyl-enzyme was cleaved by water instead of an

amine acceptor in a transamidation reaction (Fruton, 1957). With glycyl-L-tyrosinamide as the substrate, such hydrolytic cleavage appears to be considerably greater (under comparable experimental conditions) than with other dipeptide amides, notably L-alanyl-L-phenylalaninamide (Fruton *et al.*, 1953) and glycyl-L-tryptophanamide, and the yield of glycyltyrosyl polymer is much lower than with either of the other two dipeptide amides. In view of the conclusions drawn above about the cleavage of the tetrapeptide amide released from the enzyme surface, it may be inferred that, in the formation of the alanylphenylalanyl or glycyltryptophyl polymer, intermediates such as the corresponding tetrapeptide amide are utilized more effectively in the polymerization, possibly because they are released less readily from the active region of the enzyme than the oligopeptide intermediates in the formation of the glycyltyrosyl polymer.

Further studies are needed to establish more definitely whether, as suggested by these results, a "single-chain" mechansim of polymerization is operative in the action of cathepsin C. If the growth of the polymer is by addition of "activated" dipeptide units to the amino end of the growing chain (Fruton, 1957), the polymerization of glycyl-L-tyrosinamide might be expected to involve: (1) the transfer of the glycyl-L-tyrosyl unit from the dipeptide amide to an "active site" on the enzyme, presumably by the formation of an acyl-enzyme; (2) the reaction of the glycyltyrosyl-enzyme with a molecule of glycyl-L-tyrosinamide to yield a tetrapeptide amide still bound to the enzyme, but with release of the "active site" so that another molecule of the dipeptide amide can transfer its glycyltyrosyl unit to the enzyme; (3) a favorable steric relation between the α-amino group of the enzyme-bound tetrapeptide amide and the reactive carbonyl group of the glycyltyrosyl-enzyme; (4) subsequent addition of further glycyltyrosyl units, via glycyltyrosyl-enzyme intermediates, to the enzyme-bound hexapeptide amide until an insoluble polymer is formed.

It should be noted, however, that the available data do not rule out the possibility that the polymerization reactions catalyzed by cathepsin C involve the successive addition of dipeptide amide to the carboxyl end of the growing peptide chain. In such a single-chain mechanism, step 1 would be the same as suggested in the preceding paragraph, but this would be followed by: (2a) release of the "active site" by the formation of the enzyme-bound tetrapeptide amide, followed by the formation of a glycyltyrosylglycyltyrosyl-enzyme by the reaction of the $CO-NH_2$ group of the bound tetrapeptide amide with this site; (3a) a favorable steric relation between the activated carbonyl group of this acyl-enzyme and the α-amino group of the glycyltyrosinamide that is added to form an enzyme-bound hexapeptide amide; (4a) formation of an acyl-enzyme involving the carboxyl-terminal CO group of the enzyme-bound hexapeptide amide, and addition of further glycyltyrosinamide molecules until an insoluble polymer is formed.

Although a clear-cut decision between these two possible single-chain mechanisms cannot be made at present, the available data appear to favor polymerization by the addition of glycyltyrosyl units to the amino end of the growing chain. The occurrence of step 2a might be expected to yield, as a by-product, glycyl-L-tyrosylglycyl-L-tyrosine through hydrolysis of the postulated acyl-enzyme; thus far, there has been no evidence for the appearance of this tetrapeptide, although further work may reveal its presence in the reaction mixture. Furthermore, the reversibility of the reaction between glycyltyrosyl-enzyme and glycyltyrosinamide would be expected to lead to cleavage of the interior peptide bond of the enzyme-bound tetrapeptide amide, rather than the activation of the terminal $CO-NH_2$ group.

Whichever of these two possible mechanisms may be operative in the action of cathepsin C, it may be suggested, as a working hypothesis, that only a limited portion of the growing polypeptide chain (*e.g.*, the amino-terminal dipeptide unit) remains bound to the enzyme, and that the chain "rolls off" as successive glycyltyrosyl units are added by transamidation reactions. To make such "roll-off" possible, it seems necessary to assume, especially in the case of addition to the amino end of the chain, the presence of two equivalent pairs of "binding sites" in the vicinity of the "active site" of the enzyme. Each of these pairs of binding sites is thought to include one site that interacts specifically with the α-amino group of the dipeptide amide or of the growing chain, and another that interacts with some other part of the terminal dipeptide unit, the NH group of the peptide bond being suggested. The hypothetical model suggested here resembles, in principle, that suggested by Corey (1959) for the stereospecific isotactic polymerization of propylene oxide with $FeCl_3$ catalyst.

The assumption of two binding sites per dipeptide unit is based on the stereochemical specificity of cathepsin C (Izumiya and Fruton, 1956), and is consistent with the finding that L-phenylalaninamide is much more effective as a competitive inhibitor of the enzyme than is the D-isomer (Fruton and Mycek, 1956). In regard to the choice of the peptide NH group as one of two binding sites, it may be noted that glycyl-*N*-methyl-L-phenylalaninamide is resistant to the action of cathepsin C, in contrast to the ready cleavage of glycyl-L-phenylalaninamide (Izumiya and Fruton, 1956).

A point of some interest in connection with any model for the catalysis of reactions of dipeptide amides and dipeptide esters by cathepsin C is that the enzyme promotes intermolecular condensation rather than intramolecular cyclization leading to diketopiperazine formation, a process long known to be favored in nonenzymic systems at alkaline pH values (for a recent study, see Wieland and Bernhard, 1953). This difference in behavior supports the view that the α-amino group and the reactive carbonyl group of a single dipeptide unit are held by the enzyme in a steric relation not favorable for intramolecular reaction. Furthermore, the fact that intermolecular condensation occurs so readily suggests, as indicated above, that the α-amino group of the acceptor is held at a binding site near

the reactive carbonyl group. It seems likely that acceptor amines other than dipeptide amides (or polymers derived from them) may also be held at this binding site; for example, hydroxylamine is an excellent acceptor in transamidation reactions catalyzed by cathepsin C, but strongly inhibits the polymerization of dipeptide amides (Jones *et al.*, 1952).

We hope to investigate further the validity of the single-chain mechanism in the enzymic polymerization of dipeptide amides by allowing cathepsin C to act on a mixture of two dipeptide amides which differ in the rate and extent of conversion to polymer and in the chain-length of the polymer products. It may be expected that if two donors of dipeptide units differ greatly in their ability to form acyl-enzyme, or if oligopeptide intermediates are released from the active region of the enzyme to a different extent, the synthesis of one type of homopolymer will be preferred over the other; if a copolymer is formed, the dipeptide unit from the more effective donor may be expected to predominate in the polymer preparation obtained. The structural differences between substrates leading to such differences in the nature of the polymers formed are clearly related to the specificity of the enzyme in the catalysis of polymerization reactions. The experiment reported above, in which cathepsin C acted on a mixture of glycyl-L-tyrosinamide and glycyl-DL-tryptophanamide, is an initial attempt to study the specificity of enzymic polymerization from this point of view. In order to establish, however, the presence, in a polymer preparation, of a mixture of different homopolymers or of a copolymer, methods are needed for the separation of such oligopeptides so that the amino acid sequence of homogeneous products can be determined. Efforts along this line are currently in progress.

If further work should support the plausibility of the "roll-off" model suggested above for the action of cathepsin C, the principle it implies may have pertinence to the mechanism of other enzyme-catalyzed polymerization reactions, such as those catalyzed by polysaccharide phosphorylases and transglycosylases (Hestrin, 1959) and by nucleotide phosphorylases (Singer *et al.*, 1960a, 1960b). Bovey (1959) has presented evidence in favor of the view that, in the action of dextransucrase on sucrose, a single-chain mechanism is operative and that the growing polysaccharide chain remains with the enzyme as glucosyl units are added. In the transfructosylation reaction catalyzed by levansucrase, French (1959) has suggested that both the donor (*e.g.*, sucrose) and the acceptor (the nonreducing end of a levan chain) are bound to the enzyme, and that after transfer of a fructosyl unit to the acceptor, the chain "shifts" on the enzyme surface without dissociation from the active region. If two sets of complementary binding sites were operative in this polymerization, as suggested above for the process catalyzed by cathepsin C, the idea of a "shift" could be replaced by a cyclic mechanism in which a fructosyl unit from the donor would be held at the same binding sites both before and after formation of the glycosidic bond.

In addition to the above considerations, mention should be made of the possibility that the enzymic cleavage of polymers (polypeptides, polysaccharides, polynucleotides) may in some instances involve the reverse of the "roll-off" single-chain mechanism suggested above. In the degradative process catalyzed by cathepsin C, it might be assumed that the amino-terminal portion of the substrate interacts with the active region of the enzyme in such a manner as to form an acyl-enzyme to which the residual polypeptide is still held at a pair of binding sites. Hydrolysis of the acyl-enzyme to release a dipeptide (*e.g.*, glycyl-L-tyrosine) would free the active site for the next cleavage of the peptide chain, and so forth. Such a mechanism would be consistent with the observations of Waley and Watson (1953) on the action of trypsin on poly-L-lysine. The possibility that the mechanism may also apply to the enzymic hydrolysis of other homopolymers is suggested by the finding that, in the hydrolytic action of β-amylase, maltose units are released in large part by single-chain degradation (Bailey and French, 1957; Bailey and Whelan, 1957).

Experimental

Enzyme Preparation.—The preparation of cathepsin C used in these experiments was obtained in the manner described previously (de la Haba *et al.*, 1959) except that the steps after the heat treatment were omitted. The enzyme solution contained, per ml, 20 units of cathepsin C having a specific activity $[C.U.]^{GTA}_{mEN} = 29$.

Enzyme Experiments.—The substrate concentration was 0.05 M, except where otherwise noted. As enzyme activator, L-cysteine (0.004 M or 0.01 M) or β-mercaptoethylamine (0.01 M) was employed. The pH of the reaction at pH 5–6 was controlled by means of 0.1 citrate buffers, and at pH 7–7.6 by allowing the dipeptide amides to serve as buffers. The temperature was 37.5°. Ammonia liberation was measured by the Conway microdiffusion method (Johnston *et al.*, 1950), and carboxyl liberation by the method of Grassmann and Heyde (1929).

Paper Electrophoresis and Paper Chromatography.—Paper electrophoresis was conducted by use of Whatman No. 1 paper, with pyridine–glacial acetic acid–water (10:0.4:90 v/v), pH 6.4, as the buffer solution, for 5 hours at 1000 volts (20 volts per cm). Under these conditions, all the ninhydrin-positive components of interest in this investigation migrated toward the cathode. Samples (usually 0.25 ml) of the incubation mixture were diluted to 1 ml with absolute ethanol, and 0.01–0.02 ml portions were applied to the paper. For paper chromatography (descending), Whatman No. 1 paper and the following solvent mixtures were used: 1-butanol–pyridine–water (5:2:3 v/v), *sec*-butanol–formic acid–water (75:15:10 v/v), and isopropanol–ammonia–water (8:1:1 v/v) were used. In all cases, the paper was dried at room temperature before being sprayed with a 1% (w/v) solution of ninhydrin in 95% ethanol. In both electrophoresis and chromatography, appropriate reference compounds were always run on the same sheet of paper.

Glycyl-L-tyrosinamide Acetate.—This compound

was prepared as described previously (Fruton and Bergmann, 1942).

Glycyl - L - tyrosylglycyl - L - tyrosinamide Acetate.—A mixture of 2.7 g (9 mmoles) of glycyl-L-tyrosine ethyl ester hydrochloride (Izumiya and Fruton, 1956), 3.4 g (9 mmoles) of carbobenzoxyglycyl-L-tyrosine (Bergmann and Fruton, 1937), 1.8 g (9 mmoles) of dicyclohexylcarbodiimide, and 1.25 ml (9 mmoles) of triethylamine in 100 ml of tetrahydrofuran was stirred at room temperature for 20 hours. The filtrate obtained after removal of dicyclohexylurea and triethylammonium chloride was concentrated under reduced pressure, and the residue was dissolved in ethyl acetate. The solution was washed with aqueous 5% $NaHCO_3$, water, N hydrochloric acid, and water, and dried over exsiccated Na_2SO_4. After the solution had been concentrated under reduced pressure, the addition of petroleum ether gave a solid carbobenzoxytetrapeptide ethyl ester (2.4 g) that was reprecipitated from ethanol-ether. M.p., 105–115°.

Anal. Calcd. for $C_{32}H_{36}O_9N_4$ (620.6): N, 9.0. Found: N, 8.8.

The carbobenzoxytetrapeptide ester (1.0 g) was dissolved in 15 ml of absolute methanol that had been saturated with NH_3 at 0°, and the solution was kept at room temperature for 3 days in a pressure bottle. Evaporation of the solvent gave a crystalline residue that was recrystallized from ethanol-ether. M.p., 222° (decomp.); yield, 0.80 g.

Anal. Calcd. for $C_{30}H_{33}O_8N_5$ (591.7): N, 11.8. Found: 11.6.

Upon hydrogenolysis of 0.60 g of the carbobenzoxytetrapeptide amide in 30 ml of ethanol containing 0.1 ml of glacial acetic acid, with Pd black as the catalyst, the tetrapeptide amide acetate was obtained in 85% yield; $[\alpha]_D^{25} = +26.7°$ (6.45% in water).

Anal. Calcd. for $C_{24}H_{31}O_8N_5$ (517.5): C, 55.7; H, 6.0; N, 13.5. Found: C, 55.4; H, 5.9; N, 13.6 (Dumas).

For the preparation of the tetrapeptide ethyl ester HCl, 1.4 g of the carbobenzoxy derivative was subjected to hydrogenolysis in 30 ml of ethanol containing 3 mmoles of HCl. The product was recrystallized from ethanol-ether by the addition of petroleum ether. M.p. 150–155° (decomp.); yield 78%.

Anal. Calcd. for $C_{24}H_{30}O_7N_4Cl$ (523.0): N, 10.7. Found: 10.3.

Saponification of the carbobenzoxytetrapeptide ester (550 mg) with 5 ml N NaOH for 30 minutes at room temperature gave, upon acidification, a product that was extracted with ethyl acetate. The carbobenzoxytetrapeptide in the organic layer was then extracted with dilute bicarbonate, the aqueous solution was acidified, and the precipitate was again extracted with ethyl acetate. Concentration of the organic layer gave an oily product that was subjected to hydrogenolysis in the usual manner to yield the free tetrapeptide glycyl-L-tyrosylglycyl-L-tyrosine. Upon paper chromatography with 1-butanol–pyridine–water as the solvent, it gave a single ninhydrin-reactive spot of R_F 0.82.

Estimation of Amount of Tetrapeptide Amide Present in Incubation Mixture.—A solution (10 ml) containing 0.05 M glycyl-L-tyrosinamide acetate, 0.004 M cysteine hydrochloride, and 2 units of cathepsin C was adjusted to pH 7.6 by the addition of 0.1 N NaOH, and kept at 37.5° for 5 hours. A precipitate of the polymer appeared at about 80 minutes. At various times during this period, 0.1-ml samples were removed from the reaction mixture for determination of the extent of ammonia liberation, and 0.25-ml samples were diluted to 1 ml with absolute ethanol for electrophoretic analysis. The extent of ammonia liberation (in μmoles per ml of incubation mixture) was found to be: 60 minutes, 6.0; 140 minutes, 14.5; 300 minutes, 14.5. For paper electrophoresis, 0.02-ml samples of the diluted mixture were applied, and after the operation was completed, the paper was dried, and the region corresponding to that of the marker tetrapeptide amide was cut out and eluted with 5 ml of water. The absorbance of the eluate at 280 mμ was determined, with the aqueous extract of an equal amount of paper used as the blank. The measured absorbance was assumed to be due to glycyl-L-tyrosyl-glycyl-L-tyrosinamide, and the concentration of the tetrapeptide amide in the eluate was calculated from the absorbance given by the eluate obtained by paper electrophoresis of a known quantity of the synthetic tetrapeptide amide.

Experiment with C^{14}-Labeled Glycyl-L-tyrosinamide.—The labeled dipeptide amide acetate was prepared in the same manner as the unlabeled compound; uniformly C^{14}-labeled L-tyrosine (Nuclear-Chicago) was diluted with unlabeled tyrosine and used as starting material in the synthesis. For the enzyme experiment, three incubation mixtures (5 ml) were prepared, each of which contained, per ml, 50 μmoles of C^{14}-labeled glycyl-L-tyrosinamide acetate (4,560 cpm per μmole), 10 μmoles of β-mercaptoethylamine, and 0.3 units of cathepsin C; the pH was adjusted to 7.7 with 0.1 N NaOH. Solution A contained no other components; solution B also contained 10 μmoles per ml of glycyl-L-tyrosylglycyl-L-tyrosinamide acetate; solution C also contained 25 μmoles per ml of this tetrapeptide amide. After being kept at 37.5° for 2.5 hours (ammonia release after 2 hours, 17.5 to 19 μmoles per ml, or 35 to 38% of the theory for the dipeptide amide), the three suspensions containing precipitated polymer were treated in identical manner. The suspensions were chilled to 0°, and the polymer was washed with five 5-ml portions of water, the resulting suspensions being centrifuged between washings. (In some repetitions of this experiment, the polymers were separated by filtration with suction, and washed on the filter, with similar results.) The washed polymers were dried over P_2O_5 *in vacuo;* the yields from the three incubation mixtures were: (A) 9.5 mg; (B) 16.4 mg; (C) 30.3 mg. For radioactivity determinations, 1.8 to 2.0 mg of dried polymer was dissolved in 5 ml of water by the addition of 0.1 ml of 0.1 N NaOH, and 0.2 ml of the resulting clear solution was evaporated on aluminum planchets. A gas-flow counter (background, 10 ± 1 cpm) was used. To express the specific radioactivity of the polymer in figures comparable to the C^{14}-content of the labeled dipeptide amide, a portion of the solution

prepared for counting was adjusted with 0.17 N NaOH to a final concentration of 0.1 N NaOH, and the absorbance at 294 mμ was determined with a Beckman DU spectrophotometer. By use of the value of 2389 for the molecular extinction coefficient of tyrosine at this wave length (Goodwin and Morton, 1946), the number of μmoles of "tyrosine" in the polymer solution was calculated. It may be added that solutions of the glycyltyrosyl polymer in 0.1 N NaOH gave a clear maximum at 294 mμ, and the measured absorbance of a solution of carefully reprecipitated and dried polymer gave a value of 1150 for the molecular weight of the expected decapeptide amide (calculated molecular weight, 1115).

The specific radioactivity (cpm per μmole tyrosine) of the polymer from mixture A was found to be 4650 (compare with 4560 for the glycyl-L-tyrosinamide used); from mixture B, 3120; from mixture C, 1840. The residual polymer from each incubation mixture was dissolved with 0.1 N NaOH, clarified by filtration, and reprecipitated by adjusting the pH to 6–7 with 0.1 N HCl. The resulting precipitates were redissolved in dilute alkali for counting and spectrophotometric measurement; the specific radioactivity (cpm per μmole tyrosine) of the reprecipitated polymer from (A) was 4550; from (B) 3220; from (C) 1930. A further reprecipitation of the polymers in the manner described above gave samples which had the following specific radioactivity (cpm per μmole tyrosine): (A) 4650; (B) 3000; (C) 1850.

To estimate the extent to which unlabeled tetrapeptide amide remains bound to the polymer after the washing procedure described above, 2.5 mg of polymer (specific radioactivity, 4530 cpm per μmole tyrosine) was dissolved in 2.5 ml of water with 0.1 ml of 0.1 N NaOH and mixed with 0.1 ml of a solution of 6.5 mg of glycyl-L-tyrosylglycyl-L-tyrosinamide acetate. The pH of the resulting solution was about 7, and the polymer separated. It was filtered with suction, washed with three 5-ml portions of water, and redissolved in water as described above. The specific radioactivity of the product was found to be 4580 cpm per μmole tyrosine, indicating that the tetrapeptide amide is not firmly bound to the polymer and that the isotope dilution observed in the enzymic experiment was not a consequence of such tight binding.

Glycyl-DL-tryptophanamide Acetate.—The mixed anhydride was prepared in the usual manner from carbobenzoxyglycine (5 g; 0.024 mole) and isobutyl chlorocarbonate (3.8 ml; 0.028 mole) in the presence of triethylamine (3.9 ml; 0.028 mole), with chloroform (50 ml) as the solvent. A mixture of DL-tryptophan methyl ester HCl (6.1 g; 0.024 mole) and triethylamine (3.9 ml; 0.028 mole) in 40 ml of chloroform was added, and after 20 hours at room temperature the reaction mixture was washed with water, 5% $NaHCO_3$, dilute hydrochloric acid, and water. After it had been dried over Na_2SO_4, the solution was concentrated to yield the oily carbobenzoxydipeptide ester, which could not be induced to crystallize. The ester was dissolved in 50 ml of methanol that had been saturated with NH_3 at 0°, and the solution was kept at room temperature for 2 days. Crystals of the amide separated, and the filtrate was concentrated under reduced pressure to about 10 ml to yield 6.6 g of the product. After recrystallization from methanol, the substance melted at 191–193°.

Anal. Calcd. for $C_{21}H_{22}O_4N_4$ (394.4): C, 63.9; H, 5.6; N, 14.2. Found: C, 64.1; H, 5.3; N, 14.1.

Hydrogenolysis of the above product (4.0 g) in methanol (60 ml) containing 0.29 ml glacial acetic acid gave the dipeptide amide acetate, which was recrystallized from methanol–ethyl acetate. M.p., 159–160°; yield, 84%.

Anal. Calcd. for $C_{15}H_{22}O_4N_4$ (320.3): C, 56.2; H, 6.2; N, 17.5. Found: C, 55.8; H, 6.0; N, 17.5.

The dinitrophenyl derivative was prepared by treatment of glycyl-DL-tryptophanamide acetate (160 mg; 0.5 mmole) with 1-fluoro-2,4-dinitrobenzene (0.08 ml; 0.6 mmole) in the presence of triethylamine (0.17 ml; 1.2 mmole) and dimethylformamide (1 ml). After 45 minutes at room temperature, chloroform was added to yield a crystalline product (184 mg) which was recrystallized from dimethylformamide - chloroform. M.p. 263–264° (decomp.).

Anal. Calcd. for $C_{19}H_{18}O_6N_6$ (426.4): C, 53.5; H, 4.3; N, 19.7. Found: C, 53.2; H, 4.3; N, 20.1 (Dumas).

Carbobenzoxyglycyl-L-tryptophanamide.—Carbobenzoxyglycine (0.01 mole) was coupled with L-tryptophan methyl ester (0.01 mole) by the mixed anhydride method, as described above for the DL-compound. The resulting ester (oil) was converted to the amide, which was recrystallized from methanol. Yield, 2.2 g; m.p. 169–170°. Smith (1948) reported that his preparation softened at 117–121° and melted at 145°.

Anal. Calcd. for $C_{21}H_{22}O_4N_4$ (394.4): C, 63.9; H, 5.6; N, 14.2. Found: C, 63.6; H, 5.4; N, 14.1.

Isolation and Characterization of Polymer from Glycyl-DL-tryptophanamide.—The incubation mixture (50 ml) contained 0.80 g (2.5 mmoles) of glycyl-DL-tryptophanamide acetate, 0.01 M cysteine hydrochloride, 1.8 ml of N NaOH (to bring pH to 7.5), and 1 ml of cathepsin C solution (20 units). It was kept at 37.5° for 185 minutes. Removal of 0.1-ml samples for ammonia determination gave the following values (in μmoles per ml): 15 minutes, 13.3; 25 minutes, 19.5; 35 minutes, 24.6; 75 minutes, 25.3; 185 minutes, 25.3. Since the initial substrate concentration is 50 μmoles per ml, it is clear that the ammonia liberation ceases when one-half of the DL-compound has reacted. The precipitate formed in the reaction began to appear within 8 minutes of the start. At the end of the incubation period, the precipitate was separated by centrifugation, and was extracted five times with 15-ml portions of water, and centrifuged each time. The washed precipitate was dried over P_2O_5 *in vacuo* at 78°. Yield, 198 mg. This amount represents about 70% of that to be expected from the polymerization of 0.40 g of glycyl-L-tryptophanamide acetate. The product contained 0.46% ash, and on an ash-free basis it contained 62.6% C, 5.8% H, and 18.3% N (Dumas). These values are in reasonable agreement with the theory for the octa-

peptide amide glycyl-L-tryptophyl(glycyl-L-tryptophyl)$_2$glycyl-L-tryptophanamide. Calcd. for $C_{52}H_{55}O_8N_{13}$ (990): C, 63.1%, H, 5.6%, N, 18.4%. The product is optically active, having an $[\alpha]_D^{25} = +11.9°$ (1% in glacial acetic acid). The supernatant fluid obtained by centrifuging the polymer produced by the action of cathepsin C on 0.05 M glycyl-DL-tryptophanamide at pH 7.5 in the presence of β-mercaptoethylamine (in place of L-cysteine) was found to be slightly levorotatory ($\alpha = -0.20°$, 2 dm. tube, 25°, Rudolph photoelectric polarimeter).

In order to determine the amide-N of the product, 19.1 mg was subjected to hydrolysis with 2 ml of N $Ba(OH)_2$ at 100° for 16 hours, and the ammonia formed was driven over with N_2 into the boric acid solution used in the Conway microdiffusion method. From the amount of 0.01 N HCl required to neutralize the boric acid solution, it was calculated that the product contained 1.45% amide-N. The reliability of this method was checked by subjecting glycyl-DL-tryptophanamide to similar treatment, and the amide-N found corresponded to the theory within 2% (three determinations). Acid hydrolysis could not be used for the determination of amide-N of the polymeric product, since glycyltryptophanamide gives values of NH_3 release corresponding to 115–125% of theory, presumably owing to the decomposition of the indole nucleus.

The tryptophan content of the alkaline hydrolysate was determined spectrophotometrically according to the method of Goodwin and Morton (1946). The hydrolysate was neutralized with N H_2SO_4, the $BaSO_4$ was removed by filtration, and the filtrate was diluted with NaOH to a volume of 1 liter, and 0.1 N with respect to NaOH. Measurement of the absorbance at 280 mμ (molecular extinction coefficient of tryptophan, 5400) indicated that the hydrolysate from 19.1 mg of product contained 74.5 μmoles of tryptophan. Since the analysis of this hydrolysate for amide-N indicated the liberation of 19.5 μmoles of NH_3, it may be concluded that the ratio of tryptophyl residues to amide-N in the original product was approximately 4.

This result is in accord with the conclusion that the product is an octapeptide amide containing four glycyltryptophyl residues. This view is supported by the ratio of total N to amide-N: 18.3/1.45 = 12.6 (theory for the octapeptide amide, 13).

An estimate of the chain length of the polymer also was obtained by treatment of 50 mg of the above preparation with 0.06 ml of 1-fluoro-2,4-dinitrobenzene in 7.5 ml dimethylformamide, in the presence of 0.07 ml of triethylamine. After 3 hours at room temperature, ether was added. The resulting precipitate was triturated with 20% acetic acid to remove unreacted peptide. Yield, 39 mg. Spectrophotometric determination of the absorbance of a solution of 9.9 mg of the DNP-polymer in 250 ml of glacial acetic acid gave a value of 0.49 at 340 mμ, where a clear maximum was evident. The molecular extinction coefficient at 340 mμ of DNP-glycyl-DL-tryptophanamide in acetic acid was found to be 14,700. From these values it may be calculated that the molecular weight of the DNP-polymer is approximately 1185. This value is in satisfactory agreement with the value of 1156 for the calculated molecular weight of the dinitrophenyl derivative of the octapeptide amide.

Characterization of Polymer from Mixture of Glycyl-DL-tryptophanamide and Glycyl-L-tyrosinamide.—The incubation mixture (5 ml) contained 0.25 mmoles of glycyl-DL-tryptophanamide acetate, 0.125 mmoles of glycyl-L-tyrosinamide acetate, 0.01 M cysteine hydrochloride, 0.2 ml of N NaOH (to bring pH to 7.5), and 0.05 ml of cathepsin C solution (1 unit). It was kept at 37.5° for 90 minutes, at which time 25.7 μmoles NH_3 had been liberated per ml of incubation mixture. The precipitate which had formed (it appeared at about 20 minutes after the start of the incubation) was removed by centrifugation, washed five times with 2-ml portions of water, and dried over P_2O_5 *in vacuo* at 78°. Yield, 9.8 mg. Hydrolysis of 4.36 mg of this product with N $Ba(OH)_2$ in the manner described above yielded 4.55 μmoles of NH_3. The alkaline hydrolysate was neutralized with N H_2SO_4, the $BaSO_4$ was removed, and the amount of tyrosine and tryptophan in the solution was estimated spectrophotometrically by the method of Goodwin and Morton, by taking absorbance measurements of the solution (250 ml, 0.1 N in NaOH) at 280 mμ and 294 mμ. By the use of their formula, it was calculated from the observed absorbance measurements (0.290 at 280 mμ and 0.159 at 294 mμ) that the hydrolysate had contained 4.1 μmoles of tyrosine and 12.7 μmoles of tryptophan. If the assumption is made that the polymeric material obtained in the enzyme experiment is a homogeneous product, it would appear to be an octapeptide amide containing three glycyl-L-tryptophyl residues and one glycyl-L-tyrosyl residue per amide-N. The calculated molecular weight of such a polymer is 957; the molecular weights calculated from the analyses for amide-N, tyrosine, and tryptophan are approximately 960, 1060, and 1030; average, 1020. Clearly, the available data do not warrant the conclusion that the product obtained in this experiment is indeed a homogeneous octapeptide amide of the structure suggested above. Further work is needed to establish more definitely the nature of this material.

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Masked Imidazole Groups in Cyanoferrihemoglobin and Carbonylhemoglobin*

JACINTO STEINHARDT, REMEDIOS ONA, AND SHERMAN BEYCHOK†

From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts

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Earlier papers have shown that the reversible acid denaturation of ferrihemoglobin and carbonylhemoglobin at both 0° and 25° liberates approximately 22 acid-binding groups (presumably imidazole) which do not combine with acid in the native protein. The demonstration for ferrihemoglobin has depended (a) on rapid-flow measurements (2–3 seconds) of pH in solutions of sufficiently high ionic strength (0.3 M chloride) to suppress differences in acid-binding between native and denatured protein which are due only to changes in the electrostatic interaction of their charged groups; and (b) on the fact that the difference in the titration curves does not pass through a well-marked narrow maximum as the pH is lowered (as it would if only electrostatic effects were involved), but remains at a nearly constant level over a wide range of pH. At an ionic strength of 0.3 the difference is not sustained below pH 4, presumably because at lower pH values partial denaturation occurs within the 2 to 3 seconds required for pH measurement. In the present paper recourse is had to titrating cyanoferrihemoglobin (CN-hemoglobin) and carbonylhemoglobin (CO-hemoglobin)—both of which are much more stable to acid than is ferrihemoglobin—to extend the constant difference between the titration curves of native and denatured protein in solutions of high ionic strength to considerably lower pH, and thus to strengthen the interpretation of actual unmasking. The results show an extension of the pH region of constant difference up to 2 pH units, and confirm the earlier conclusions that the basic function of the imidazole groups in 22 histidines is masked in native horse hemoglobin, and that other less basic groups, such as carboxylate, are completely reactive. The results also show that the rate of unmasking in cyanohemoglobin is approximately the same as the rate of denaturation of this protein measured spectrophotometrically.

In acid-denatured carbonylhemoglobin (CO-hemoglobin) (Steinhardt and Zaiser, 1951) or ferrihemoglobin (Steinhardt and Zaiser, 1953) the number of prototropic groups titrating between pH 4.5 and 7.5 exceeds by about thirty-six the number titrating between these limits of pH in the corresponding native proteins. In the earliest report (Steinhardt and Zaiser, 1951) the possibility was advanced that at least part of these "masked" groups were in histidine residues, but weak carboxyls were also considered a strong possibility. In subsequent papers other possibilities were put forward (Zaiser and Steinhardt, 1956; for summary of earlier work, see Steinhardt and Zaiser, 1955). More recently Tanford (1955) suggested that no actual unmasking occurred, but that the effects observed could be attributed quantitatively to changes brought about by denaturation in the electrostatic interaction term w in the familiar titration equation:

$$\mathrm{pH} = \mathrm{pK_{int}} + \log \frac{h}{n - h} - 0.868wz \qquad (1)$$

in which h represents the number of sites of a set n having intrinsic dissociation constant K_{int}, which have dissociated a hydrogen ion at each pH, and z is the net charge at that pH. However, the validity

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† Present address: Department of Neurology, College of Physicians and Surgeons, Columbia University, New York.