DISCUSSION

The progressive loss of activity of the enzyme preparations during dialysis suggested that some cofactor was being removed from the enzyme by the treatment. This was tested by adding a concentrate of the diffusate to the enzyme preparations, but no reactivation of the enzyme preparations was obtained. This suggests that, if loss of activity of the enzymes is due to a loss of a cofactor, then this cofactor must be labile under the conditions of dialysis and subsequent concentration of the aqueous diffusate.

The bases tested as acceptors in the transphosphorylation reaction may be divided into two classes: those containing acidic groups such as phosphate or sulphate and those without acidic groups. It may be of significance that only the acid-containing bases (lombricine, guanidinoethanol phosphate and taurocyamine) were phosphorylated.

SUMMARY

1. Lombricine has been isolated in good yield from *Lumbricus terrestris* L. with ion-exchange resins. Lombricine flavianate has been prepared.

2. Transphosphorylation from adenosine triphosphate to lombricine has been achieved with dialysed enzyme preparations from worm homogenates or acetone-dried powders. The addition of Mg^{2+} ions was necessary for maximum enzyme activity.

3. Of the bases other than lombricine tested (guanidinoethanol phosphate, taurocyamine, arginine, creatine, guanidine, guanidinoethanol and glycocyamine) as phosphate acceptors, only guanidinoethanol phosphate and taurocyamine were active.

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Pepsin-Catalysed Transpeptidation of the Amino-Transfer Type

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Some *N*-acyldipeptides containing tyrosine or phenylalanine or both are known to be hydrolysed by pepsin (Fruton & Bergmann, 1939; Harington & Pitt-Rivers, 1944; Dekker, Taylor & Fruton, 1949; Casey & Laidler, 1950; Baker, 1951). Direct cleavage of the peptide bond, producing an acylamino acid and a free amino acid, was the only reaction assumed to take place. Analysis of the products formed by peptic digestion of substrates of the above type, containing a *C*-terminal tyrosine, showed that both transpeptidation and hydrolysis occur. It could furthermore be demonstrated that the transpeptidation involves an amino transfer, where the amino moiety of the tyrosine peptides used as substrates is transferred to an acceptor with a free carboxyl group (Hanes, Hird & Isherwood, 1950).

EXPERIMENTAL

Materials

Twice-crystallized pepsin from Mann Research Laboratories Inc., New York, was used throughout.

The following substances were prepared according to the literature: *N*-acetyl-L-tyrosine (du Vigneaud & Meyer, 1932); *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine and *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine (Bergmann, Zervas, Salzmann & Schleich, 1934); *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosyl-L-tyrosine (*N*-benzyloxycarbonyltri-L-tyrosine, Barkdoll & Ross, 1944); *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine amide (Fruton & Bergmann, 1939); *N*-benzyloxycarbonyl-L-glutamic acid (Bergmann & Zervas, 1932); L-tyrosyl-L-tyrosine (Barkdoll & Ross, 1944); *N*-benzyloxycarbonyl-L-tyrosine (Bergmann & Zervas, 1932); L-tyrosine amide (Blau & Waley, 1954). *NO*-Dibenzyloxycarbonyl-L-tyrosine was prepared according to Katchalski & Sela (1953); good yields were obtained when care was taken to maintain pH 9–10 during the coupling. The amount of 4N-NaOH (40 ml.) given by Katchalski & Sela (1953) is in error. Only about 15 ml. was necessary for the coupling of 11·5 g. of tyrosine.

NO-*Dibenzyloxycarbonyl-L-tyrosyl-L-tyrosine ethyl ester* (I). This compound was prepared from *NO*-dibenzyloxycarbonyl-L-tyrosine (4·5 g.), *iso*butyl chlorocarbonate (1·37 g.), triethylamine (1 g.) and L-tyrosine ethyl ester (2·1 g.) in ethyl acetate, by the procedure of Vaughan & Osato (1952). Yield was 5 g. Recrystallization from ethanol gave 4·2 g. of *NO*-dibenzyloxycarbonyl-L-tyrosyl-L-tyrosine ethyl ester, m.p. 178–179° (Found: C, 67·5; H, 5·7; N, 4·3. $C_{36}H_{36}N_2O_9$ requires C, 67·5; H, 5·7; N, 4·4%).

L-*Tyrosyl-L-tyrosine ethyl ester hydrobromide* (II). Compound (I) (2·5 g.) was treated with anhydrous HBr (30% in acetic acid) at room temperature (Ben-Ishai & Berger, 1952) in the presence of phenol (1 g.). On addition of anhydrous ether, after 20 min. an oil precipitated which was repeatedly washed with ether. Reprecipitation with ether from ethanol solution gave 1·4 g. of (II), m.p. 218–220° (Found: C, 52·7; H, 5·5; N, 5·9; Br, 17·7. $C_{20}H_{24}N_2O_5$,HBr requires C, 53·0; H, 5·6; N, 6·2; Br, 17·6%).

NOO′-*Triacetyl-L-tyrosyl-L-tyrosine ethyl ester* (III). Compound (II) (3 g.) was dissolved in anhydrous pyridine, and acetic anhydride (10 ml.) was added. After 24 hr. the reaction mixture was poured into ice water and acidified with conc. HCl. The triacetyl-L-tyrosyl-L-tyrosine ethyl ester was extracted from the above mixture with ethyl acetate, dried over anhydrous Na_2SO_4 and reprecipitated with light petroleum. Recrystallization from methanol gave 2·6 g. of (III), m.p. 99° (Found: C, 63·2; H, 5·9; N, 5·9. $C_{26}H_{30}N_2O_8$ requires C, 62·6; H, 6·1; N, 5·6%).

N-*Acetyl-L-tyrosyl-L-tyrosine*. Compound (III) (2·5 g.) was dissolved in ethanol, and N-NaOH (25 ml.) was added. After 30 min. the solution was acidified with conc. HCl to pH 5, concentrated *in vacuo* and the residue dissolved in ethyl acetate. On precipitation with light petroleum an oil was obtained, which after crystallization from methanol gave 0·8 g. of *N*-acetyl-L-tyrosyl-L-tyrosine. $[\alpha]_D^{25}+19°$ in ethanol (*c*, 1·5), m.p. 240° [Baker (1951) gives $[\alpha]_D^{25}+19°$ in ethanol, m.p. 241°] (Found: C, 62·0; H, 5·7; N, 7·2; CH_3·CO, 12·0. $C_{20}H_{22}N_2O_6$ requires C, 62·2; H, 5·7; N, 7·2; CH_3·CO, 11·2%).

N-p-*Nitrobenzoyl-L-tyrosine ethyl ester* (IV). This compound was prepared by coupling *p*-nitrobenzoyl chloride (9·2 g.) with L-tyrosine ethyl ester hydrochloride (12·2 g.) in 500 ml. of chloroform, in the presence of 100 ml. of aqueous N-Na_2CO_3. Yield was 13 g., m.p. 139–140° (Found: C, 60·2; H, 4·9; N, 7·8; C_2H_5O, 13·0. $C_{18}H_{18}N_2O_6$ requires C, 60·3; H, 5·1; N, 7·8; C_2H_5O, 12·6%).

N-p-*Nitrobenzoyl-L-tyrosine hydrazide* (V). Compound (IV) (7 g.) was dissolved in anhydrous ethanol (50 ml.) and refluxed for 2 hr. with hydrazine hydrate (2 g.). *N-p*-Nitrobenzoyl-L-tyrosine hydrazide (5 g.) precipitated, m.p. 265° (Found: C, 55·8; H, 4·7; N, 16·3; equiv. wt. 355 by titration with perchloric acid in acetic acid and methyl violet as indicator. $C_{16}H_{16}N_4O_5$ requires C, 55·8; H, 4·7; N, 16·3%; equiv. wt. 344).

N-p-*Nitrobenzoyl-L-tyrosyl-L-tyrosine ethyl ester* (VI). A solution of (V) (4 g.) in acetic acid was mixed with 100 ml. of ethyl acetate, 20 ml. of 2N-HCl and 50 ml. of water. To the ice-cooled mixture $NaNO_2$ (900 mg.) in 2 ml. of cold water was added with shaking. The organic layer, containing *p*-nitrobenzoyltyrosine azide, was separated, washed with water and aqueous bicarbonate, and dried over anhydrous Na_2SO_4. The dried azide solution was mixed with an ice-cooled solution of tyrosine ethyl ester (3 g.) in ethyl acetate (20 ml.). After standing overnight, *p*-nitrobenzoyl-L-tyrosyl-L-tyrosine ethyl ester (1·6 g.) precipitated, m.p. 185–187° (Found: C, 62·0; H, 5·3; N, 8·1; C_2H_5O, 8·5. $C_{27}H_{27}N_3O_8$ requires C, 62·2; H, 5·2; N, 8·1, C_2H_5O, 8·6%).

N-p-*Nitrobenzoyl-L-tyrosyl-L-tyrosine* (VII). This compound was prepared by shaking (VI) with 3 equiv. of N-NaOH until dissolved (the solution became red). After 1 hr. *p*-nitrobenzoyl-L-tyrosyl-L-tyrosine was precipitated by acidification. It was extracted into ethyl acetate, the solution dried and the product was precipitated by light petroleum, m.p. 218–219° (Found: C, 58·8; H, 4·9; N, 8·2. $C_{25}H_{23}N_3O_8$,H_2O requires C, 58·7; H, 4·9; N, 8·2%).

N-p-*Aminobenzoyl-L-tyrosyl-L-tyrosine*. This compound was obtained by catalytic reduction of (VII) in ethanol at room temperature and 2 atm. pressure in the presence of Pd (5% on charcoal). The reaction mixture was filtered, and concentrated *in vacuo*. On precipitation with water *N-p*-aminobenzoyl-L-tyrosyl-L-tyrosine, m.p. 246–249°, was obtained (Found: C, 62·3; H, 5·4; N, 8·7. $C_{25}H_{25}N_3O_6$,H_2O requires C, 62·4; H, 5·6; N, 8·7%).

N-p-*Nitrobenzoyl-L-tyrosine* (VIII). This compound was prepared by alkaline hydrolysis of (IV), with 2 equiv. of 2N-NaOH, analogously to the preparation of (VII). On acidification *p*-nitrobenzoyl-L-tyrosine was precipitated, m.p. 176–177° (Found: C, 57·8; H, 4·2; N, 8·5. $C_{16}H_{14}N_2O_6$ requires C, 58·2; H, 4·3; N, 8·5%).

N-p-*Aminobenzoyl-L-tyrosine*. This compound was obtained by catalytic reduction of (VIII) in ethanol at room temperature and 1 atm. in the presence of Pd (5% on charcoal). The ethanol was evaporated *in vacuo* and the residue dried in a desiccator. When the crude product was treated with hot ethyl acetate it first dissolved and then crystallized out on further heating. *N-p*-Aminobenzoyl-L-tyrosine was recrystallized from aq. 85% (v/v) ethanol, m.p. 267° (Found: C, 63·5; H, 5·0; N, 9·5. $C_{16}H_{16}N_2O_4$ requires C, 64·0; H, 5·4; N, 9·3%).

N-*Benzyloxycarbonyl-L-phenylalanyl-L-tyrosine* (IX). This compound was obtained by alkaline (2 moles of 2N-NaOH) hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine ethyl ester (Vaughan & Osato, 1952) in ethanol, m.p. 183–184° (Found: C, 67·4; H, 5·6; N, 6·1. $C_{26}H_{26}N_2O_6$ requires C, 67·5; H, 5·7; N, 6·1%).

L-*Phenylalanyl-L-tyrosine hydrobromide*. Compound (IX) was treated with anhydrous HBr (Ben-Ishai & Berger, 1952). The peptide was isolated as the hydrobromide by precipitation with anhydrous ether, followed by repeated

washing with ether (Found: Br, 19·2. $C_{18}H_{20}N_2O_4$,HBr requires Br, 19·5%).

N-Benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-tyrosyl-L-tyrosine ethyl ester (X). This compound was prepared from *N*-benzyloxycarbonyl-γ-benzyl-L-glutamate (740 mg.) (Hanby, Waley & Watson, 1950), triethylamine (400 mg.), *iso*butyl chlorocarbonate (275 mg.) and tryosyltyrosine ethyl ester hydrobromide (900 mg.), by the procedure of Vaughan & Osato (1952). Yield was 1·2 g., m.p. 141–143° (Found: C, 65·9; H, 6·2; N, 5·7. $C_{40}H_{43}N_3O_{10}$ requires C, 66·2; H, 5·9; N, 5·8%).

N-Benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine. Compound (X) was dissolved in a minimal amount of ethanol and a slight excess (4·5 moles) of 2N-NaOH was added. After half an hour the solution was diluted with water and acidified, whereupon benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine was precipitated, m.p. 177–178° (Found: C, 61·2; H, 5·5; N, 7·0. $C_{31}H_{33}N_3O_{10}$ requires: C, 61·3; H, 5·5; N, 6·9%).

NO-Dibenzyloxycarbonyl-L-tyrosyl-L-tyrosyl-L-tyrosine ethyl ester (XI). Dibenzyloxycarbonyl-L-tyrosine (4·5 g.) (Katchalski & Sela, 1953) was treated with triethylamine (2 g.), *iso*butyl chlorocarbonate (1·37 g.) and L-tyrosyl-L-tyrosine ethyl ester hydrobromide (4·5 g.), by the procedure of Vaughan & Osato (1952). Dibenzyloxycarbonyltrityrosine ethyl ester thus obtained had m.p. 143–144°, yield 5·2 g. (Found: C, 67·2; H, 5·8; N, 5·1. $C_{45}H_{45}N_3O_{11}$ requires C, 67·2; H, 5·6; N, 5·2%).

Tri-L-tyrosine ethyl ester hydrobromide (XII). This compound was prepared from (XI) and anhydrous HBr, by the procedure of Ben-Ishai & Berger (1952); m.p. 167–170° after precipitation from ethanol by means of ether (Found: Br, 12·8. $C_{29}H_{33}N_3O_7$,HBr requires Br, 13·1%).

N-Benzyloxycarbonyl-γ-benzyl-L-glutamyltri-L-tyrosine ethyl ester (XIII). This compound was prepared by the mixed-anhydride method (Vaughan & Osato, 1952) from *N*-benzyloxycarbonyl-γ-benzyl-L-glutamate (3·5 g.) (Hanby *et al.* 1950), triethylamine (1 g.), *iso*butyl chlorocarbonate (1·37 g.) and trityrosine ethyl ester (4·3 g.). Yield was 60%, m.p. 167–169° (Found: C, 65·8; H, 5·8; N, 5·9. $C_{49}H_{52}N_4O_{12}$ requires C, 66·2; H, 5·9; N, 6·3%).

N-Benzyloxycarbonyl-L-glutamyltri-L-tyrosine. Compound (XIII) (0·5 g.) was shaken with 2·5 ml. of N-NaOH for half an hour. The solution was filtered and acidified with 2N-HCl. The *N*-benzyloxycarbonylglutamyltrityrosine which precipitated was dissolved in ethyl acetate and re-precipitated with light petroleum; m.p. 155–157°. Recrystallization from 85% (v/v) ethanol gave m.p. 179–180° (Found: C, 61·8; H, 5·8; N, 7·6. $C_{40}H_{42}N_4O_{12}$ requires C, 62·3; H, 5·5; N, 7·3%).

Methods

Buffers. In the pH range 3·7–5·0, acetic acid–sodium acetate buffers with the following composition were used: 0·85M-acetic acid, 0·1M-sodium acetate (pH 3·7); 0·28M-acetic acid, 0·1M-sodium acetate (pH 4·2); 0·15M-acetic acid, 0·1M-sodium acetate (pH 4·5); 0·09M-acetic acid, 0·1M-sodium acetate (pH 4·7); 0·05M-acetic acid, 0·1M-sodium acetate (pH 5·0).

In the pH range 1·7–3·5, HCl–sodium acetate buffers with the following composition were used: 0·22M-HCl, 0·20M-sodium acetate (pH 1·7); 0·21M-HCl, 0·20M-sodium acetate (pH 2·0); 0·204M-HCl, 0·20M-sodium acetate (pH 2·3); 0·199M-HCl, 0·20M-sodium acetate (pH 2·7); 0·194M-HCl, 0·20M-sodium acetate (pH 3·1); 0·18M-HCl, 0·20M-sodium acetate (pH 3·5).

The following phosphate buffers were used in the electrophoresis experiments: 0·05M-Na_2HPO_4, 0·05M-NaH_2PO_4 (pH 6·8); 0·10M-Na_2HPO_4 (pH 8·5).

Enzymic reaction. Reaction mixtures contained a total of 15–50 μmoles of substrate, and from 3 to 20 mg. of pepsin, per ml. The solvents were acetate buffers at pH 3·5–5·0, or HCl–sodium acetate buffers at pH 1·7–3·5. In the control experiments either the substrate or the enzyme was omitted. Because of the low solubility of most of the substrates in the pH range employed, they were dissolved in ethanol, or in the minimal amount of 0·2M-NaOH, before adjusting the pH with 0·2M-HCl and diluting the solution to the final volume with buffer. The final concentrations of ethanol did not exceed 10%. In some cases the substrates precipitated upon adjustment of the pH but redissolved during the course of incubation. The enzymic reactions were carried out at 37 ± 1°.

Analysis of reaction products. For chromatographic analysis samples of 10–20 μl. of the incubation mixture were placed on Whatman no. 1 filter paper and developed with either butanol–acetic acid–water (25:6:25, by vol.) or propanol–water–conc. aq. NH_3 soln. ammonia (100:50:1, by vol.).

Paper electrophoresis was carried out in a Consden & Stanier (1952) apparatus with a potential gradient of 10 v/cm., with phosphate buffer, pH 8·5 for acetyltyrosyltyrosine and pH 6·8 for the other substrates.

Ninhydrin was used to locate the products containing free amino groups. Tyrosine and its derivatives were detected with diazotized sulphanilic acid in aq. 10% (w/v) Na_2CO_3 (Cramer, 1955), and *p*-aminobenzoyl derivatives with 2% (w/v) *p*-dimethylaminobenzaldehyde in 5% (w/v) HCl. This reagent produces a yellow with aromatic amines, which is strongly fluorescent in ultraviolet light (Dalgliesh, 1952).

As benzyloxycarbonyl-L-glutamic acid and benzyloxycarbonyl-L-phenylalanine could not be detected by these reagents, they were decarbobenzoxylated on the paper with HBr gas, and the free glutamic acid and phenylalanine formed were located with ninhydrin as follows: the paper was placed in a desiccator, which was evacuated and then filled with HBr gas, for 15–30 min. After evacuation to remove the HBr, the paper was washed with ether, dried in a stream of warm air (50–60°) and sprayed with ninhydrin containing pyridine. The blue spots obtained faded within 2 days. Benzyloxycarbonyl-L-glutamyl-L-tyrosine and benzyloxycarbonyl-L-phenylalanyl-L-tyrosine gave faint spots with ninhydrin under these conditions.

Abbreviations

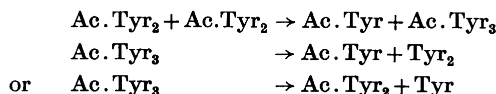
The following abbreviations are used: Tyr for L-tyrosine and L-tyrosyl residues, Phe for L-phenylalanyl residues, Glu for L-glutamyl residues, Ac for acetyl, Z for benzyloxycarbonyl, PAB for *p*-aminobenzoyl and Am for amide groups.

RESULTS

N-Acetyl-L-tyrosyl-L-tyrosine (Ac.Tyr_2). When Ac.Tyr_2 was incubated with pepsin for varying time intervals (up to 48 hr.) at pH 2·0–4·7 and the mixture was analysed chromatographically with

butanol–acetic acid–water as developer, two ninhydrin-positive spots appeared, whose intensity increased with time. One spot had R_F 0·45, identical with that of a sample of tyrosine, and the other spot had R_F 0·68, identical with that of an authentic sample of Tyr_2. The eluted spot with R_F 0·68 yielded only tyrosine on acid hydrolysis. In experiments conducted at pH 2·0–3·5 (HCl–NaCl in the absence of sodium acetate) no formation of acetic acid could be detected either by the method of Hutchens & Kass (1949), or by chromatography according to Kennedy & Barker (1951). This excludes the possibility that Tyr_2 is formed by the hydrolysis of the *N*-acetyl bond of $Ac.Tyr_2$. (Pepsin did not hydrolyse *N*-acetyl-L-tyrosine in the pH range 2·0–4·7.)

The formation of Tyr_2 in the above experiments may be explained by the following sequence of reactions, involving transpeptidation as well as hydrolysis:



$$Ac.Tyr_2 + Ac.Tyr_2 \rightarrow Ac.Tyr + Ac.Tyr_3$$
$$Ac.Tyr_3 \rightarrow Ac.Tyr + Tyr_2$$
or
$$Ac.Tyr_3 \rightarrow Ac.Tyr_2 + Tyr$$

N-p-*Aminobenzoyl*-L-*tyrosyl*-L-*tyrosine* ($PAB.Tyr_2$). This compound was chosen because of the ease with which *p*-aminobenzoic acid can be detected, even in traces, on paper chromatograms.

Chromatograms of 24 hr. incubation mixtures of $PAB.Tyr_2$ and pepsin at pH 1·7–4·7 developed with butanol–acetic acid–water, revealed, with ninhydrin, tyrosine (R_F 0·45) as well as tyrosyltyrosine (R_F 0·68). Parallel chromatograms with propanol–water–conc. aq. NH_3 soln. as developer revealed with *p*-dimethylaminobenzaldehyde two yellow spots with R_F 0·76 and 0·83, corresponding to those of the synthetic PAB.Tyr and $PAB.Tyr_2$ respectively. No trace of free *p*-aminobenzoic acid (R_F 0·51 in propanol–water–conc. aq. NH_3 soln.) was detected. Electrophoretic papers (pH 6·8) run for 2 hr. revealed with the same reagent two yellow spots, 5·3 cm. and 7·5 cm. from the origin towards the cathode, corresponding to those of the synthetic PAB.Tyr and $PAB.Tyr_2$ respectively. No traces of *p*-aminobenzoic acid, which moves 11 cm. from the origin towards the cathode under these conditions, was detected.

The absence of *p*-aminobenzoic acid in the peptic digest shows that the formation of Tyr_2 involved a transpeptidation reaction corresponding to that formulated above for $Ac.Tyr_2$, and did not result from the hydrolysis of the $PAB.Tyr_2$.

N-*Benzyloxycarbonyl*-L-*tyrosyl*-L-*tyrosine* ($Z.Tyr_2$). Chromatographic analysis of a 24 hr. peptic digest of $Z.Tyr_2$ (pH 4·7), with butanol–acetic acid–water as developer, revealed the formation of Tyr and Tyr_2. Since Z.Tyr is resistant to pepsin it may be assumed that the formation of Tyr_2 proceeds by a mechanism analogous to the one suggested to explain its formation from $Ac.Tyr_2$ and $PAB.Tyr_2$.

N-*Benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosine* (Z.Glu.Tyr). Chromatographic analysis of the reaction mixture of pepsin and Z.Glu.Tyr at pH 4·5, with butanol–acetic acid–water as developer, revealed with ninhydrin the presence of Tyr (R_F 0·45) and Tyr_2 (R_F 0·68) in the digest. No spot corresponding to Glu.Tyr (Bergmann *et al.* 1934) (R_F 0·52) could be detected on the chromatogram.

The appearance in a proteolytic digest of a product with an amino acid sequence not present in the substrate is evidence for the occurrence of transpeptidation. Special care was therefore taken to prove unequivocally the identity of the assumed Tyr.Tyr spot. The spot was eluted and hydrolysed with 6N-HCl and found to yield tyrosine only. In addition, the ratio between the absorption at 2420 Å (in 0·1N-NaOH) and the intensity of the ninhydrin colour (at 5700 Å) was determined in the eluted intact material, and found to be close to the corresponding value for authentic Tyr_2. This number is a measure of the ratio between tyrosine residues and terminal amino groups in the substance tested. The ratios obtained from similar measurements carried out with tyrosine, Phe.Tyr and Glu.Tyr were, as expected, approximately half of that found for Tyr_2.

Closer inspection of the enzymic reaction at shorter time intervals gave a significant clue to the reaction mechanism. In this, as well as in the following experiments, advantage was taken of the fact that peptide derivatives containing the glutamic acid residue show rather large mobilities and good separations on paper electrophoresis (pH 6·8), that tyrosine and Tyr_2 separate sufficiently

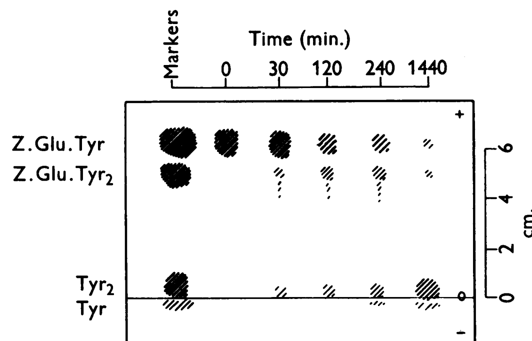


Fig. 1. Electrophoresis diagram showing the action of pepsin on benzyloxycarbonyl-L-glutamyl-L-tyrosine. The reaction mixture contained 13·5 mg. of Z.Glu.Tyr and 9 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37°. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

well at this pH, the former moving to the cathode, the latter to the anode and that tyrosine-containing molecules can be specifically detected by the diazo reagent. An electrophoresis diagram of samples withdrawn from an enzymic reaction mixture at different times is shown in Fig. 1. It can be seen that the substrate (Z.Glu.Tyr) spot decreases steadily and the Tyr_2 spot increases. A small quantity of tyrosine is also formed. There appears, however, a new spot identified as $Z.Glu.Tyr_2$ by its mobility. It shows up clearly at the initial stages of the reaction, when almost no Tyr_2 is yet formed. It is present in a constant concentration while the amount of Tyr_2 is increasing, and disappears towards the end of the reaction together with the substrate (Z.Glu.Tyr). Z.Glu (detected as described in the Methods section), on the other hand, appeared already at the very start of the enzymic reaction, and its amount increased steadily with time.

The above results, where Z.Glu.Tyr yields Z.Glu and Tyr_2 as well as some Tyr, and where $Z.Glu.Tyr_2$ appears as an intermediate, might be explained by the following set of reactions:

(a) $2Z.Glu.Tyr \rightarrow Z.Glu.Tyr_2 + Z.Glu$

(b) $Z.Glu.Tyr \rightarrow Z.Glu + Tyr$

(c) $Z.Glu.Tyr_2 \rightarrow Z.Glu + Tyr_2$

The following experiment indicates, however, that the actual reaction mechanism is more complex.

N-*Benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosyl*-L-*tyrosine* ($Z.Glu.Tyr_2$). This experiment was performed in order to clarify the behaviour towards pepsin of $Z.Glu.Tyr_2$, which appears as intermediate in the enzymic experiment with Z.Glu.Tyr. Comparison of the rate of peptic digestion of $Z.Glu.Tyr_2$ (pH 4·7) with that of Z.Glu.Tyr, under similar conditions, showed that the former is digested initially much more rapidly than the latter. $Z.Glu.Tyr_2$ yielded, at the early stages of enzymic incubation (1–2 hr.), mainly Tyr_2 and Z.Glu.Tyr, as detected electrophoretically (Fig. 2). After 2 hr. the amount of Z.Glu.Tyr formed already exceeds that of the remaining substrates. The latter stages of the reaction resemble therefore those observed in the enzymic experiment with Z.Glu.Tyr. The final reaction mixture was shown to contain Z.Glu, Tyr_2 and tyrosine.

As only traces of tyrosine appear at the beginning of the reaction the formation of Z.Glu.Tyr can be explained only by a transpeptidation reaction followed by hydrolysis:

$$2\,Z.Glu.Tyr_2 \rightarrow Z.Glu.Tyr + Z.Glu.Tyr_3$$
$$Z.Glu.Tyr_3 \rightarrow Z.Glu.Tyr + Tyr_2$$

The scheme suggested explains also the appearance, at the initial phase of the reaction, of relatively large amounts of Tyr_2 not accompanied by corresponding quantities of Z.Glu. The compound $Z.Glu.Tyr_3$ could be detected on the electrophoresis pattern in small quantitities as an intermediate of the enzymic reaction. The peptic digestion of this intermediate is described in the following experiment.

N-*Benzyloxycarbonyl*-L-*glutamyltri*-L-*tyrosine* ($Z.Glu.Tyr_3$). Electrophoretic analysis of the reaction products, carried out as in the previous cases (see Fig. 3), showed that digestion by pepsin proceeds rapidly, yielding initially Tyr_2, Z.Glu.Tyr and $Z.Glu.Tyr_2$, but no tyrosine. The first two substances are probably formed by the direct hydrolysis of the substrate. Direct hydrolysis can,

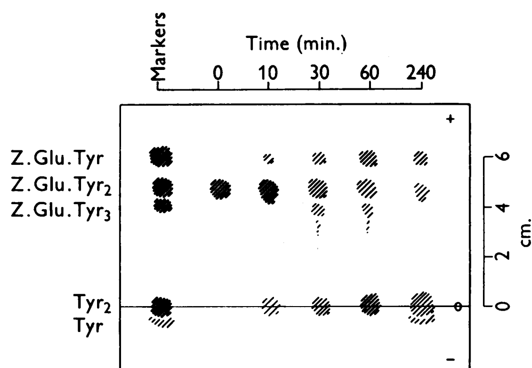


Fig. 2. Electrophoresis diagram showing the action of pepsin on benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine. The reaction mixture contained 7·6 mg. of $Z.Glu.Tyr_2$ and 6·7 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37°. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

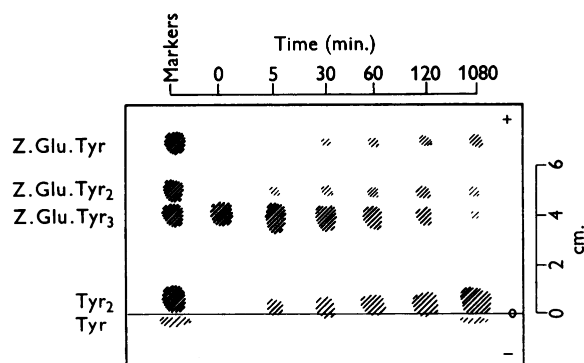
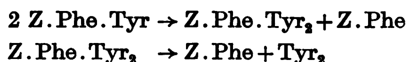


Fig. 3. Electrophoresis diagram showing the action of pepsin on benzyloxycarbonyl-L-glutamyl-L-trityrosine. The reaction mixture contained 6·3 mg. of $Z.Glu.Tyr_3$ and 4·3 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37°. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

however, not be the cause of the formation of Z.Glu.Tyr_2, as no tyrosine was formed. Analogous to the case of Z.Glu.Tyr_2, a transpeptidation reaction involving Z.Glu.Tyr_4, yielding Z.Glu.Tyr_2 and Tyr_2 on hydrolysis, has to be assumed.

N-*Benzyloxycarbonyltri*-L-*tyrosine* (Z.Tyr_3). In this case, too, hydrolysis to Z.Tyr and Tyr_2 is fast. Transpeptidation, however, occurs here also to a certain extent, as is evident from the appearance of Z.Tyr_2, not accompanied by a parallel appearance of tyrosine.

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-L-*tyrosine* (Z.Phe.Tyr). Analysis of the peptic digest (at pH 4·7) of this substrate was carried out similarly to that of Z.Glu.Tyr. Tyr and Tyr_2 (identified as above) were formed, but no spot corresponding to Phe.Tyr (R_F 0·80 in butanol–acetic acid–water) could be detected. Tyr_2 is obviously the result of a transpeptidation reaction followed by hydrolysis:



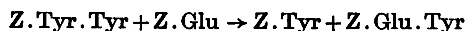
2 Z.Phe.Tyr → Z.Phe.Tyr_2 + Z.Phe
Z.Phe.Tyr_2 → Z.Phe + Tyr_2

N-*Benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosine amide* (Z.Glu.Tyr.Am). This substrate was hydrolysed in the presence of pepsin at pH 4·5 to yield Z.Glu and Tyr.Am, the latter giving the only ninhydrin-positive spot (R_F 0·79 in propanol–water–conc. aq. NH_3 soln.; R_F 0·45 in butanol–acetic acid–water) on a chromatogram of a 48 hr. peptic digest.

The absence of a transpeptidation reaction in the present case indicates that pepsin-catalysed transpeptidation reactions require an acceptor with a free α-carboxyl group.

Mixture of N-*benzyloxycarbonyl*-L-*tyrosyl*-L-*tyrosine* (Z.Tyr_2) *and benzyloxycarbonyl*-L-*glutamic acid* (Z.Glu). A reaction mixture consisting of Z.Tyr_2 (15 μmoles), Z.Glu (40 μmoles), pepsin (6 mg.), acetate buffer, pH 4·5 (0·9 ml.), and ethanol (0·1 ml.) was incubated at 37° for 2 days. In a control experiment the Z.Glu was omitted. Paper electrophoresis (2 hr.) of the control digest showed, with the diazo reagent, four constituents, located at −0·4 cm., +1·2 cm., +3·1 cm. and +4·5 cm. from the origin. By comparison with synthetic markers they were identified as Tyr, Tyr_2, Z.Tyr_2 and Z.Tyr respectively. With the peptic digest of the mixture containing Z.Glu an additional anionic diazo-positive spot was detected +6·5 cm. from the origin after electrophoresis for 2 hr. It was identified as Z.Glu.Tyr by comparison with a synthetic marker, and by the fact that on acid hydrolysis it gave rise to approximately equimolar quantities of tyrosine and glutamic acid. Z.Glu, which does not react with the diazo reagent, was detected with ninhydrin after treatment of the paper with HBr (see Methods section). At the end of 2 hr. it had travelled a distance of 9·5 cm. towards the anode, thus being clearly separated from Z.Glu.Tyr. No reaction occurred when Z.Glu and Tyr were incubated with pepsin.

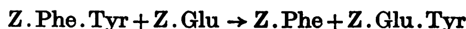
The formation of the Z.Glu.Tyr may be formulated as follows:



Z.Tyr.Tyr + Z.Glu → Z.Tyr + Z.Glu.Tyr

The fact that Z.Glu can serve as an acceptor for the terminal tyrosine moiety of Z.Tyr.Tyr proves the occurrence of a transpeptidation reaction of the amino transfer type.

Mixture of N-*benzyloxycarbonyl*-L-*phenylalanyl*-L-*tyrosine* (Z.Phe.Tyr) *and benzyloxycarbonyl*-L-*glutamic acid* (Z.Glu). A reaction mixture consisting of Z.Phe.Tyr (16 μmoles), Z.Glu (40 μmoles) pepsin (8 mg.), acetate buffer, pH 4·5 (0·9 ml.), and ethanol (0·1 ml.) was incubated for 1 day. In the control experiment Z.Glu was omitted. As in the preceding experiment, electrophoresis papers sprayed with the diazo reagent showed the appearance of Z.Glu.Tyr in the reaction mixture:



Z.Phe.Tyr + Z.Glu → Z.Phe + Z.Glu.Tyr

A relatively large amount of the transpeptidation product (Z.Glu.Tyr) was formed within 1 day.

Mixture of N-*benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosine* (Z.Glu.Tyr) *and* N-*benzyloxycarbonyl*-L-*tyrosine* (Z.Tyr). A reaction mixture consisting of Z.Glu.Tyr (18 μmoles), Z.Tyr (30 μmoles) and pepsin (6·5 mg.) in acetate buffer, pH 4·5 (1 ml.),

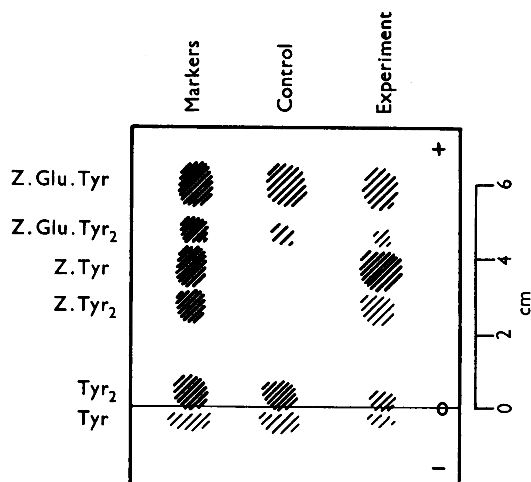


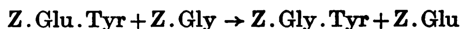
Fig. 4. Electrophoresis diagram showing the action of pepsin on a mixture of benzyloxycarbonyl-L-glutamyl-L-tyrosine and benzyloxycarbonyl-L-tyrosine. The reaction mixture contained 7 mg. of Z.Glu.Tyr, 9·5 mg. of Z.Tyr and 6·5 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37° for 24 hr. In the control experiment the Z.Tyr was omitted. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

was incubated for 1 day. In the control experiment the Z.Tyr was omitted.

Electrophoretic analysis of the peptic digest showed a strong spot, which was identified as Z.Tyr_2 by its mobility (Fig. 4). The amounts of tyrosine and Tyr_2 formed were much smaller than in the control experiment. The formation of the Z.Tyr_2 is the result of a transpeptidation reaction in which Z.Tyr acts as acceptor:

$$Z.Glu.Tyr + Z.Tyr \rightarrow Z.Glu + Z.Tyr_2$$

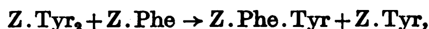
Mixture of benzyloxycarbonyl-L-glutamyl-L-tyrosine (Z.Glu.Tyr) *and benzyloxycarbonylglycine* (Z.Gly). A similar experiment was made with Z.Gly as the acceptor and Z.Glu.Tyr as the tyrosine donor. After 2 days a new spot corresponding to that of Z.Gly.Tyr was located on an electrophoresis paper on spraying with the diazo reagent:



$$Z.Glu.Tyr + Z.Gly \rightarrow Z.Gly.Tyr + Z.Glu$$

Mixture of N-benzyloxycarbonyl-L-tyrosyl-L-tyrosine (Z.Tyr_2) *and N-benzyloxycarbonyl-L-phenylalanine* (Z.Phe). The reaction mixture consisted of Z.Tyr_2 (25 μmoles), Z.Phe (30 μmoles), pepsin (7·5 mg.), acetate buffer, pH 4·5 (0·5 ml.), and ethanol (0·1 ml.). It was incubated for 3 days at 37° before analysis. In the control experiment Z.Phe was omitted.

The expected formation of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine (Z.Phe.Tyr), according to the scheme



$$Z.Tyr_2 + Z.Phe \rightarrow Z.Phe.Tyr + Z.Tyr,$$

could not be demonstrated by either chromatography or electrophoresis of the reaction mixture, since Z.Phe.Tyr cannot be separated by these techniques from Z.Tyr_2. The presence of Z.Phe.Tyr in the reaction mixture could, however, be shown by the isolation of Phe.Tyr after decarbobenzoxylation. The peptic digest was brought to dryness and the residue treated with 1 ml. of 30% (w/v) HBr in acetic acid. The precipitate formed on the addition of ether (20 ml.) was taken up in water (1 ml.) and analysed chromatographically with butanol–acetic acid–water as developer. In addition to the spots of tyrosine, Tyr_2 and phenylalanine (originating from excess of Z.Phe), with R_F values 0·45, 0·68 and 0·65 respectively, a spot with R_F 0·80, identical with that of authentic Phe.Tyr, appeared. The identity of the spot was confirmed by total hydrolysis, which gave rise to approximately equimolar quantities of tyrosine and phenylalanine.

DISCUSSION

In all the experiments with pepsin described in this paper, no 'energy-rich' compounds were present. The formation of new peptide bonds reported here may thus be explained only by a transpeptidation mechanism. The pepsin-catalysed transpeptidations could be demonstrated by the appearance of products with either an increased number of amino acid residues, or with a new amino acid sequence.

Elongation of the peptide chain was found with Z.Glu.Tyr and Z.Glu.Tyr_2, where, after short incubation with pepsin, Z.Glu.Tyr_2 and Z.Glu.Tyr_3 respectively were formed. The formation of new sequences was observed on peptic digestion of Z.Glu.Tyr and Z.Phe.Tyr, both of which yielded Tyr_2 as one of the final products. New sequences were also obtained when, in addition to the substrate, a suitable acceptor was present. The new sequences formed under these conditions are summarized in Table 1.

In some of the enzymic experiments products were formed which could have arisen by simple hydrolysis. Investigation of the other reaction products pointed, however, to a more complicated set of reactions. Thus Ac.Tyr_2 and PAB.Tyr_2 both gave a high yield of Tyr_2, but neither acetic acid nor *p*-aminobenzoic acid was formed. This led to the conclusion that the Tyr_2 must have been formed by the hydrolysis of an intermediate (Ac.Tyr_3 or PAB.Tyr_3) resulting from a transpeptidation reaction similar to the case of Z.Glu.Tyr, and not by a direct hydrolysis of the substrates.

An indirect argument for the occurrence of transpeptidation was used for Z.Glu.Tyr_3. Here the formation of Z.Glu.Tyr_2 in the first stages of the reaction was not accompanied by the corresponding amount of tyrosine. This indicated the formation of Z.Glu.Tyr_4 by the transpeptidation, followed by hydrolysis to Z.Glu.Tyr_2. In this case the intermediate Z.Glu.Tyr_4 could, however, not be clearly detected.

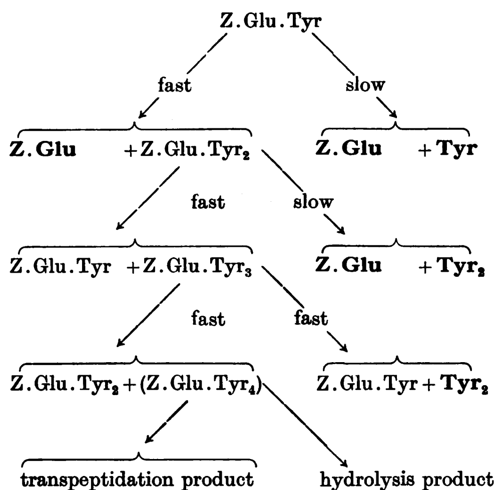
The possible complexity of the reactions taking place when certain low-molecular-weight peptides are digested by pepsin may be illustrated by the following reaction scheme describing the main hydrolysis and transpeptidation pathways in the peptic digestion of Z.Glu.Tyr. The scheme was constructed from the results obtained with Z.Glu.Tyr itself, as well as with the successive intermediates Z.Glu.Tyr_2 and Z.Glu.Tyr_3, synthesized

Table 1. *Products formed by a pepsin-catalysed transpeptidation in the presence of acceptors*

For explanation of abbreviation see Experimental section.

Substrate	Acceptor	Product
Z.Tyr_2	Z.Phe	Z.Phe.Tyr
Z.Tyr_2	Z.Glu	Z.Glu.Tyr
Z.Phe.Tyr	Z.Glu	Z.Glu.Tyr
Z.Glu.Tyr	Z.Tyr	Z.Tyr_2
Z.Glu.Tyr	Z.Gly	Z.Gly.Tyr

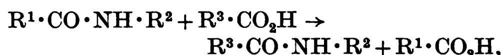
for this purpose. The arrows pointing to the right and left indicate hydrolysis and transpeptidation respectively. Final products are in bold type.



In the substrates investigated, the hydrolysis of the terminal peptide bond adjacent to a tyrosine residue bearing a free carboxyl group was slow as compared with transpeptidation. Fast enzymic hydrolysis was observed only in Z.Glu.Tyr_3 and Z.Tyr_3. The bond hydrolysed in these was the Tyr.Tyr bond not involving the *C*-terminal tyrosine. These findings recall similar observations of the behaviour of exo- and endo-peptide bonds of lysine peptides towards trypsin (Levin, Berger & Katchalski, 1956). With trypsin, as in the present case, digestion of Lys_2 and Lys_3, which are not hydrolysed readily, occurs via the synthesis of higher oligopeptides formed by a transpeptidation mechanism.

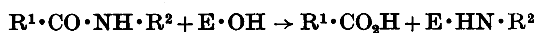
In the attempt to formulate the mechanism of the pepsin-catalysed transpeptidation discussed here, it was borne in mind that: (*a*) transpeptidation occurred only with substrates, such as Z.Glu.Tyr, Z.Phe.Tyr and Z.Tyr_2, possessing a free terminal α-carboxyl group; (*b*) blocking of the α-carboxyl group, as in Z.Glu.Tyr.Am, prevented transpeptidation; (*c*) all compounds acting as acceptors contained a free α-carboxyl group to which a tyrosine residue was transferred.

The overall reaction scheme thus involves the transfer of the tyrosine amino moiety of the donor $R^1{\cdot}CO{\cdot}NH{\cdot}R^2$ [$R^2 = CH(CO_2H){\cdot}CH_2{\cdot}C_6H_4{\cdot}OH$] to an acceptor with a free carboxyl group, $R^3{\cdot}CO_2H$:

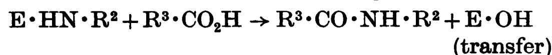


$$R^1{\cdot}CO{\cdot}NH{\cdot}R^2 + R^3{\cdot}CO_2H \rightarrow R^3{\cdot}CO{\cdot}NH{\cdot}R^2 + R^1{\cdot}CO_2H,$$

and is therefore of the amino transfer type (Hanes *et al.* 1950). The detailed mechanism of the enzymic transfer and hydrolysis reactions might be envisaged as follows:



$$R^1{\cdot}CO{\cdot}NH{\cdot}R^2 + E{\cdot}OH \rightarrow R^1{\cdot}CO_2H + E{\cdot}HN{\cdot}R^2$$



$$E{\cdot}HN{\cdot}R^2 + R^3{\cdot}CO_2H \rightarrow R^3{\cdot}CO{\cdot}NH{\cdot}R^2 + E{\cdot}OH \quad \text{(transfer)}$$

$$E{\cdot}HN{\cdot}R^2 + H_2O \rightarrow H_2N{\cdot}R^2 + EOH \quad \text{(hydrolysis)}$$

The enzyme (E·OH) cleaves the susceptible bond of the substrate to yield an enzyme–tyrosine compound ($E{\cdot}HN{\cdot}R^2$), which is decomposed either by the acceptor ($R^3{\cdot}CO_2H$), with the formation of a new peptide bond, or by water, yielding free tyrosine.

Most of the transpeptidation reactions investigated occurred at about pH 4·5. This pH was chosen to facilitate solution of the substrates. It should be noted, however, that whenever tested it was found that pepsin could catalyse transpeptidation also at pH values as low as 1·8–2·0. In this low pH range, carboxyl groups are unionized. It appears therefore that it is the un-ionized carboxyl group that serves as the acceptor.

The possible existence of transpeptidation of the amino transfer type was pointed out by Hanes *et al.* (1950). It seems, however, that the present work constitutes the first experimental proof for such a reaction.

SUMMARY

1. The synthesis of the following compounds is described: *N*-*p*-nitrobenzoyl-L-tyrosine; *N*-*p*-nitrobenzoyl-L-tyrosine ethyl ester; *N*-*p*-nitrobenzoyl-L-tyrosine hydrazide; *N*-*p*-aminobenzoyl-L-tyrosine; L-tyrosyl-L-tyrosine ethyl ester hydrobromide; *N*-acetyl-L-tyrosyl-L-tyrosine; *NOO*-triacetyl-L-tyrosyl-L-tyrosine ethyl ester; *NO*-dibenzyloxycarbonyl-L-tyrosyl-L-tyrosine ethyl ester; *N*-*p*-nitrobenzoyl-L-tyrosyl-L-tyrosine; *N*-*p*-nitrobenzoyl-L-tyrosyl-L-tyrosine ethyl ester; *N*-*p*-aminobenzoyl-L-tyrosyl-L-tyrosine; L-phenylalanyl-L-tyrosine hydrobromide; *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine; tri-L-tyrosine ethyl ester hydrobromide; *NO*-dibenzyloxycarbonyl-L-tyrosyl-L-tyrosyl-L-tyrosine; *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine; *N*-benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-tyrosyl-L-tyrosine ethyl ester; *N*-benzyloxycarbonyl-L-glutamyltri-L-tyrosine and *N*-benzyloxycarbonyl-γ-benzyl-L-glutamyltri-L-tyrosine ethyl ester.

2. A method for the detection of *N*-benzyloxycarbonylamino acids on paper chromatograms was developed.

3. Acetyl-L-tyrosyl-L-tyrosine, *p*-aminobenzoyl-L-tyrosyl-L-tyrosine and *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine yielded tyrosine and tyrosyltyrosine on peptic digestion. Evidence is presented to show that the tyrosyltyrosine was formed, not by a direct hydrolysis of the substrate but as a result

of a transpeptidation reaction, leading to the formation of an *N*-substituted trityrosine intermediate, followed by hydrolysis.

4. Peptic digestion of *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine led to the formation of tyrosine, tyrosyltyrosine and *N*-benzyloxycarbonyl-L-glutamic acid. *N*-Benzyloxycarbonylglutamyltyrosyltyrosine appeared as intermediate. A scheme describing the hydrolysis and transpeptidation reactions taking place in this case was constructed with the help of results obtained with *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine and *N*-benzyloxycarbonyl-L-glutamyltri-L-tyrosine on digestion with pepsin.

5. Peptic digestion of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine yielded tyrosine and tyrosyltyrosine, the latter being the result of a transpeptidation reaction.

6. Incubation of benzyloxycarbonyl-L-glutamyl-L-tyrosine amide with pepsin yielded *N*-benzyloxycarbonylglutamic acid and tyrosine amide. No transpeptidation was observed with this substrate.

7. The compounds *N*-benzyloxycarbonyl-L-phenylalanine, *N*-benzyloxycarbonyl-L-glutamic acid, *N*-benzyloxycarbonyl-L-tyrosine and *N*-benzyloxycarbonylglycine were shown to act as acceptors for the terminal tyrosine residue of the substrates, *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine, *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine, acting as donors in the presence of pepsin.

8. Analysis of the pepsin-catalysed transpeptidation reaction showed that it involves the transfer of a tyrosine amino moiety of a donor to an acceptor with a free carboxyl group. It may thus be classified as a transpeptidation of the amino transfer type.

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Biochemical Adaptation as a Response to Exercise

1. EFFECT OF SWIMMING ON THE LEVELS OF LACTIC DEHYDROGENASE, MALIC DEHYDROGENASE AND PHOSPHORYLASE IN MUSCLES OF 8-, 11- AND 15-WEEK-OLD RATS

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(*Received* 10 *November* 1958)

Although adaptation to physical training has received extensive study from the aspects of performance and physiological effects (Åstrand, 1956), there is little information on this effect at the cellular level. It is significant that the comprehensive review of enzyme adaptation by Knox, Auerbach & Lin (1956) makes no reference whatever to enzymic changes accompanying athletic training.

Most of the published work in this field stems from the Leningrad Physical Culture Research Institute (Yakovlev, 1950; Yampolskaya & Yakovlev, 1951; Yampolskaya, 1952). These workers claim that muscle analyses after training have