Hydrolysis and Transpeptidation of Lysine Peptides by Trypsin

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The study of the tryptic digestion of poly-L-lysine (Katchalski, 1951; Waley & Watson, 1953) has shown that lysine oligopeptides appear during the reaction. A better understanding of the mode of action of trypsin on the high-molecular-weight peptide might be gained by a study of the action of the enzyme on a homologous series of lysine peptides (for a preliminary note see Katchalski, Berger & Levin, 1954). In the present article the behaviour of the following substrates towards trypsin is reported: L-lysine amide, di-L-lysine, tri-L-lysine, di-L-lysine amide, glycyl-L-lysine amide, L-lysylglycine amide, benzoyl-L-lysine, benzoyldi-L-lysine and benzoyltri-L-lysine. In agreement with the results of Waley & Watson (1954), it will be shown that trypsin mediates transpeptidation as well as hydrolysis of many of the substrates investigated. The effect of free α-amino and α-carboxyl groups on hydrolysis and transpeptidation will also be shown.

In the synthesis of the lysine peptides and their derivatives the mixed anhydride method of Wieland & Sehring (1950) as well as the azide method was used. As it has been shown (Vaughan, 1952; Vaughan & Eichler, 1953), that racemization may accompany peptide synthesis when mixed anhydrides of acylated amino acids are used, the azide method was employed in the synthesis of benzoyldi-L-lysine and benzoyltri-L-lysine.

EXPERIMENTAL

Materials

Melting points are uncorrected.

L-Lysine amide dihydrochloride. A stream of dry HCl was passed through a solution of ϵ-benzyloxycarbonyl-L-lysine amide hydrochloride (Bergmann, Zervas & Ross, 1935) (2 g.) in glacial acetic acid (20 ml.) for 3 hr. at 80°. The precipitation of the L-lysine amide dihydrochloride formed was brought to completion by the addition of anhydrous ether (100 ml.). The precipitate was collected, washed with anhydrous ether and dried *in vacuo* (10^{-2} mm.) at 100° over solid KOH and P_2O_5. Yield, 85%; m.p. 250–260° (decomp.). Waley & Watson (1954) give m.p. 217–218° (decomp.). (Found: C, 33·6; H, 8·1; N, 19·0; amino-N, 12·6; amide-N, 6·8; Cl, 31·6; $C_6H_{17}ON_3Cl_2$ requires C, 33·0; H, 7·9; N, 19·3; amino-N, 12·8; amide-N, 6·4; Cl, 32·5%.)

α-Benzoyl-L-lysine. This compound was prepared according to Ross & Green (1941).

α-Benzoyl-L-lysine amide hydrochloride. This compound was prepared according to Hofmann & Bergmann (1939).

$\alpha\epsilon$-Dibenzyloxycarbonyl-L-lysylglycine methyl ester (I). This compound was prepared from $\alpha\epsilon$-dibenzyloxycarbonyl-L-lysine (4·0 g.) (Bergmann *et al.* 1935), *iso*butyl chlorocarbonate (1·35 g.), triethylamine (2·0 g.) and glycine methyl ester hydrochloride (1·2 g.) in ethyl acetate, following the procedure of Vaughan & Osato (1952). Recrystallization from benzene gave 3 g. of *$\alpha\epsilon$-dibenzyloxycarbonyl-L-lysylglycine methyl ester*, m.p. 122–123°. (Found: C, 62·2; H, 6·8; N, 8·7. $C_{25}H_{31}N_3O_7$ requires C, 61·9; H, 6·4; N, 8·7%.)

$\alpha\epsilon$-Dibenzyloxycarbonyl-L-lysylglycine amide (II). A solution of the ester (I) (0·5 g.) in anhydrous methanol (5 ml.) was saturated with NH_3 at 0° and kept in a tightly closed vessel for 4 hr. at room temp. *$\alpha\epsilon$-Dibenzyloxycarbonyl-L-lysylglycine amide* (0·35 g., m.p. 135°) was obtained after evaporation of solvent and recrystallization from ethyl acetate. (Found: C, 61·3; H, 6·4; N, 12·0. $C_{24}H_{30}N_4O_6$ requires C, 61·3; H, 6·4; N, 11·9%.)

L-Lysylglycine amide dihydrobromide. Compound (II) (10 g.) was treated with anhydrous HBr (30%) in glacial acetic acid (4 ml.) at room temp. (Ben-Ishai & Berger, 1952). The material dissolved with simultaneous evolution of CO_2. The dihydrobromide separated out within a few minutes. After an additional 10 min. the precipitate was washed with glacial acetic acid and finally with ether. Purification was effected by precipitation from a concentrated methanolic solution by means of glacial acetic acid and ether. Yield, 80%. Dried *in vacuo* at 80° over KOH and P_2O_5 before analysis. (Found: N, 15·7; amino-N, 7·45; Br, 43·6. $C_8H_{20}N_4O_2Br_2$ requires N, 15·4; amino-N, 7·7; Br, 43·9%.)

Benzyloxycarbonylglycyl-ϵ-benzyloxycarbonyl-L-lysine benzyl ester (III). This compound was prepared from benzyloxycarbonylglycine (2·1 g.) (Bergmann & Zervas, 1932), *iso*butyl chlorocarbonate (1·36 g.), triethylamine (2·0 g.) and ϵ-benzyloxycarbonyl-L-lysine benzyl ester (4·1 g.) (Erlanger & Brand, 1951), in ethyl acetate, following the procedure of Vaughan & Osato (1952). Recrystallization from benzene–ether and from ethyl acetate–light petroleum gave 4 g. of *benzyloxycarbonylglycyl-ϵ-benzyloxycarbonyl-L-lysine benzyl ester*, m.p. 87–89°. (Found: C, 66·1; H, 6·5; N, 7·7. $C_{31}H_{35}N_3O_7$ requires C, 66·3; H, 6·3; N, 7·5%.)

Benzyloxycarbonylglycyl - ϵ - benzyloxycarbonyl - L - lysine amide (IV). This compound was prepared from the benzyl ester (III) (2 g.) analogously to the preparation of II. Recrystallized from methanol and ether. Yield 1·5 g., m.p. 134–138° (Bergmann *et al.* 1935 give m.p. 130–134°.)

Glycyl-L-lysine amide dihydrobromide. Compound (IV), on treatment with anhydrous HBr in glacial acetic acid at room temp. analogously to the preparation of L-lysylglycine amide dihydrobromide, was converted into *glycyl-L-lysine*

amide dihydrobromide (yield 78%). It was dried *in vacuo* over KOH and P_2O_5 at room temp. before analysis. (Found: N, 14·8; Br, 44·2. $C_8H_{20}N_4O_2Br_2$ requires N, 15·4; Br, 43·9%.)

Glycyl-L-lysine dihydrochloride. Compound (III) was treated with anhydrous HBr in glacial acetic acid at room temp. for 30 min. according to Ben-Ishai & Berger (1952). The material obtained gave two spots on a paper chromatogram developed with *n*-butanol:acetic acid:pyridine:water (30:6:20:24, by vol.). It was, therefore, treated at room temp. with N-NaOH for 2 hr. to hydrolyse any remaining benzyl ester groups. The hydrolysate was acidified with HCl and evaporated to dryness, and the residue extracted with ethanol. The glycyl-L-lysine dihydrochloride obtained after the evaporation of solvent was found to be chromatographically pure and in the solvent mixture described above showed an R_F value equal to that of a L-lysylglycine prepared according to Bergmann, Zervas, Rinke & Schleich (1934). It also moved as a single spot on paper electrophoresis with a rate equal to that of L-lysylglycine. On acid hydrolysis equimolar amounts of lysine and glycine were obtained.

αε-Dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine benzyl ester (V). Dibenzyloxycarbonyl-L-lysine (1·40 g.) (Bergmann *et al.* 1935) was treated with *iso*butyl chlorocarbonate (1·35 g.), triethylamine (1·00 g.) and ε-benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (4·00 g.) (Erlanger & Brand, 1951) in ethyl acetate (25 ml.) following the procedure of Vaughan & Osato (1952). After the addition of 50 ml. of ethyl acetate, triethylamine hydrochloride was filtered off and extracted with hot ethyl acetate (20 ml.). To the combined ethyl acetate solutions light petroleum (500 ml.) was added and the crystalline precipitate was collected and washed with light petroleum and ether. After recrystallization from ethanol–ether *αε-dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine benzyl ester* (6·8 g. m.p. 139–142°) was obtained. (Found: C, 67·4; H, 6·6; N, 7·2. $C_{43}H_{50}N_4O_9$ requires C, 67·3; H, 6·6; N, 7·3%.)

αε-Dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine amide (VI). This compound was prepared from (V) (2·00 g.) analogously to the preparation of (II). Recrystallization from glacial acetic acid–ether gave 1·70 g. of *αε-dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine amide*, m.p. 203–204°. (Found: C, 64·4; H, 6·7; N, 10·5. $C_{36}H_{45}N_5O_8$ requires C, 64·0; H, 6·7; N, 10·4%.)

Di-L-lysine amide trihydrobromide. Compound (VI) was treated with anhydrous HBr in glacial acetic acid at room temp. analogously to the preparation of L-lysylglycine amide dihydrobromide. The product was further purified by precipitation from a concentrated methanolic solution with glacial acetic acid. *Di-L-lysine amide trihydrobromide* was obtained as a white hygroscopic powder in 70% yield. (Found: N, 12·9; amino N, 7·8; amide N, 2·2; Br, 46·3. $C_{12}H_{30}N_5O_2Br_3$ requires N, 13·5; amino N, 8·1; amide N, 2·7; Br, 46·4%.)

α-Benzoyl-ε-benzyloxycarbonyl-L-lysine benzyl ester (VII). ε-Benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (4·0 g.) (Erlanger & Brand, 1951) was treated with benzoyl chloride (2·0 ml.) at 0°, in a mixture of ethyl acetate (20 ml.), 1N-$KHCO_3$ (20 ml.) and 4N-NaOH (2·5 ml.). The crystalline product was washed with ether and recrystallized from ethyl acetate and light petroleum. Yield of *α-benzoyl-ε-benzyloxycarbonyl-L-lysine benzyl ester* 2·5 g., m.p. 120°. (Found: C, 70·5; H, 6·1; N, 5·9. $C_{28}H_{30}N_2O_5$ requires C, 70·9; H, 6·4; N, 5·9%.) On treatment with methanol saturated with ammonia (VII) was converted into α-benzoyl-ε-benzyloxycarbonyl-L-lysine amide (Bergmann *et al.* 1935).

α-Benzoyl-ε-benzyloxycarbonyl-L-lysine hydrazide (VIII). Compound (VII) (2·5 g.) was refluxed with hydrazine hydrate (0·5 ml.) in ethanol (10 ml.) for 3 hr. The reaction mixture was cooled and ether (20 ml.) added. The precipitate (1·6 g.) which had a m.p. of 137°, was recrystallized from water. The analytical sample of *α-benzoyl-ε-benzyloxycarbonyl-L-lysine hydrazide* melted at 155°. (Found: C, 63·7; H, 6·4; N, 14·2. $C_{21}H_{26}N_4O_4$ requires C, 63·3; H, 6·6; N, 14·1%.)

α-Benzoyl-di-(ε-benzyloxycarbonyl)-di-L-lysine benzyl ester (IX). A solution of VIII (2·0 g.) in glacial acetic acid (2 ml.) was mixed with ethyl acetate (40 ml.) and 6N-HCl (10 ml.). To the ice-cooled mixture sodium nitrite (0·40 g. in 10 ml. of water) was added with shaking. The organic layer containing α-benzoyl-ε-benzyloxycarbonyl-L-lysine azide, was separated and the aqueous layer extracted with ethyl acetate (20 ml.). The combined extracts were washed with water, aqueous bicarbonate and water, and finally dried over anhydrous sodium sulphate. The dried azide solution was mixed with an ice-cooled solution of ε-benzyloxycarbonyl-L-lysine benzyl ester (4·0 g.) (Erlanger & Brand, 1951) in ethyl acetate (20 ml.). After standing overnight at room temp., ether (40 ml.) was added and a yield of 2·0 g. of *α-benzoyl-εε-dibenzyloxycarbonyl-di-L-lysine benzyl ester*, m.p. 105°, obtained. Recrystallization from ethanol raised the m.p. to 128°. (Found: C, 68·6; H, 6·4; N, 7·4. $C_{42}H_{48}N_4O_8$ requires C, 68·5; H, 6·6; N, 7·6%.)

α-Benzoyl-di-L-lysine dihydrobromide. Compound (IX) (0·5 g.) was dissolved in glacial acetic acid (10 ml.) and a stream of anhydrous HBr was passed through the solution at 80° during 30 min. Anhydrous ether (20 ml.) was added and the precipitate formed was washed repeatedly with anhydrous ether. It was dissolved in 10 ml. of water, the solution extracted with ether, concentrated to a small volume and *α-benzoyl-di-L-lysine dihydrobromide* precipitated by the addition of absolute ethanol and ether. The white powder thus obtained was washed with ether and dried *in vacuo* over KOH and P_2O_5. (Found: N, 9·8; amino-N, 5·1; Br, 29·8. $C_{19}H_{32}N_4O_4Br_2$ requires N, 10·4; amino-N, 5·2; Br, 29·6%.)

α-Benzoyl-di-L-lysine dihydrochloride. This compound was obtained by catalytic hydrogenation of (IX) in methanol at room temp. and 2 atm. pressure in the presence of Pd (5% on charcoal) and 2 equiv. of HCl. The product obtained had an R_F identical with that of the dihydrobromide.

α-Benzoyl-di-(ε-benzyloxycarbonyl)-di-L-lysine hydrazide (X). This compound was prepared in a 70% yield from (IX) analogously to the preparation of (VIII). After recrystallization from ethanol–ether it had m.p. 185–189°. (Found: C, 63·8; H, 6·7; N, 13·1. $C_{35}H_{44}N_6O_7$ requires C, 63·6; H, 6·7; N, 12·7%.)

α-Benzoyl-tri-(ε-benzyloxycarbonyl)-tri-L-lysine benzyl ester (XI). This compound was prepared in a 58% yield from (X) and ε-benzyloxycarbonyl-L-lysine benzyl ester, analogously to the preparation of (IX). M.p. 120–125°. (Found: C, 67·4; H, 7·0; N, 8·4. $C_{56}H_{66}N_6O_{11}$ requires C, 67·3; H, 6·7; N, 8·4%.)

α-Benzoyl-tri-(ε-benzyloxycarbonyl)-tri-L-lysine (XII). This compound was prepared by alkaline hydrolysis of (XI) in ethanol. It was dissolved in ethyl acetate and

precipitated by light petroleum. M.p. 100–115°. (Found: equiv. wt., 916, by titration with sodium methoxide, with thymol blue as indicator. $C_{49}H_{60}N_6O_{11}$ requires equiv. wt. 909.)

α-Benzoyltri-L-lysine trihydrobromide. Removal of the benzyloxycarbonyl groups of (XII) with anhydrous hydrogen bromide as described in the preparation of (IX) gave *α-benzoyltri-L-lysine trihydrobromide.* Yield 85%. (Found: N, 10·6; amino-N, 5·6; Br, 32·3. $C_{25}H_{45}N_6O_5Br_3$ requires N, 11·1; amino-N, 5·6; Br, 32·0%.)

α-Benzoyldibenzyloxycarbonyl-dilysine benzyl ester was also prepared by coupling ε-benzyloxycarbonyl-L-lysine benzyl ester with the mixed anhydride derived from α-benzoyl-ε-benzyloxycarbonyl-L-lysine and *iso*butyl chloroformate according to the procedure of Vaughan & Osato (1952). The α-benzoyldibenzyloxycarbonyl-di-lysine formed on alkaline hydrolysis was again coupled with *iso*butyl chloroformate and the mixed anhydride obtained reacted with ε-benzyloxycarbonyl-L-lysine benzyl ester. The α-benzoyltri-(benzyloxycarbonyl)trilysine benzyl ester thus obtained was hydrolysed with NaOH and the benzyloxycarbonyl groups of the free acid were removed with anhydrous HBr in glacial acetic acid. The α-benzoyltrilysine isolated from the reaction mixture moved on a chromatogram at a rate identical with that of the α-benzoyltri-L-lysine prepared by the azide method. As it was found, however, that the α-benzoyltrilysine prepared by the azide method is quantitatively digested by trypsin to benzoyllysine and lysyllysine, whereas the α-benzoyltrilysine prepared by the mixed anhydride technique is hydrolysed by the same enzyme only to a small extent, it was concluded that partial racemization occurred during the synthesis of the α-benzoyltrilysine by the mixed-anhydride technique.

Di-, tri-, tetra- and penta-L-lysine. These compounds were prepared according to Waley & Watson (1953).

Enzyme. Crystalline trypsin (50% $MgSO_4$) from Mann Research Laboratories, New York, Inc., was used throughout.

Methods

Enzymic reactions. A weighed amount of substrate (about 5 mg.) was dissolved in 0·25 ml. of 0·1 M phosphate buffer, pH 7·6, and 0·25 ml. of a solution of trypsin (0·004 to 0·8 mg. of N/ml.) in the same buffer was added. The mixture was incubated at 25° or 40°, and samples of 8·0 μl. were withdrawn at given intervals for chromatographic analysis. Blanks were taken on 4·0 μl. samples before the addition of the enzyme.

Qualitative chromatographic analysis. Chromatographic analyses of the enzymic incubation mixtures were carried out according to Waley & Watson (1953), *n*-butanol–acetic acid–pyridine–water (30:6:20:24, by vol.) being used as chromatographic solvent. Table 1 gives the R_F values for the various materials used.

Quantitative chromatographic analysis. Chromatograms were dried in a hood at room temp. for several hours and heated to 100° during 15 min., and the spots were located in ultraviolet light (Fowden, 1951). The paper was sprayed with a 1% solution of KOH in absolute methanol and heated for 15 min. at 60°. The spots, as well as paper blanks, were then cut out and the ninhydrin colour was developed and read (Boissonnas, 1950) on a Hilger Uvispek spectrophotometer H 700/303. Calibration curves giving the optical density at 5700 Å as a function of concentration were obtained from chromatograms containing known amounts of substance whenever the material was found to be chromatographically pure. Otherwise the materials were subjected to large-scale paper chromatography employing about 20 mg. of substance. After elution of the appropriate parts of the paper the concentrations (in mg. of N/ml.) were determined by micro-Kjeldahl analysis, and calibration curves constructed as above.

Paper electrophoresis. Paper electrophoresis analyses were carried out in a Consden & Stanier (1952) apparatus with a potential gradient of 10 v/cm. A 0·1 M acetate buffer (pH 4·0) was used and the electrical current usually passed for 2 hr.

Table 1. *R_F values of reference compounds*

The compounds were chromatographed on Whatman no. 1 paper with *n*-butanol–acetic acid–pyridine–water (30:6:20:24, by vol.).

Substance	R_F	Substance	R_F
L-Lysine	0·20	α-Benzoyl-L-lysine	0·74
Di-L-lysine	0·14	α-Benzoyldi-L-lysine	0·48
Tri-L-lysine	0·10	α-Benzoyltri-L-lysine	0·32
Tetra-L-lysine	0·07	Glycine amide	0·26
Penta-L-lysine	0·05	Glycyl-L-lysine	0·18
L-Lysine amide	0·25	L-Lysylglycine	0·18
Di-L-lysine amide	0·15	Glycyl-L-lysine amide	0·20
		L-Lysylglycine amide	0·20

RESULTS

The following abbreviations are used: Lys for L-lysine and L-lysyl residues, Gly for glycyl residues, and Am for amide groups.

α-Benzoyl-L-lysine. The reaction mixture contained 2·5 mg. of trypsin and 10 mg. of benzoyllysine/ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°. Within 48 hr. no reaction products could be detected chromatographically, the spot of benzoyllysine remaining unchanged.

α-Benzoyldi-L-lysine. The reaction mixture contained 2·5 mg. of trypsin, 10 mg. of α-benzoyldilysine dihydrochloride in 1 ml. of 0·1 M phosphate buffer (pH 7·6), and was incubated at 25°. A very slow hydrolytic reaction occurred. Benzoyllysine and lysine could be detected after 24 hr. and their amounts increased on further incubation for 72 hr.

α-Benzoyltri-L-lysine. The course of the reaction is shown in Fig. 1. A significant hydrolysis to α-benzoyllysine and dilysine was detected already within 2 min. After an hour the hydrolysis was almost complete and the only products formed were α-benzoyllysine and dilysine.

Lysine amide. The reaction mixture contained 2·5 mg. of trypsin and 10 mg. of lysine amide dihydrochloride/ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°. Although the concentration of substrate was considerably lower than that used by Waley & Watson (1954), a course of reaction similar to that reported by these authors was observed. The main products were lysine and dilysine; traces of trilysine appeared only after several days. The appearance of dilysine and tri-

lysine proves that a transpeptidation reaction occurred. The absence of dilysine amide in most of our experiments shows that this substance, which is probably the first product formed as a result of transpeptidation: 2 Lys.Am → Lys_2.Am + NH_3, rapidly undergoes further reactions to give either dilysine by hydrolysis or higher lysine peptide amides by transpeptidation. The experiments to be reported below show that Lys_2.Am is rapidly hydrolysed by trypsin. The possible formation of lysine by direct hydrolysis of lysine amide cannot be excluded.

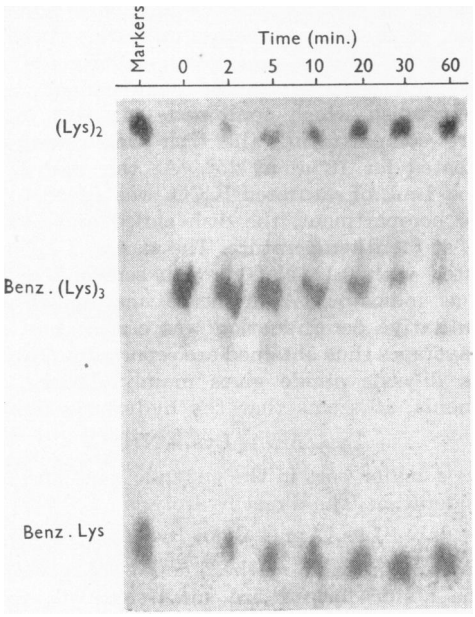

Fig. 1. Chromatogram showing the action of trypsin on α-benzoyltri-L-lysine trihydrobromide. The reaction mixture contained 10 mg. of substrate and 0·25 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

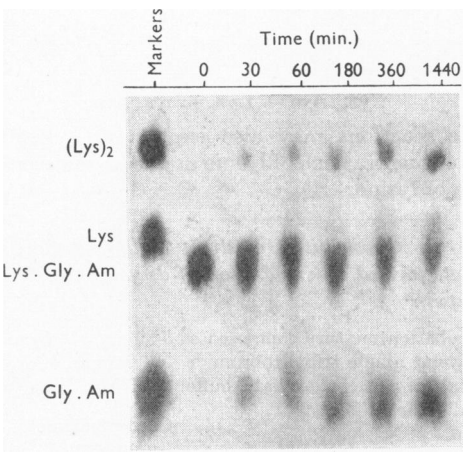

Fig. 2. Chromatogram showing the action of trypsin on L-lysylglycine amide dihydrobromide. The reaction mixture contained 10 mg. of substrate and 2·5 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

L-*Lysylglycine amide.* The course of the reaction is shown in Fig. 2. After half an hour of incubation the chromatogram revealed distinct spots of glycine amide and of dilysine, the amount of which increased with time. Lysylglycine amide and lysine could not be separated by paper chromatography. A larger quantity of a substance with an R_F equal to that of lysylglycine amide was therefore separated from a 24 hr. enzymic mixture by paper chromatography; the spot obtained was eluted with 0·1 N acetic acid and analysed by paper electrophoresis. Two distinct spots corresponding to lysine and lysylglycine amide were obtained. The latter moved towards the cathode considerably faster than the former. Additional characterization of the various spots was effected by extraction, acid hydrolysis and subsequent chromatography or paper electrophoresis which allowed the identification of glycine and lysine. Although some intact lysylglycine amide remained in the reaction mixture even after an incubation period of 48 hr., the amounts of glycine amide, lysine and dilysine increased and traces of trilysine appeared.

The formation of dilysine during the reaction suggests a transpeptidation reaction followed by a rapid hydrolysis:

$$2\ \text{Lys.Gly.Am} \rightarrow \text{Lys}_2\text{.Gly.Am} + \text{Gly.Am},$$
$$\text{Lys}_2\text{.Gly.Am} \rightarrow \text{Lys}_2 + \text{Gly.Am}.$$

Lys_2.Am (see below), as well as Lys_2.Tyr.Am (cf. Waley & Watson, 1954), is rapidly hydrolysed by trypsin to Lys_2 and NH_3 or Lys_2 and Tyr.Am respectively. This may explain the absence of Lys_2.Gly.Am on the chromatogram. Lysine may be formed either by direct hydrolysis of Lys.Gly.Am, or from Lys_2 as discussed above.

Glycyl-L-lysine amide. The behaviour of glycyl-L-lysine amide towards trypsin is given in Fig. 3. Rapid hydrolysis of the substrate occurred, yielding glycyllysine exclusively. Within 24 hr. the hydrolysis was almost complete. The spot of glycyllysine formed yielded after elution and total acid hydrolysis equimolar amounts of glycine and lysine which were identified by paper chromatography, with butanol–acetic acid–water (4:1:5, by vol.) as developing solvent, or by paper electrophoresis.

Di-L-lysine. The reaction mixture contained 2·5 mg. of trypsin and 10 mg. of dilysine trihydrochloride/ml. of 0·1 M phosphate buffer (pH 7·6) and

was incubated at 25°. The results obtained were in agreement with those given by Waley & Watson (1954), i.e. similar amounts of lysine and trilysine were formed.

Di-L-lysine amide. The action of trypsin on dilysine amide is given in Fig. 4. The chromatogram revealed that dilysine amide gives on incubation with trypsin mainly dilysine; however, lysine amide, lysine, as well as trilysine and probably traces of tetralysine, appeared in the mixture. It was observed that tetralysine disappeared after 24 hr. and the amount of lysine amide formed was considerably larger than that of lysine.

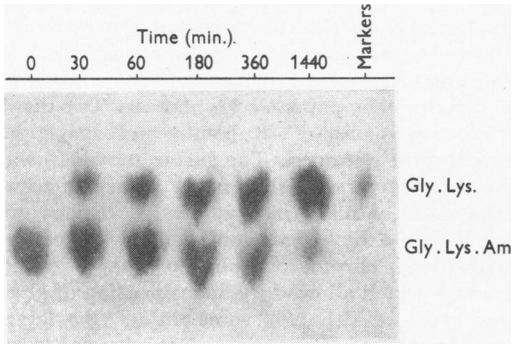

Fig. 3. Chromatogram showing behaviour of glycyl-L-lysine amide dihydrobromide towards trypsin. The reaction mixture contained 10 mg. of substrate and 0·5 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

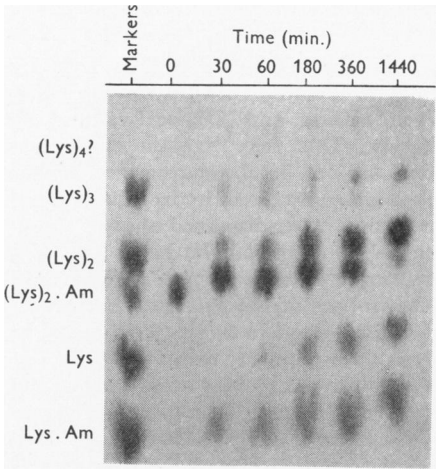

Fig. 4. Chromatogram showing the action of trypsin on di-L-lysine amide. The reaction mixture contained 10 mg. of dilysine amide trihydrobromide and 0·5 mg. of trypsin in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

A quantitative analysis of the products present in an incubation mixture after 18 hr. is given in Table 2. The amounts of dilysine amide, dilysine, lysine amide, lysine and trilysine were determined by quantitative chromatography. For the determination of the amount of ammonia liberated 0·4 ml. of the initial reaction mixture, containing 2·38 mg. of dilysine amide trihydrobromide, was transferred into the outer compartment of a Conway dish which contained 0·1 N-HCl in the centre compartment; the dish was closed and incubated for 18 hr. at 25°. At the end of this period 1 ml. of saturated K_2CO_3 was added to the outer compartment, the dish closed and left for 3 hr. at room temperature. The excess of HCl was titrated with 0·1 N-NaOH, with screened methyl red as indicator. A duplicate analysis of each quantitative determination was carried out, and the averages thus obtained are reported in Table 2.

As dilysine amide gives mainly dilysine and ammonia, it seems that the hydrolytic reaction

$$Lys_2 . Am \rightarrow Lys_2 + NH_3$$

plays a major part in the enzymic reaction under consideration. The direct hydrolysis

$$Lys_2 . Am \rightarrow Lys + Lys . Am$$

seems less probable as the amounts of lysine and lysine amide formed are small, and the molar concentration of lysine amide exceeds considerably that of lysine. The formation of trilysine and tetralysine indicates that a transpeptidation reaction also occurs. The appearance of trilysine may be explained, for example, by the following two successive steps:

$$2Lys_2 . Am \rightarrow Lys_3 . Am + Lys . Am,$$

and $$Lys_3 . Am \rightarrow Lys_3 + NH_3 .$$

Trilysine amide may, of course, be hydrolysed by other routes such as

$$Lys_3 . Am \rightarrow Lys_2 + Lys . Am.$$

These reactions may also explain the relatively high concentrations of lysine amide as compared to those of lysine.

Table 2. *Quantitative analysis of the reaction products formed on incubation of dilysine amide with trypsin*

Incubation mixture composed of 5·95 mg. (11·5 μmoles) of dilysine amide trihydrobromide and 0·5 mg. of trypsin in 1 ml. of 0·1 M phosphate buffer (pH 7·6). Incubation period 18 hr. at 25°.

Material	Concentration (μmoles/ml.)
Dilysine amide	2·0
Dilysine	6·5
Lysine amide	1·9
Trilysine	0·5
Lysine	1·1
Ammonia	7·2

The appearance of tetralysine may be explained by a transpeptidation reaction:

$$2Lys_2.Am \rightarrow Lys_4.Am + NH_3,$$

followed by a hydrolytic reaction:

$$Lys_4.Am \rightarrow Lys_4 + NH_3.$$

As tetralysine disappears rapidly in the presence of trypsin, its concentration in the reaction mixture is small.

Tri-L-lysine. Fig. 5 shows that tetra-, penta-, and hexa-lysine appear in an incubation mixture of trilysine and trypsin within a few minutes, and their concentration stays practically constant for several hours. The concentration of trilysine decreases, while that of dilysine increases, during this period. A strikingly small amount of lysine was formed.

A quantitative chromatographic analysis of an incubation mixture of trilysine and trypsin at different time intervals is given in Table 3. The amounts of tetra-, penta- and hexa-lysine were too small for a reliable quantitative analysis.

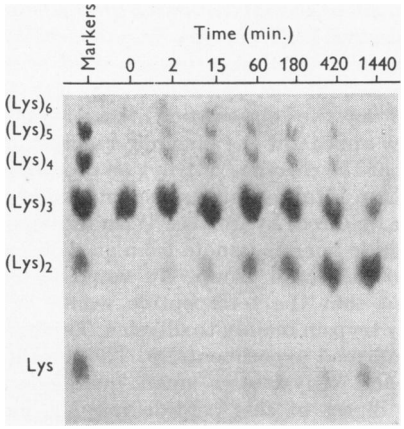

Fig. 5. Chromatogram showing the action of trypsin on tri-L-lysine tetrahydrochloride. The reaction mixture contained 10 mg. of substrate and 0·5 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

The appearance of tetra-, penta- and hexa-lysine in the reaction mixture proves that a transpeptidation reaction took place. Their formation may be explained by reactions such as:

$$2Lys_3 \rightarrow Lys_4 + Lys_2, \quad Lys_4 + Lys_3 \rightarrow Lys_5 + Lys_2$$
$$2Lys_3 \rightarrow Lys_5 + Lys, \quad Lys_4 + Lys_3 \rightarrow Lys_6 + Lys$$
$$Lys_5 + Lys_3 \rightarrow Lys_6 + Lys_2.$$

In all our experiments (e.g. Table 3) the relative amounts of lysine were small and the trilysine which disappeared from the reaction mixture was transformed almost quantitatively into dilysine. This indicates that a direct hydrolysis of trilysine to dilysine and lysine does not proceed to a considerable extent. As the higher lysine peptides are hydrolysed more rapidly than the lower ones (Waley & Watson, 1953) it seems probable that the tetralysine and the higher lysine peptides serve as intermediates in the transformation of trilysine to the terminal products of the enzymic reaction.

Transpeptidations of trilysine leading to the liberation of dilysine appear to dominate those leading to the formation of lysine. The tetralysine which was formed by transpeptidation may give dilysine by hydrolysis: $Lys_4 \rightarrow 2Lys_2$. The pentalysine, on the other hand, may give dilysine and trilysine. A more complicated route of hydrolysis would be expected for hexalysine.

As the concentration of trypsin in these experiments was considerably lower than that required to effect noticeable cleavage of dilysine, it seems that the amount of dilysine accumulated approximates the amount formed. This conclusion is supported by the small amounts of lysine found in the tryptic digests of trilysine.

The results show that the cleavage of trilysine by trypsin involves a transpeptidation reaction leading to the formation of higher lysine peptides, which yield mainly dilysine on hydrolysis.

DISCUSSION

The minimum structural requirements for trypsin substrates were summarized by Neurath & Schwert (1950) and by Green & Neurath (1954). It was shown that the only peptide or ester bonds

Table 3. *Quantitative analysis of an incubation mixture of trilysine and trypsin*

The incubation mixture contained 10·23 mg. (18·7 μmoles) of trilysine tetrahydrochloride and 0·26 mg. of trypsin in 1 ml. of 0·1 M phosphate buffer (pH 7·6), and was incubated at 40°. Samples (8·2 μl.) were withdrawn at given intervals and the amounts of trilysine, dilysine and lysine determined as described above. Concentrations are given in μmoles of lysine residue × 10^2 per 8·2 μl. of reaction mixture.

Material	Time (min.) 0	15	45	100	180	360	1440
Trilysine	46·2	44·0	40·0	37·8	35·8	29·7	18·4
Dilysine	0·0	1·6	3·9	8·2	10·0	17·1	28·8
Lysine	0·0	0·0	0·0	0·5	1·0	1·6	2·2
Trilysine decomposed	—	2·2	4·0	8·4	10·5	16·5	27·9

which are hydrolysed are those in which the carbonyl group is contributed by arginine or lysine, possessing a free ϵ-amino or a free δ-guanido group respectively. The blocking of the α-amino group of lysine or arginine by an acyl group facilitates considerably hydrolysis at alkaline pH values. Our findings with benzoyltrilysine, benzoyldilysine and benzoyllysine clearly indicate that in these substrates, in which no transpeptidation takes place, an α-carboxyl group adjacent to a lysyl peptide bond inhibits its tryptic digestion. Benzoyltrilysine which contains two lysyl peptide bonds, the first adjacent to a free α-carboxyl group and the second remote from it, is hydrolysed rapidly, in the presence of trypsin, at the remote peptide bond to give benzoyllysine and dilysine. Benzoyldilysine, on the other hand, which contains only one lysine bond adjacent to a free α-carboxyl group, is practically resistant to tryptic digestion.

The elucidation of the effect of an α-amino group adjacent to a lysyl peptide bond on the availability of this bond to tryptic digestion is considerably more difficult than in the case of the carboxyl group, since the presence of a free amino group enables transpeptidation to occur, leading to the formation of new compounds, some of which might be formed by direct hydrolysis of the substrate. The simplest compound for the investigation of the influence of an adjacent α-amino group on the rate of hydrolysis of a lysyl peptide bond is lysine amide. The evidence advanced by Waley & Watson (1954), as well as the results reported above, show that the disappearance of lysine amide, in the presence of trypsin, takes place to a significant extent only at relatively high enzyme concentration. Furthermore, it seems probable that, under the experimental conditions used, most of the lysine formed was obtained as a result of transpeptidation. These observations support the conclusion that a free α-amino group adjacent to a lysyl amide bond retards its hydrolysis by trypsin. The findings of Waley & Watson (1954) that dilysine ethyl ester is rapidly hydrolysed in the presence of low concentrations of trypsin to dilysine and ethanol, as well as our findings that dilysine amide is hydrolysed by trypsin mainly to dilysine and ammonia, also indicate that while the amide or ester bonds which are remote from the free α-amino group of the dipeptide undergo relatively rapid hydrolysis, the lysyl amide bonds which are adjacent to the α-amino group remain intact or undergo only slow hydrolysis. The observation of Waley & Watson (1954) that dilysyltyrosine amide yields in the presence of trypsin mainly dilysine and tyrosine amide, as well as our own observation that glycyllysine amide is hydrolysed considerably faster than lysylglycine amide, the former yielding glycyllysine and ammonia and the latter lysine and glycine amide, are also in accord with the interpretation given above for the inhibitory effect of the α-amino group. The findings of Werbin & Palm (1951) and Goldenberg & Goldenberg (1950) that lysine and arginine esters are hydrolysed rapidly by trypsin seem to contradict the conclusions drawn concerning the inhibitory action of the α-amino group. However, esters usually undergo tryptic hydrolysis considerably more rapidly than the respective amides and the optimum tryptic hydrolysis of the arginine methyl ester takes place at pH 5·8, whereas all the enzymic experiments reported in the present paper were carried out at pH 7·6. The data of Goldenberg & Goldenberg (1950) show that at this alkaline pH the tryptic hydrolysis of arginine ester is very slow.

The only peptide bond in lysyllysine is adjacent to a terminal α-carboxyl as well as a terminal α-amino group; as expected, it is hydrolysed extremely slowly by trypsin. At relatively high enzyme concentrations the dipeptide gives lysine and trilysine as a result of transpeptidation. In trilysine two peptide bonds are present, one adjacent to a free terminal carboxyl group and the other to a free terminal α-amino group. The tryptic hydrolysis of both peptide links should therefore be inhibited. The observation that trilysine is transformed almost quantitatively into dilysine, as well as the observation that higher lysine peptides appear in the enzymic reaction mixture, show that the cleavage of trilysine proceeds mainly through an intermediate transpeptidation reaction and not by direct hydrolysis. With tetralysine only one peptide bond is remote from an α-amino group and an α-carboxyl group. It would therefore be expected that the tetrapeptide would be hydrolysed by trypsin mainly to dilysine. This conclusion was confirmed experimentally. The appearance of lysine and trilysine in small quantities in the tryptic digest of this peptide may be explained either by transpeptidation or by direct hydrolysis. Pentalysine possesses two peptide bonds which are not affected by the two terminal groups, and their hydrolytic cleavage should obviously lead to the formation of equimolar amounts of dilysine and trilysine. In fact Waley & Watson (1953) found that at low enzyme concentration, when the transpeptidation reactions of dilysine and trilysine in the presence of trypsin are negligible, pentalysine yielded the di- and tri-peptide in equimolar amounts.

In order roughly to estimate the relative rates at which the various substrates are degraded by trypsin, the time $t_{\frac{1}{4}}$, required for the disappearance of approximately 50% of substrate was determined chromatographically in a number of cases. The molar concentration of the lysine residue in each of the substrate solutions tested was 50 μM.

The results given in Table 4 confirm the general conclusions drawn above. In addition, it is demonstrated that benzoyltrilysine is hydrolysed at a rate comparable with that of tetralysine, and considerably more rapidly than benzoyllysine amide. This suggests that the number of amide bonds in the substrate has a pronounced influence on the rate of tryptic digestion.

In the present work, as well as in the work of Waley & Watson (1954), transpeptidation was recognized by the formation of products which cannot be derived from the substrate by direct hydrolysis. The appearance of peptides with a number of lysine residues greater than that present in the original substrate, or in some cases the appearance of peptides with a sequence of amino acids different from that to be found in the substrate, proved that transpeptidation occurred. All the substrates in which transpeptidation reactions occurred contained a free α-amino group. The blocking of the α-amino group by a benzoyl group eliminated transpeptidation. This suggests that the transpeptidation reactions investigated are of the carboxyl-transfer type, in which the carboxyl moiety of the donor peptide is transferred to new linkage with the free amino group of the acceptor (Hanes, Hird & Isherwood, 1950). In several transpeptidation reactions with lysine peptides or their derivatives the substrates served simultaneously as donors and acceptors. Transpeptidation occurs at a relatively low concentration of peptides participating in the reaction. Hydrolysis, on the other hand, takes place between a substrate at a relatively low concentration, and water at high concentration (55·5 moles/l.). The appearance of transpeptidation products in the reaction mixture indicates therefore that the specific rate constants of the transpeptidation reactions are considerably higher than the specific rate constants of hydrolysis.

Table 4. *Time of disappearance of* 50% *substrate by tryptic digestion*

The reaction mixtures tested were composed of the substrate in a concentration of 50 μM lysine residue, and trypsin, in a concentration specified below, in 0·1 M phosphate buffer (pH 7·6). Incubation temp. 25°.

Substrate	Enzyme concn. (mg. of protein/ml.)	$t_{\frac{1}{2}}$ (min.)
α-Benzoyltrilysine	0·025	40
α-Benzoyllysine amide	0·25	120
Dilysine amide	0·25	288
Glycyllysine amide	0·25	258
Lysylglycine amide	5·00	230
Lysine amide	5·00	540
Tetralysine	0·025	100
Trilysine	0·26	720

SUMMARY

1. The synthesis of L-lysine amide, L-lysylglycine amide, glycyl-L-lysine amide, di-L-lysine amide, α-benzoyldi-L-lysine and α-benzoyltri-L-lysine is described.

2. α-Benzoyltri-L-lysine is rapidly hydrolysed by trypsin to α-benzoyllysine and dilysine. α-Benzoyldi-L-lysine is almost resistant to trypsin.

3. Chromatographic analysis showed that although glycyl-L-lysine amide is hydrolysed rapidly by trypsin to glycyllysine and ammonia, L-lysylglycine amide is attacked by trypsin slowly, yielding dilysine in addition to glycyllysine amide and lysine.

4. The chromatographic analysis of the incubation mixtures with trypsin of L-lysine amide, di-L-lysine amide and tri-L-lysine proved that transpeptidation as well as hydrolysis take place.

5. A quantitative chromatographic analysis of the reaction products formed on incubation of tri-L-lysine with trypsin showed that the tripeptide is transformed almost quantitatively into dilysine. Tetra-, penta-, and hexa-lysine appear in the incubation mixture.

6. The inhibitory effect of α-amino and α-carboxyl groups on the trypsin-catalysed hydrolysis of an adjacent lysyl peptide bond is discussed.

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Some Components of Tobacco Mosaic Virus Preparations made in Different Ways

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Tobacco mosaic virus (TMV) is being studied in many different laboratories for many different reasons, and with results that are not always identical. Some of the diversity is probably caused by the existence of many virus strains, but much of it comes from differences in the conditions under which the host plants are grown and in the technique used for isolating the virus. Bawden & Pirie (1945) demonstrated that several different types of fraction could be made from infected leaves, each containing material related serologically to TMV but differing in infectivity, physical properties and chemical composition.

The different fractions in extracts of the infected leaf differ in their rate of sedimentation in the ultracentrifuge and in the readiness with which they form a coherent pellet in the tube. If therefore the leaf extract is ultracentrifuged for the minimum time needed to sediment most of the virus, and if as much as possible of the supernatant fluid is poured off, the preparation will contain only part of the total material related to TMV, but that part will be relatively uniform because it will contain only one of the fractions described by Bawden & Pirie. The preparations of TMV used in most laboratories are of this type, but even with them there are some inconsistencies, and changes go on during storage.

The object of this paper is to describe some of these changes and to suggest techniques for testing TMV preparations to determine the extent to which they have occurred. These changes had gone nearly to completion in preparations made by the original brutal methods (Bawden & Pirie, 1937*a*, 1943); the gentler ultracentrifugal methods that are now generally used leave components in the preparations that are not essential for infectivity. Significance can be attached to differences in the physical properties or analytical composition of virus strains only if it is certain that all are in the same state. It is clearly convenient that this state should be the simplest with all dispensable material removed, even though there is reason to think (cf. Pirie, 1949, 1953) that complexes between the simplest form of TMV and other materials are not artifacts but exist in the infected plant.

EXPERIMENTAL

Materials and methods

All the preparations of TMV used in these experiments were the pellets made when obtaining ultracentrifuge supernatant fluid for an examination of the properties of the very slowly sedimenting forms of TMV. The technique of growing and harvesting the plants is described in another paper (Bawden & Pirie, 1956). Three virus strains—type strain, *Datura* and U2—were used; their relationships to one another are discussed in that paper. The technique of mincing and ultracentrifuging was that used for making normal leaf nucleoprotein (NP) (Pirie, 1950). Unless otherwise stated, the preparations were sedimented for 30 min. at 65 000–80 000 *g*.

The serological activities and infectivities of these preparations were measured (Bawden & Pirie, 1956) and no preparation used here was less than half as active as the best preparations. Tests were also made on material that had been exposed to the various forms of incubation. These showed that there had been no significant fall in infectivity, and the serological activities, when measured after the aggregation procedure described by Bawden & Pirie (1956), were also unaltered.

The techniques of precipitating nucleic acid with uranyl nitrate and trichloroacetic acid (TCA) and of determining total P were those described by Holden & Pirie (1955*c*).

The measurements of ultraviolet absorption were made at about 20° in 1 cm. quartz cells on a spectrophotometer (Unicam Instrument Co.).