Trypsin-Catalysed Transpeptidations

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The way in which proteins are formed in nature is so far obscure. It is generally considered that transpeptidation reactions may play a part in the process (Borsook, 1952; Campbell & Work, 1953), and recently a variety of enzymes have been found to catalyse transamidations. The transamidation reaction may be written:

Thus in an early example (Bergmann & Fraenkel-Conrat, 1937), $\text{X} = \text{C}_6\text{H}_5 \cdot \text{CO}$, $\text{R} = \text{Y} = \text{H}$, $\text{Z} = \text{C}_6\text{H}_5$; many of the reactions studied recently by Fruton and his co-workers have also been of this type with the acceptor molecule ($\text{Z} \cdot \text{NH}_2$) an amino acid or peptide, so that the reaction leads to the lengthening of a peptide chain (Fruton, Johnston & Fried, 1951; Jones, Hearn, Fried & Fruton, 1952). The same molecule may serve as both donor and acceptor when a dipeptide amide is used, leading to polymer formation; thus glycyl-L-phenylalanine amide polymerized in the presence of cathepsin-C (Jones *et al.* 1952). True peptides (as opposed to amides) can also undergo transpeptidation; this has been especially studied with γ -glutamyl peptides (Hanes, Hird & Isherwood, 1952; Kinoshita & Ball, 1953; Fodor, Miller & Waelsch, 1953).

Little, however, is known of the scope of transpeptidation reactions, nor is it known whether they are to be expected from the action of proteolytic enzymes on peptides. We have examined the transferase activity of a typical, crystalline, proteolytic enzyme, trypsin; both true peptides and amides have been used as substrates. The formation of dilysine by the action of a mixture of trypsin and chymotrypsin on the tripeptides lysyltyrosyllysine and lysyltyrosylleucine was reported earlier (Waley & Watson, 1951). It has now been found that trypsin alone will effect this transpeptidation and also catalyses transpeptidations with the following substrates: dilysine, lysine amide, lysyllysyltyrosine amide and pentalysine.

Several proteolytic enzymes have recently been found to catalyse the hydrolysis of esters which are related to their usual substrates (Schwert, Neurath,

Kaufman & Snoke, 1948) and condensation reactions (analogous to transpeptidations) of the type

might therefore be expected. Brenner, Müller & Pfister (1950) found that, in the presence of chymotrypsin, esters of methionine (and some other amino acids) gave the di- and tri-peptides, some higher polymers, and also the amino acid itself. We have observed that trypsin catalyses both the polymerization of lysyllysine ethyl ester and its reaction with tyrosine amide, phenylalanine amide, tyrosyltyrosine amide and tyrosylleucine.

As in our earlier work (Waley & Watson, 1953*a*), the mixed anhydride method of Wieland & Sehring (1950) was much used for the synthesis of peptides. Recently, however, Vaughan (1952) has shown that racemization may accompany peptide synthesis when the mixed anhydride of an acylated dipeptide (e.g. I) is used; the extent of racemization depended

greatly on the solvent. No racemization was observed when the mixed anhydride derived from an acylated amino acid was used. Now a tripeptide may be prepared either by coupling a protected dipeptide with an amino acid ester or by coupling a protected amino acid with a dipeptide ester. The latter method has been used to minimize the danger of racemization. We wished also to avoid the saponification of benzyloxycarbonyl peptide esters, which may be attended by side reactions (Waley & Watson, 1953*a*), and so used benzyl esters extensively. It was convenient in some cases to use formyl as a protective group, since it may be removed without splitting peptide bonds or benzyl esters (Waley, 1953). The azide method of peptide synthesis was also employed and the two routes used for the synthesis of lysyltyrosylleucine are shown below.

Similarly, two routes were used for the preparation of lysyltyrosyllysine; there was little to choose between the two methods for the preparation of the tripeptides, but the mixed anhydride method is simpler for the preparation of dipeptides.

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One dipeptide, tyrosylleucine, was also prepared by two methods; the first was hydrogenation of the benzyl ester (II) and the second was hydrolysis of *O*-acetyltyrosylleucine methyl ester (from reduction of *O*-acetyl-*N*-benzyloxycarbonyltyrosylleucine methyl ester). The alkaline hydrolysis of peptide esters is completed in a few minutes in 0.1N alkali, so that there is usually little danger of racemization. The esters of lysine peptides, however, were shown chromatographically to give by-products in this reaction.

EXPERIMENTAL

Materials

General. The values of the *pK* of the α -amino group of the peptides and their α -amino N as a fraction of the total N were determined by electrometric titration in 0.083M-KCl at 25.8° (Waley & Watson, 1953a). The microanalyses were carried out by Mr G. Ingram of these laboratories. The preparation of the peptides of lysine has been described already (Waley & Watson, 1953a). Crystalline trypsin (50% MgSO_4 , 7H₂O; Armour Laboratories, Lindsey Street, London, E.C. 1) was used. L-Amino acids were used throughout. The error of the optical rotations is the standard deviation, calculated according to the method of Dean & Dixon (1951) for a small number (about ten) of observations. All the peptides prepared were chromatographically homogeneous in the solvent employed (p. 532). The m.p.'s are uncorrected.

Lysine amide. ϵ -Benzyloxycarbonyllysine amide hydrochloride (Bergmann, Zervas & Ross, 1935) was hydrogenated in aqueous methanol containing one mol. prop. of HCl, using Pd black as catalyst. *Lysine amide dihydrochloride*, crystallized from methanol, had m.p. 217–218° (decomp.). (Found: N, 18.5; Cl, 32.3. $\text{C}_8\text{H}_{13}\text{ON}_3$, 2HCl requires N, 19.3; Cl, 32.6%.)

Dibenzoyloxycarbonyllysyltyrosine amide. The peptide synthesis of Wieland & Sehring (1950) was used. Benzoyl chloride (0.58 ml.) was added to dibenzoyloxycarbonyllysine (Bergmann *et al.* 1935) (2.07 g.) and *N*-ethylpiperidine (0.67 ml.) in tetrahydrofuran (5 ml.) at 0°. After 2 min. the mixture was added to tyrosine amide (Blau & Waley, 1954) (0.9 g.) suspended in ethyl acetate (10 ml.) and 1.24N- KHCO_3 (6 ml.). The mixture was stirred for 40 min. and the product collected and recrystallized from methanol; yield

2 g. (70%); m.p. 183–184°. (Found: C, 65.0; H, 6.5; N, 9.8. $\text{C}_{31}\text{H}_{36}\text{O}_7\text{N}_4$ requires C, 64.6; H, 6.25; N, 9.7%.)

Lysyltyrosine amide dihydrochloride. Dibenzoyloxycarbonyllysyltyrosine amide was reduced by the method described for the preparation of lysine amide, except that 2 mol. prop. of HCl were used. The product crystallized on trituration under acetone; m.p. 250–253° (decomp.). (Found: Cl, 17.9; $\text{C}_{15}\text{H}_{24}\text{O}_3\text{N}_4$, 2HCl, H_2O requires Cl, 17.8%.)

Dibenzoyloxycarbonyllysyl- ϵ -N-benzyloxycarbonyllysyltyrosine amide. Dibenzoyloxycarbonyllysyl- ϵ -N-benzyloxycarbonyllysine was condensed with tyrosine amide, using the procedure described above. The product was recrystallized from methanol (yield 58%); m.p. 188°. (Found: C, 64.3; H, 6.4; N, 9.7. $\text{C}_{45}\text{H}_{54}\text{O}_{10}\text{N}_6$ requires C, 64.4; H, 6.4; N, 10.0%.)

Lysyllysyltyrosine amide trihydrochloride. Dibenzoyloxycarbonyllysyl- ϵ -N-benzyloxycarbonyllysyltyrosine amide was hydrogenated in the presence of 3 mol. prop. of HCl, using 5% Pd-BaSO₄ as catalyst. The product crystallized under ethanol, and was recrystallized from methanol: ethanol:ether. (Found: C, 45.3; H, 7.7; Cl, 19.0. $\text{C}_{21}\text{H}_{36}\text{O}_4\text{N}_6$, 3HCl, H_2O requires C, 44.7; H, 7.3; Cl, 18.9%.)

N-Formyltyrosine. This was prepared by a modification of the methods of formylation of du Vigneaud, Dorfmann & Loring (1932) and Builmann, Jensen & Jensen (1934). Acetic anhydride (280 ml.) was added to tyrosine (90 g.) in 98–100% formic acid (840 ml.) and the solution kept at 35° for 30 min. After this time the ninhydrin test was faint (a portion of the solution was brought to pH 5 by diluting with aqueous sodium acetate and heated with aqueous ninhydrin). Water (90 ml.) was added, the solution evaporated under reduced pressure, and the residue recrystallized from 400 ml. of water (charcoal). *N*-Formyltyrosine hydrate (89 g., 79%) had $[\alpha]_D^{25} + 84.2 \pm 0.2^\circ$ in ethanol (c, 2.6; based on anhydrous material). Fischer (1907) gives $[\alpha]_D^{20} + 84.9^\circ$.

Optically pure tyrosine methyl ester was obtained in 68% yield by the action of methanolic HCl on *N*-formyltyrosine.

O-Acetyl-N-formyltyrosine. Acetic anhydride (25 ml.) was added to *N*-formyltyrosine hydrate (50 g.) in 1.175N-NaOH (425 ml.) at 0°. After 10 min. the solution was acidified and the acetyl compound isolated by extraction with ethyl acetate and recrystallized from the same solvent (35 g., 63%), m.p. 163.5–164.5° (decomp.); $[\alpha]_D^{19} + 73.5 \pm 0.3^\circ$ in ethanol (c, 1.9). (Found: C, 57.6; H, 5.3; N, 6.0. $\text{C}_{13}\text{H}_{13}\text{O}_5\text{N}$ requires C, 57.4; H, 5.2; N, 5.6%.)

O-Acetyl-*N*-formyltyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester. *O*-Acetyl-*N*-formyltyrosine was condensed with ϵ -benzyloxycarbonyllysine benzyl ester (Erlanger & Brand, 1951) in the usual way. After recrystallization from ethyl acetate and then from 75% (v/v) aqueous ethanol the product was obtained in 48% yield, m.p. 157°. (Found: C, 65.5; H, 6.3; N, 7.3. $C_{33}H_{37}O_6N_3$ requires C, 65.6; H, 6.2; N, 7.0%.)

Tyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester. Acetyl chloride (2.7 ml.) in dry benzyl alcohol (20 ml.) was added to *O*-acetyl-*N*-formyltyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester (7.5 g.) in benzyl alcohol (47 ml.). After 40 hr., the hydrochloride was precipitated with dry ether (500 ml.), digested with ethyl methyl ketone (25 ml.), and recrystallized from water (20 ml.) containing 0.5*N*-HCl (1 ml.); yield 5.1 g. (67%), m.p. 158.5–160°. (Found: C, 63.5; H, 6.3; N, 7.2. $C_{30}H_{35}O_6N_3$, HCl requires C, 63.2; H, 6.3; N, 7.4%.) Treatment of the hydrochloride with aqueous $NaHCO_3$ gave the free base, which crystallized from aqueous ethanol, m.p. 119–120°. (Found: C, 68.0; H, 6.8; N, 7.4; equiv. wt. 543. $C_{30}H_{35}O_6N_3$ requires C, 67.7; H, 6.4; N, 7.9%; equiv. wt. 532.)

Dibenzoyloxycarbonyllysyltyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester. (a) Dibenzoyloxycarbonyllysine was condensed with tyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester to give the tripeptide ester, which, after recrystallization from 75% (v/v) aqueous ethanol, and then ethyl acetate, had m.p. 128.5–129.5° (yield 55%). The analytical sample melted at 131–132°. (Found: C, 67.3; H, 6.5; N, 7.6. $C_{53}H_{59}O_{11}N_5$ requires C, 67.1; H, 6.4; N, 7.5%.)

(b) Condensation of dibenzoyloxycarbonyllysine with tyrosine methyl ester by the method of Wieland & Sehrling (1950) gave *dibenzoyloxycarbonyllysyltyrosine methyl ester* (73%), which, after recrystallization from ethyl acetate: light petroleum (b.p. 60–80°) had m.p. 131–133°. (Found: C, 64.9; H, 6.6; N, 7.15. $C_{33}H_{37}O_7N_3$ requires C, 65.0; H, 6.3; N, 7.1%.) After keeping the methyl ester (13 g.) in methanol (50 ml.) containing hydrazine hydrate (10 ml.) for 24 hr., the product which had separated was washed with methanol and water and then digested with hot methanol. *Dibenzoyloxycarbonyllysyltyrosine hydrazide* (90%) had m.p. 220–221°. (Found: C, 62.6; H, 6.4; N, 11.8. $C_{31}H_{37}O_7N_5$ requires C, 63.0; H, 6.3; N, 11.85%.) The hydrazide (5.9 g.) in acetic acid (25 ml.) and 2*N*-HCl (5 ml.) at 0° was treated with $NaNO_2$ (1 g.) in water (3 ml.). The mixture was diluted with ice-water and the azide extracted with cooled ethyl acetate. The ethyl acetate solution was washed with cooled aqueous $KHCO_3$, dried (Na_2SO_4) and added to ϵ -benzyloxycarbonyllysine benzyl ester (from 4.1 g. of the hydrochloride) in ethyl acetate; the solution was diluted with an equal volume of ether and kept at 0° overnight. After filtration, the mixture was washed with 0.1*N*-HCl, aqueous $KHCO_3$ and aqueous NaCl, dried (Na_2SO_4) and the solvents were removed by distillation. The residue was triturated under ether and recrystallized from 80% (v/v) aqueous methanol; yield 2.7 g. (29%), m.p. 128–130°. Mixed m.p. with tripeptide ester prepared by method (a), 130–132°.

Lysyltyrosyllysine dihydrochloride. Dibenzoyloxycarbonyllysyltyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester was hydrogenated in acetic acid in the presence of 2 mol. prop. of HCl, using Pd black as catalyst. The tripeptide dihydrochloride crystallized under ethanol and was precipitated from 90% (v/v) aqueous methanol by ethanol and ether in 40% yield. The material was dried *in vacuo* at 60° for

analysis. (Found: C, 49.6; H, 7.2; N, 13.4; Cl, 14.0; α -amino N/total N, 20. $C_{21}H_{35}O_6N_5$, 2HCl requires C, 49.3; H, 7.3; N, 13.7; Cl, 13.9; α -amino N/total N, 20.0%); $[\alpha]_D^{20} = +22.4 \pm 0.6$ in 0.5*N*-HCl (c, 1.58); pK 7.25.

Tyrosyllysine. Hydrogenation in 75% (v/v) aqueous acetic acid of tyrosyl- ϵ -benzyloxycarbonyllysine benzyl ester hydrochloride (using 10% Pd-charcoal as catalyst) gave the peptide; satisfactory analytical values were not obtained, but the material was chromatographically homogeneous.

Leucine benzyl ester hydrochloride was prepared by the method of Miller & Waelsch (1952); later, this method was simplified by using the benzenesulphonate directly: it was recrystallized from water, and had m.p. 171°. (Found: C, 60.4; H, 6.7; N, 3.8. $C_{13}H_{19}O_4N$, $C_6H_5O_2S$ requires C, 60.2; H, 6.6; N, 3.7%.)

O-Acetyl-*N*-formyltyrosylleucine benzyl ester. *O*-Acetyl-*N*-formyltyrosine was condensed with leucine benzyl ester. The reaction mixture was extracted with ethyl acetate and the extracts were washed with 1.2*N*- $KHCO_3$, 0.1*N*-HCl and finally with water. The dried solution was concentrated and the product collected (yield 61%). After recrystallization from ethyl acetate, it had m.p. 156–157°. (Found: C, 66.1; H, 6.6; N, 6.35. $C_{25}H_{30}O_6N_2$ requires C, 66.05; H, 6.7; N, 6.15%.)

Tyrosylleucine benzyl ester hydrochloride. Acetyl chloride (2.8 ml.) in benzyl alcohol (14 ml.) was added to *O*-acetyl-*N*-formyltyrosylleucine benzyl ester (7 g.) in benzyl alcohol (20 ml.). After 48 hr., the product was precipitated with ether:light petroleum (b.p. 60–80°) (2:3), digested with ethyl acetate (27 ml.), and recrystallized from ethyl methyl ketone:CHCl₃, giving 3.6 g. (56%) of colourless needles, m.p. 169–172°; $[\alpha]_D^{20} = -16.7 \pm 0.3$ in ethanol (c, 1.6). (Found: C, 63.0; H, 6.7; N, 6.6. $C_{25}H_{30}O_6N_2$, HCl requires C, 62.8; H, 6.9; N, 6.65%.)

Dibenzoyloxycarbonyllysyltyrosylleucine benzyl ester. (a) Dibenzoyloxycarbonyllysine was condensed with tyrosylleucine benzyl ester; the product was isolated by the method described under *O*-acetyl-*N*-formyltyrosylleucine benzyl ester. After recrystallization from ethyl acetate:light petroleum (b.p. 60–80°), it melted at 130.5–132° (yield 65%). (Found: C, 67.9; H, 6.7. $C_{44}H_{53}O_9N_4$ requires C, 67.7; H, 6.7%.)

(b) Dibenzoyloxycarbonyllysyltyrosine hydrazide was converted into the azide and condensed with leucine benzyl ester, using the procedure described above; the product was obtained in 56% yield, m.p. 126–128°. Mixed m.p. with sample prepared by method (a), 127–129°.

Lysyltyrosylleucine dihydrochloride. Dibenzoyloxycarbonyllysyltyrosylleucine benzyl ester was hydrogenated in acetic acid containing 3 mol. prop. of 0.5*N*-HCl, using Pd black as catalyst. Evaporation of the solvent gave the crystalline tripeptide dihydrochloride monohydrate, which was recrystallized from methanol:acetone; pK 7.32. (Found: C, 49.1; H, 7.2; Cl, 13.6; α -amino N/total N, 26. $C_{21}H_{34}O_6N_4$, 2HCl, H_2O requires C, 49.1; H, 7.4; Cl, 13.8; α -amino N/total N, 25.0%.)

Tyrosylleucine. (a) Tyrosylleucine benzyl ester hydrochloride was treated with aqueous $NaHCO_3$ and the free base isolated by extraction with ethyl acetate. Hydrogenation in acetic acid using 10% Pd-charcoal gave the dipeptide hydrate, which crystallized from 80% (v/v) aqueous ethanol in colourless needles, m.p. 271–275° (decomp.); $[\alpha]_D^{21} + 6.3 \pm 0.4$ in 0.5*N*-HCl (c, 3.2). (Found:

C, 57.4; H, 7.8; α -amino N/total N, 53. $C_{15}H_{23}O_4N_3$, H_2O requires C, 57.7; H, 7.7; α -amino N/total N, 50.0%.) The pK was 7.46.

(b) *O*-Acetyl-*N*-benzyloxycarbonyltyrosine (Blau & Waley, 1954) was condensed with leucine methyl ester to give *O*-acetyl-*N*-benzyloxycarbonyltyrosylleucine methyl ester (76%), which, after recrystallization from isopropyl ether: CCl_4 , had m.p. 143°. (Found: C, 64.6; H, 6.6; N, 6.2; OMe, 6.5. $C_{36}H_{53}O_7N_3$ requires C, 64.5; H, 6.6; N, 5.8; OMe, 6.4%.) Hydrogenation in 80% (v/v) aqueous acetic acid containing 1.5 mol. prop. of HCl using 10% Pd-charcoal as catalyst gave *O*-acetyltyrosylleucine methyl ester hydrochloride, which was recrystallized from ethyl acetate: ether, m.p. 174–175° (decomp.). (Found: C, 55.6; H, 6.7; Cl, 9.7; OMe, 8.1. $C_{18}H_{28}O_5N_2$, HCl requires C, 55.9; H, 7.0; Cl, 9.2; OMe, 8.0%.) The methyl ester hydrochloride (0.66 g.) was dissolved in 0.74 N- $KHCO_3$ (5 ml.) and the free base isolated with ethyl acetate. After shaking for 30 min. with 0.1015 N- $Ba(OH)_2$ (67.4 ml.) the solution was neutralized with H_2SO_4 , filtered and evaporated. Recrystallization from 80% ethanol gave tyrosylleucine hydrate, m.p. 278° (decomp.); $[\alpha]_D^{25} + 6.9 \pm 0.1^\circ$ in 0.5 N-HCl (c, 9.3). (Found: C, 57.4; H, 7.8; α -amino N/total N, 51. Calc. for $C_{15}H_{23}O_4N_3$, H_2O : C, 57.7; H, 7.7; α -amino N/total N, 50.0%.) The pK was 7.44.

ϵ -Benzyloxycarbonyllysine ethyl ester hydrochloride. *ϵ* -Benzyloxycarbonyl- α -carboxyllysine anhydride (Bergmann *et al.* 1935) (12.2 g.) in ethanolic HCl (85 ml. of N) was heated to the b.p. and then allowed to cool. After distillation of the solvent, the hydrochloride monohydrate was recrystallized from ethyl acetate; yield 8.2 g., 60%, m.p. 115.5–116.5°; $[\alpha]_D^{17} + 12.2 \pm 0.2^\circ$ in 0.1 N-HCl (c, 2.1). (Found: N, 7.45; Cl, 9.8. $C_{16}H_{24}O_4N_2$, HCl, H_2O requires N, 7.7; Cl, 9.8%.)

Dibenzoyloxycarbonyllysyl- ϵ -benzyloxycarbonyllysine ethyl ester. Dibenzoyloxycarbonyllysine was condensed with the *ϵ* -benzyloxycarbonyllysine ethyl ester. The product was collected, and recrystallized from ethyl acetate: ether; yield 71%; m.p. 117–117.5°. $[\alpha]_D^{18} - 5.6 \pm 0.2^\circ$ in acetone (c, 2.46). (Found: C, 64.8; H, 6.9; N, 8.0. $C_{38}H_{48}O_8N_4$ requires C, 64.8; H, 6.8; N, 7.95%.)

Lysyllysine ethyl ester. Dibenzoyloxycarbonyllysyl- *ϵ* -benzyloxycarbonyllysine ethyl ester was hydrogenated in ethanolic HCl, using Pd black as catalyst. The product could not be crystallized, but was chromatographically homogeneous.

Enzymic reactions

The enzymic reactions, carried out at 25.8°, were followed by transferring samples (approx. 10 μ l.) at various times to the starting line of a chromatogram; a drop of 2 N acetic acid had previously been applied to this position. The chromatographic solvent was *n*-butanol:acetic acid:pyridine:water (30:6:20:24, v/v); Whatman no. 1 paper was customarily employed. The R_F values of the compounds studied are given in Table 1. Markers were used in 1% solution in 50% (v/v) aqueous isopropanol, and the chromatograms were developed by spraying with a solution of ninhydrin (0.4%) and cobalt chloride (0.2%) in isopropanol, followed by heating at 110° for 10 min. (Wiggins & Williams, 1952). Since both the α - and ϵ -amino groups of lysine react with ninhydrin, especially small amounts of lysine peptides are detectable, varying from 0.1 μ g. in the case of dilysine to 0.9 μ g. in the case of Lys.Tyr.Leu. Peptides containing tyrosine were revealed by spraying chromatograms with either Pauly reagent (Block, Le Strange & Zweig, 1952) or

Table 1. R_F values of reference compounds

The compounds were chromatographed on Whatman no. 1 paper using *n*-butanol:acetic acid:pyridine:water (30:6:20:24, v/v).

Substance	R_F
Lysine	0.24
Dilysine	0.17
Trilysine	0.12
Tetralysine	0.09
Pentalysine	0.06
Lysine amide	0.29
Lysyltyrosine amide	0.51
Lysyllysyltyrosine amide	0.36
Tyrosine	0.56
Leucine	0.66
Tyrosyllysine	0.42
Tyrosylleucine	0.83
Lysyltyrosyllysine	0.33
Lysyltyrosylleucine	0.64
Lysyllysine ethyl ester	0.38

Folin's reagent (Cornforth, Cornforth, Dalglish & Neuberger, 1951); with the latter reagent, the smallest quantity of peptide which could be detected was the amount which contained about 0.3 μ g. of tyrosine.

Experiments with dilysine, lysine amide, Lys.Lys.Tyr amide, Lys.Tyr.Lys and Lys.Tyr.Leu, under the same conditions of concentration, temperature and pH as were used in the enzymic reactions, showed that no decomposition could be detected within 24 hr. The case of dilysine ethyl ester is discussed in detail on p. 536.

RESULTS AND DISCUSSION

The following abbreviations are used: Lys for lysine and lysyl residues, Tyr for tyrosine and tyrosyl residues, Leu for leucyl residues, Gly for glycyl residues and Am and Et for amide and ethyl ester groups respectively.

Dilysine. Although it has been reported that trypsin does not attack dilysine (Waley & Watson, 1953b), it has now been found that at relatively high concentrations of the enzyme reaction does take place; the course of the reaction is shown in Fig. 1.

As far as one can tell by visual inspection, comparable amounts of lysine and trilysine are formed initially and only in the later stages of the reaction, when trilysine will have decomposed somewhat (equation 2), is the amount of lysine appreciably greater than the amount of trilysine. The trilysine is formed by the transpeptidation reaction 1.

Equation 1 gives equimolecular amounts of lysine and trilysine. Now if any direct hydrolysis of dilysine (equation 3) occurred, the amount of lysine would always be greater than the amount of trilysine; this does not appear to be the case, so that direct hydrolysis does not take place.

Transpeptidations leading to higher peptides of lysine also occur to a small extent and at higher substrate concentrations (10%) greater amounts of tetralysine are formed. Experiments carried out at pH values from 6 to 10 showed a broad optimum about pH 8. The intermediate trilylsine is relatively stable and thus transpeptidation is readily detected

Fig. 1. Chromatogram showing the action of trypsin on dilysine dihydrochloride. A 4% (w/v) solution of trypsin in 0.001N-HCl (0.25 ml.), which had been dialysed against 0.001N-HCl for 1 hr., was added to dilysine dihydrochloride (19.6 mg.) in 0.27N-KHCO₃ (0.75 ml.). If the MgSO₄ is not removed from the trypsin solution by dialysis, the lysine spot on paper chromatograms is split into two spots. The MgSO₄ does not interfere at low concentrations.

Fig. 2. Chromatogram showing the action of trypsin on lysine amide dihydrochloride. A 4% solution of trypsin in 0.001N-HCl (0.06 ml.), which had been dialysed against 0.001N-HCl for 1 hr., was added to lysine amide dihydrochloride (5.0 mg.) in phosphate buffer, pH 7.1 (Clark & Lubs, 1916) (0.14 ml.) containing 0.2N-NaOH (0.055 ml.).

in this case, but the pattern of reactions 1 and 2, which together furnish a mode of hydrolysis, may be quite common and if the second step in such a sequence is considerably faster than the first the intermediate formed by transpeptidation will never be present in sufficient quantity to be detected. Thus transpeptidation may occur even when it is not detected. Since many important biological compounds are built up and broken down by cyclic processes, it is interesting that the decomposition of dilysine should be a cyclic process of a particularly simple kind; the second molecule of dilysine may be considered to act as a cosubstrate (Behrens & Bergmann, 1939).

Lysine amide. The course of the reaction is shown in Fig. 2.

The main products are lysine and dilysine (which are formed in comparable amounts). Trilylsine is also formed and an intermediate whose R_f is slightly higher than dilysine; this is believed to be dilysine amide. Thus, the reactions taking place may be

Alternatively, the trilylsine may be formed by transpeptidation from two molecules of dilysine, instead of from lysine amide and dilysine. There are several other reactions which may be postulated, but as there is no definite evidence of their occurring they will not be mentioned. The chief point is that dilysine, which is a major product, must arise by transpeptidation.

The pH optimum was about 7; at higher pH values, relatively more trilylsine and dilysine were formed; at lower pH values the reaction was very slow.

Lysyllysyltyrosine amide. The course of the reaction is shown in Fig. 3. As the substrate decomposed, dilysine, tetralysine and tyrosine amide accumulated. Then the tetralysine disappeared. A separate chromatogram was sprayed with Folin's reagent: this revealed only the Lys. Lys. Tyr amide and tyrosine amide spots. The spot between di- and tetra-lysine (Fig. 3) did not contain any tyrosine residues and is assumed to be trilylsine. No lysine spot is observable; a small amount of Lys. Tyr amide was also formed; it is not detectable in the photograph (Fig. 3).

One of the main reactions is apparently

The tetralysine may arise as follows:

The trilyisine cannot arise from decomposition of the tetralysine since no lysine is formed; it must therefore arise by transpeptidation reactions, such as

or

The posulated intermediate, Lys.Lys.Lys.Tyr amide, would be expected to be detectable at a concentration of approximately 0.01% (by spraying with Folin reagent), but no tyrosine-containing peptide was observed of R_f lower than that of the starting material. If the rate of decomposition of Lys.Lys.Lys.Tyr amide were much greater than its rate of formation, however, its concentration would always be very low.

The action of trypsin on a mixture of Lys.Lys.-Tyr amide (2%) and tyrosine amide (5%) showed several differences. Tetralysine was no longer formed, more Lys.Tyr amide appeared, and the starting material disappeared more slowly. The Lys.Tyr amide may arise from

Trilyisine presumably arises from reaction 13, followed by reactions 11 and 12. This sequence is

Fig. 3. Chromatogram showing the action of trypsin on lysyllysyltyrosine amide trihydrochloride. Trypsin (0.22 mg.) in water (0.22 ml.) was added to Lys.Lys.Tyr amide trihydrochloride (8.8 mg.) in 0.4N-KHCO₃ (0.22 ml.).

suggested to account for more trilyisine being formed when tyrosine amide is present; the other reaction 10, which gives rise to trilyisine, depends on the participation not of tyrosine amide but of dilysine.

There are two possible explanations of the fact that the addition of tyrosine amide causes the lysyllysyltyrosine amide to decompose more slowly. The tyrosine amide may combine with the trypsin, but since trypsin is only known to combine with amino acid residues bearing a basic side chain this appears unlikely. The other explanation is that a transpeptidation reaction 14 occurs:

This transpeptidation is analogous to reaction 15 which Johnston, Mycek & Fruton (1950) discovered, using isotopically labelled glycine amide (Gly*.Am).

That the transpeptidation reaction 14 would decrease the rate of disappearance of Lys.Lys.Tyr amide can be shown as follows. If we restrict ourselves to the effect of reaction 14 on the rate of the direct hydrolysis (reaction 8), then if the Lys.Lys.Tyr amide is written as A , the tyrosine amide as B , the dilysine as C , and the trypsin as E , the reaction scheme may be written

We will assume that the stationary-state approximation applies to the enzyme-substrate complex, and that the concentration of tyrosine amide (which was present in excess in our experiments) remains constant. Then it may readily be shown that the half-life ($t_{1/2}$) for the disappearance of Lys.Lys.Tyr amide is given by

$$t_{1/2} = \{(K_m + k_4 B_0 / k_1) \log 2\} / k_3 e + A_0 / 2k_3 e,$$

where e is the total concentration of trypsin, A_0 and B_0 are the initial concentrations of Lys.Lys.Tyr amide and tyrosine amide, and $K_m = (k_2 + k_3) / k_1$. Thus the half-life increases with the initial concentration of tyrosine amide.

The following transpeptidation between Lys.-Lys.Tyr amide and phenylalanine amide, which is analogous to reaction 14, has also been observed.

Pentyllysine and tyrosine amide. Without added tyrosine amide, trypsin breaks pentyllysine down to dilysine and trylsine in equimolecular amounts. When tyrosine amide was added, initially more dilysine than trylsine was formed; in addition, two new compounds (R_f values 0.36 and 0.29) appeared, also in unequal amounts. Since lysyllysyltyrosine amide has R_f 0.36, this is taken to be one of the new compounds; the other, formed in greater amount, is probably lysyllysyllysyltyrosine amide. These two compounds decomposed at later stages of the reaction, and the amounts of dilysine and trylsine became equal.

Thus, we postulate the transpeptidations 17 and 18 and apparently reaction 18 is faster.

Similar results were obtained by the action of trypsin on polylysine in the presence of tyrosine amide: di-, tri- and tetra-lysine were formed as usual (Waley & Watson, 1953b) and in addition lysyltyrosine amide and lysyllysyltyrosine amide appeared. Higher members of the series (lysyl) $_n$ -tyrosine amide were probably also present, as several tyrosine-containing compounds with relatively low R_f values were detected by spraying with Pauly reagent.

Lysyltyrosyllysine. The course of the reaction is shown in Fig. 4.

After 1300 min. all the lysyltyrosyllysine had reacted and the products were dilysine, lysine and

tyrosyllysine; the dilysine must arise by a transpeptidation reaction. In the early stages of the reaction (up to 40 min.) a product was formed with R_f intermediate between lysine and dilysine (Fig. 4); this product, which was shown to contain tyrosine by spraying with Pauly reagent, is, together with tyrosyllysine, clearly a primary product. This substance is believed to be lysyllysyltyrosyllysine, arising by reaction 19 and decomposing to give dilysine by reaction 20.

The lysine formed might arise by hydrolysis of the starting material:

However, Fig. 4 shows that lysine only appears after 40 min., when the dilysine appears. Since the dilysine arises by break-down of the tetrapeptide lysyllysyltyrosyllysine, it seems likely that the lysine is similarly formed (reaction 22).

The sequence of reactions 19 and 22 constitute a cyclic process, analogous to reactions 1 and 2.

No new products appear to be formed by the addition of leucine, or arginine, to the reaction mixture; but in the presence of tyrosylleucine, some lysyltyrosylleucine is formed, and in the presence of tyrosine amide some lysyltyrosine amide.

Lysyltyrosylleucine. This substrate does not react with trypsin as fast as lysyltyrosyllysine does; dilysine, lysine and tyrosylleucine are formed. In this case no tetrapeptide intermediate could be detected, but it seems likely that dilysine is formed by reactions 23 and 24, analogous to reactions 19 and 20.

If the second step takes place much more rapidly than the first, the concentration of lysyllysyltyrosylleucine will always be low.

Dilysine ethyl ester. The course of the reaction is shown in Fig. 5.

The first product of the reaction is dilysine; subsequently tetralysine is formed. Dilysine ethyl ester is very readily attacked by trypsin and at the low trypsin concentrations used the tetralysine formed does not decompose at all rapidly. Traces of a substance X (R_f 0.29) also appear; its structure has not yet been determined, but it may well be the diketopiperazine of lysine.

Fig. 4. Chromatogram showing the action of trypsin on lysyltyrosyllysine dihydrochloride. Trypsin (0.88 mg.) in water (0.22 ml.) was added to lysyltyrosyllysine dihydrochloride (8.6 mg.) in 0.2N-KHCO₃ (0.22 ml.).

There are two ways in which the tetralysine might arise (reactions 25 and 26 or 27 and 28):

No evidence for the presence of tetralysine ethyl ester has been found. It might be unstable and only present in low concentrations, or its R_f might be the same as that of dilysine so that its presence would remain undetected. Again, dilysine might arise by direct hydrolysis of dilysine ethyl ester or by reaction 29.

Variation of pH altered the nature of the products formed, but the rate did not vary greatly between pH values of 6 and 11. At pH 6 the sole product was dilysine. At pH 10 more tetralysine was formed than dilysine and some products with lower R_f values appeared; these are believed to be hexalysine and octalysine; similar products were formed at pH 11.

Some experiments were also carried out without any enzyme. At pH 8.3, the main product was X,

and the only other product was dilysine; the dilysine ethyl ester was decomposed about 50 times more slowly in the absence of the enzyme than in the experiment at pH 8.3 described above. At higher pH values, the reactions in the absence of the enzyme were more rapid than those at pH 8.3, X and dilysine were again the main products (but relatively more dilysine was formed) and traces of tetra- and hexa-lysine were discernible.

The course of an experiment in which tyrosyl-leucine was added to dilysine ethyl ester is shown in Fig. 6.

The main products were dilysine, tetralysine and a further substance (R_f 0.53) which contained tyrosine; this substance was not stable, and was nearly all decomposed after 1000 min.; it is believed to be lysyllysyltyrosylleucine, formed by reaction 30.

When tyrosyltyrosine amide replaced tyrosyl-leucine in the experiment described above, the products were dilysine, tetralysine and a substance

Fig. 5. Chromatogram showing the action of trypsin on dilysine ethyl ester trihydrochloride. Trypsin (12.5 $\mu\text{g.}$) in water (0.05 ml.) was added to dilysine ethyl ester trihydrochloride (9.4 mg.) in 0.2N-KHCO₃ (0.45 ml.). The pH of the reaction mixture was 8.3.

Fig. 6. Chromatogram showing the action of trypsin on dilysine ethyl ester trihydrochloride and tyrosylleucine. Trypsin (20 $\mu\text{g.}$) in water (0.08 ml.) was added to dilysine ethyl ester trihydrochloride (15.3 mg.) and tyrosylleucine (20.6 mg.) in 0.2N-KHCO₃ (0.72 ml.).

(R_F 0.57) which is probably lysyllysyltyrosyltyrosine amide:

Similarly, lysyllysine ethyl ester reacted with tyrosine amide to give lysyllysyltyrosine amide (in this case a synthetic specimen was available for comparison):

When the reaction was carried out with excess of tyrosine amide (0.049M dilysine ester, 0.28M tyrosine amide), the dilysine ester reacted rapidly and completely with tyrosine amide; no dilysine or tetralysine were formed. The lysyllysyltyrosine amide so formed then decomposed slowly as described on p. 533. In the absence of enzyme, dilysine ester decomposed slowly in the presence of tyrosine amide at pH 8.3 to give some lysyllysyltyrosine amide; the main product was X.

Dilysine ethyl ester and lysyllysyltyrosine amide have the same R_F , but can easily be distinguished by spraying with Pauly reagent, which is specific for tyrosine residues. Phenylalanine amide reacted in the same way as tyrosine amide; in this case the formation of lysyllysylphenylalanine amide, which also had the same R_F as dilysine ester, was demonstrated chromatographically by elution of the material and partial acid hydrolysis: dilysine, lysine and phenylalanine were present.

Frequency of occurrence of transpeptidation. Our experiments with trypsin show that transpeptidation occurs frequently. The substrates used, arranged in order of decreasing reactivity, are: dilysine ethyl ester, pentalysine, lysyllysyltyrosine amide, lysyltyrosyllysine, lysine amide, lysyltyrosylleucine and dilysine. A wide range of reactivity is covered by these compounds; dilysine ethyl ester is about 10^4 times as reactive as dilysine, so transpeptidation is not restricted to the less reactive substrates. Furthermore, both the least and the most reactive substrates can, under certain conditions, undergo transfer reactions without any concomitant hydrolysis.

Role of transpeptidations in vivo. It seems reasonable to suppose that transpeptidations may play a part in the metabolism of proteins. There are two effects which transpeptidation may have: (a) to lengthen one peptide chain at the expense of another, (b) to produce novel sequences of amino acids. One of the chief reasons for considering that the first effect may mediate in protein synthesis is that the peptide chain may be lengthened with relatively little change in free energy. The second effect may accelerate the enzymic hydrolysis of proteins by substituting a more readily hydrolysed peptide bond for a resistant one.

SUMMARY

1. Some of the methods available for the synthesis of peptides are discussed. The azide method and the anhydride method of Wieland & Sehring (1950) have been used to prepare lysyltyrosyllysine and lysyltyrosylleucine; several other peptides of lysine, tyrosine and leucine have also been synthesized.

2. The action of trypsin on dilysine, lysine amide, lysyllysyltyrosine amide, lysyltyrosyllysine and lysyltyrosylleucine has been examined chromatographically. In all these cases, transpeptidations take place. Trypsin also catalyses transpeptidation between pentalysine and tyrosine amide.

3. Dilysine ethyl ester has been found to be a very reactive substrate for trypsin. Both hydrolysis and condensation to give polymers took place, as did condensation with tyrosine amide, phenylalanine amide, tyrosyltyrosine amide and tyrosylleucine.

4. In some cases hydrolysis of a peptide apparently takes place by way of an intermediate formed by transpeptidation. Some of the results are interpreted in terms of cyclic reactions.

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Chymotrypsin-Catalysed Transpeptidations

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The transferase activity of the proteolytic enzyme trypsin has recently been investigated (see preceding paper, Waley & Watson, 1954); this work has now been extended to chymotrypsin. The substrates used were tyrosine amide, phenylalanine amide, tyrosyltyrosine amide, phenylalanylphenylalanine amide, tyrosylphenylalanine amide, phenylalanyltyrosine amide, and tyrosyltyrosine. Most of these substrates were originally investigated by Fruton & Bergmann (1942), who implicitly assumed that hydrolysis was the sole chymotrypsin-catalysed reaction.

Hydrolysis is indeed the final result, but we have shown chromatographically that transpeptidation also occurs. Moreover, the initial reaction may be entirely transpeptidation, with subsequent hydrolysis of the intermediates so formed.

EXPERIMENTAL

General. Crystalline chymotrypsin (not more than 50% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and crystalline pepsin were obtained from Armour Laboratories Ltd., Lindsey Street, London, E.C. 1. Papain (Hopkin & Williams Ltd.) was purified as described by Grassmann (1935). Ficin (L. Light and Co. Ltd.) was used as supplied. Microanalyses were carried out by Mr G. Ingram of these laboratories. Melting points are uncorrected. The error of the optical rotations is the standard deviation (Dean & Dixon, 1951). The amino acids used all had the L configuration.

Materials

Tyrosine amide. Tyrosine methyl ester (Fischer & Schrauth, 1907) was allowed to stand for 48 hr. in methanol saturated with NH_3 . After evaporation of the solution, the residue was recrystallized from ethanol (yield, 82%). After

two further recrystallizations the tyrosine amide had m.p. 153–154°; $[\alpha]_D^{20} + 19.2 \pm 0.1^\circ$ in water (c, 4.1). Koenigs & Mylo (1908) give m.p. 153–154°; $[\alpha]_D^{20} + 19.5^\circ$. (Found: C, 60.3; H, 7.0; N, 15.6; equiv. wt. 180. Calc. for $\text{C}_9\text{H}_{11}\text{O}_2\text{N}_2$: C, 60.0; H, 6.7; N, 15.5%; equiv. wt. 180.)

N-Benzoyloxycarbonyltyrosine methyl ester. The method of Bergmann & Zervas (1932) was applied to tyrosine methyl ester. After recrystallization from diisopropyl ether, N-benzoyloxycarbonyltyrosine methyl ester had m.p. 94–95°; $[\alpha]_D^{20} - 6.1 \pm 0.1^\circ$ in ethanol (c, 2.0). (Found: C, 66.0; H, 5.9. $\text{C}_{18}\text{H}_{19}\text{O}_6\text{N}$ requires C, 65.6; H, 5.8%.)

O-Acetyl-N-benzoyloxycarbonyltyrosine. N-Benzoyloxycarbonyltyrosine methyl ester was hydrolysed and acetylated by the method of Bergmann, Zervas, Salzmann & Schleich (1934). The crude product, isolated by extraction with ethyl acetate, was recrystallized from diisopropyl ether. O-Acetyl-N-benzoyloxycarbonyltyrosine had m.p. 112–113°; $[\alpha]_D^{20} + 3.2 \pm 0.2^\circ$ in ethanol (c, 2.3). Bergmann, Zervas, Salzmann & Schleich (1934) give m.p. 120–121° (not sharp); the m.p. is variable unless the specimen is carefully dried. (Found: C, 63.8; H, 5.2; N, 4.1. Calc. for $\text{C}_{19}\text{H}_{19}\text{O}_6\text{N}$: C, 63.9; H, 5.3; N, 3.9%.)

O-Acetyl-N-benzoyloxycarbonyltyrosyltyrosine amide. The peptide synthesis of Wieland & Sehring (1950) was used. Benzoyl chloride (4.6 ml.) was added to O-acetyl-N-benzoyloxycarbonyltyrosine (14.3 g.) and N-ethylpiperidine (5.4 ml.) in tetrahydrofuran (50 ml.) at 0°. After 10 min. the mixture was added to tyrosine amide (7.2 g.) suspended in ethyl acetate (50 ml.) and 1.3N- KHCO_3 (50 ml.). The mixture was stirred for 1 hr., and the product collected, washed with water and ether and recrystallized from methanol. O-Acetyl-N-benzoyloxycarbonyltyrosyltyrosine amide (10.7 g., 51%) had m.p. 209–211°; $[\alpha]_D^{20} - 14.9 \pm 0.2^\circ$ in dioxan (c, 2.4). (Found: C, 64.8; H, 5.7; N, 8.1. $\text{C}_{28}\text{H}_{33}\text{O}_7\text{N}_3$ requires C, 64.7; H, 5.6; N, 8.1%.)

Tyrosyltyrosine amide. O-Acetyl-N-benzoyloxycarbonyltyrosyltyrosine amide was suspended in 0.2N methanolic HCl and hydrogenated using 10% Pd-charcoal. The use of acid in the reduction medium removes the acetyl as well as the benzoyloxycarbonyl group (Barkdoll & Ross, 1944). The filtered solution was evaporated, the residue dissolved in the minimum amount of water and the pH brought to 7.0. The product crystallized slowly. Tyrosyltyrosine amide had m.p. 193–194°; $[\alpha]_D^{20} + 4.7 \pm 0.6^\circ$ in methanol (c, 1.7).

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