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The Synthesis of Cysteine-(Cystine-) Tyrosine Peptides and the Action Thereon of Crystalline Pepsin

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In connexion with some projected immunochemical work it became desirable to have peptides containing both cystine and tyrosine. Since such compounds, none of which has hitherto been available, might also be expected to be of some intrinsic interest, the syntheses of cysteyl- and cystyltyrosine and of tyrosylcysteine and -cystine were undertaken and are here described.

The paper also contains an account of the action of crystalline pepsin on these dipeptides and their acylated derivatives, the study of which has led to results of unexpected interest.

Syntheses of l(−)*cysteyl*-l(−)*tyrosine and* l(−)*tyrosyl*-l(−)*cysteine*

The syntheses have followed conventional lines and call for little comment. It was found necessary to work throughout with the *S*-benzyl derivative of cysteine and to remove the *S*-benzyl group at the final stage.

Cysteyltyrosine. The *N*-carbobenzyloxy derivative of *S*-benzylcysteine ethyl ester was converted through the hydrazide into the azide; the latter was coupled in ethereal solution with *O*-benzoyltyrosine ethyl ester. The resulting ethyl ester of *S*-benzyl-*N*-carbobenzyloxycysteyl-*O*-benzoyltyrosine on treatment with alkali in the cold gave *S*-benzyl-*N*-carbobenzyloxycysteyltyrosine from which the desired peptide was obtained by reduction with sodium in liquid ammonia.

Tyrosylcysteine. *N*-Carbobenzyloxytyrosyl azide was coupled with *S*-benzylcysteine ethyl ester and the product was hydrolyzed with alkali to give *N*-carbobenzyloxy-*S*-benzylcysteine; sodium and liquid ammonia were again used to remove carbobenzyloxy and benzyl groups, yielding tyrosylcysteine.

The yields throughout both syntheses were good. The peptides were both readily converted into the corresponding cystine derivatives by aeration in slightly alkaline solution, the cystine peptides thus obtained being more soluble in water than either of the component amino-acids.

Both the cysteine peptides crystallized easily as did also tyrosylcystine; cystyltyrosine had first to be converted into the well-characterized hydrochloride from which the peptide could be recovered in crystalline form. It may be noted that tyrosylcystine and cystyltyrosine appear to represent the first crystalline cystine peptides to be recorded, although crystalline peptides of cysteine have been obtained by other workers.

The *N*-carbobenzyloxy derivatives of the peptides required for the enzymic experiments described below were prepared by reaction of the cystine peptides with benzylchloroformate, the corresponding

N-carbobenzyloxy cysteyl peptides being obtained by reduction with zinc dust and sulphuric acid.

Action of pepsin on acylated and non-acylated cysteine-(cystine-) tyrosine peptides

The action of crystalline pepsin was studied on (*a*) the *N*-carbobenzyloxy derivatives of cysteyl- and cystyltyrosine and of tyrosylcysteine and -cystine and (*b*) on the corresponding free peptides, the experiments being carried out both at pH 4·0 and at pH 1·8.

The results are summarized in Table 1, from which the following facts emerge: (1) cysteine-(cystine-) tyrosine peptides afford good examples of simple synthetic substrates for peptic action; (2) the action of pepsin on these substrates is much more marked at pH 4·0 than at pH 1·8 which is the commonly accepted optimum for the hydrolysis of proteins by pepsin; (3) although the *N*-carbobenzyloxy derivatives are the most readily hydrolyzed, the action of pepsin extends in this series to the free peptides, which thus represent the first true peptides shown to be attacked by this enzyme; (4) all the substrates, whether acylated or not, are more susceptible to the action of pepsin when they are in the reduced form. These points may be discussed in relation to the work of Bergmann on the nature of the action of pepsin.

(1) Hitherto the only synthetic substrates shown to be attacked by pepsin have been a series of derivatives of peptides containing tyrosine or phenylalanine and glutamic acid in which the amino group of the aromatic amino-acid was combined with the α-carboxyl group of an acylated glutamic acid (Fruton & Bergmann, 1939).

(2) Our substrates behave similarly to those of Fruton & Bergmann in that their hydrolysis by pepsin is much more rapid at pH 4·0 than at pH 1·8, although in the case of cysteyltyrosine and its *N*-carbobenzyloxy derivative we have observed a significant degree of splitting at the latter pH.

(3) As has been noted, the substrates employed by Fruton & Bergmann were all acylated (*N*-carbobenzyloxy-) peptides, with the one exception of glycylglutamyltyrosine; the free glutamyl peptides of tyrosine and phenylalanine were not attacked at all; moreover, it was necessary that the second carboxyl of the glutamic acid residue should be free, since the corresponding glutaminyl derivatives were much more resistant to peptic hydrolysis. In discussing the matter Bergmann & Fruton (1941) lay considerable emphasis on these points, concluding (*a*) that the hydrolysis of a peptide linkage by pepsin is conditional on the absence of a free amino group from the immediate neighbourhood of the linkage attacked and (*b*) that substrates for peptic action must contain more than one carboxyl group.

It is difficult to see how these conclusions can stand in the light of the results which we now record. As to (*a*), reference to Table 1 shows that, whilst acylation of the free amino groups of our peptides undoubtedly favours peptic hydrolysis, the degrees of splitting observed with some of the free peptides are by no means insignificant; moreover, in one case (cysteyltyrosine) splitting is observed at pH 1·8, a reaction at which the free amino group will be charged. In respect to (*b*) it is only necessary to note that none of our reduced substrates, which are the most readily split, has more than one free carboxyl group, and that the rate of hydrolysis of one of them (*N*-carbobenzyloxycysteyltyrosine) is comparable with that of the most sensitive substrate used by Fruton & Bergmann (1939), namely *N*-carbobenzyloxyglutamyltyrosine.

(4) The observation that our substrates are attacked by pepsin much more rapidly in the reduced (—SH) than in the oxidized (—SS—) form

Table 1. *Percentage splitting of acylated and non-acylated cysteine-(cystine-) tyrosine peptides by crystalline pepsin at* 38°

	pH 4·0			pH 1·8	
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.
A. *Acylated peptides:*					
N-Carbobenzyloxytyrosylcysteine	22	39	46	0	10
N-Carbobenzyloxytyrosylcystine	6	7	11	0	0
N-Carbobenzyloxycysteyltyrosine	33	53	85	23	32
N-Carbobenzyloxycystyltyrosine	9	21	29	0	6
N-Carbobenzyloxy-*S*-benzylcysteyltyrosine	—	26	30	—	—
B. *Non-acylated peptides:*					
Tyrosylcysteine	—	22	29	0	8
Tyrosylcystine	—	5	8	0	8
Cysteyltyrosine	—	31	55*	13	21
Cystyltyrosine	—	5	7	0	0

* In a larger experiment cysteyltyrosine (570 mg.) was hydrolyzed with pepsin at pH 4·0 for 60 hr. On removal of pepsin and concentration of the solution crystalline material separated; when recrystallized this gave 119 mg. of pure tyrosine, i.e. 30% of the theoretical yield for complete splitting of the peptide.

appears to raise a point of considerable interest. It had already been shown by Fruton & Bergmann (1939) that the action of crystalline pepsin on *N*-carbobenzyloxyglutamyltyrosine was unaffected by the presence of cysteine. We have ourselves performed the additional control experiment of incubating the twice crystallized pepsin employed by us in the presence of cysteine on the one hand and cystine on the other; under these conditions we have found that at a given pH the 'pepsin blank', i.e. the amount of free amino-nitrogen contained in the original pepsin together with any that may be formed by autolysis of the latter is, within the limits of error, the same whether cysteine or cystine has been present; we are therefore satisfied that the apparently greater susceptibility of the —SH peptides to peptic action is a genuine phenomenon.

This being accepted, it is of interest to recall (*a*) that pepsin is commonly recognized to act much more vigorously on denatured than on native proteins and (*b*) that denaturation of proteins is accompanied by the appearance of —SH groups. The possible interrelation of these facts with our observation is evident; taking into account also the fact that free tyrosine can be liberated from proteins by pepsin it seems not unlikely that at least one point of attack of a denatured protein by pepsin may be a cysteyltyrosine or tyrosylcysteine linkage.

A point of minor interest which appears from the results recorded in Table 1 is that throughout the whole series and at both hydrogen-ion concentrations the cysteyltyrosine derivatives are more sensitive to peptic action than those of tyrosylcysteine. It may also be noted that the rate of splitting of *S*-benzyl-*N*-carbobenzyloxycysteyltyrosine is similar to that of *N*-carbobenzyloxycystyltyrosine and much less than that of the corresponding cysteyl compound; this again emphasizes the importance of the —SH group for the reaction.

EXPERIMENTAL

A. *Synthesis of cysteyltyrosine*

S-Benzylcysteine ethyl ester hydrochloride. *S*-Benzylcysteine (Wood & du Vigneaud, 1939) (4·4 g.) was suspended in ethanol (60 ml.) and the mixture saturated with a vigorous stream of hydrogen chloride; the clear solution which resulted was cooled, resaturated with hydrogen chloride and evaporated under diminished pressure. The crystalline residue was taken up in hot ethanol and the solution mixed with dry ether; the material which separated on cooling was collected and a further small crop was obtained by evaporating the mother liquor, dissolving the residue in the minimum of ethanol and precipitating with ether. The total yield was 4·72 g. (96 %).

The compound formed plates from ethanol-ether, m.p. 156–157°. (Found: N, 5·3; Cl, 12·9 %. $C_{13}H_{18}O_2NSCl$ requires N, 5·1; Cl, 12·9 %.)

S-Benzyl-N-carbobenzyloxycysteine ester. *S*-Benzylcysteine ethyl ester hydrochloride (13·77 g.) was suspended in chloroform (125 ml.); the suspension was cooled in ice-salt and shaken vigorously with 2N-sodium hydroxide (25 ml.); benzyl chloroformate (5·5 ml.) was then added with shaking. This was followed by 2N-sodium carbonate (25 ml.) and a further 5·5 ml. of benzyl chloroformate, vigorous shaking and intermittent cooling being maintained. The chloroform solution was separated, washed twice with dilute hydrochloric acid, twice with aqueous sodium bicarbonate and twice with water; it was dried and evaporated and the residue was redissolved in chloroform; addition of light petroleum caused separation of the ester, which was collected and dried. The yield was 15·65 g. (84 %).

The ester crystallized from chloroform-light petroleum in needles, m.p. 52–53°. (Found: N, 3·7; S, 8·0 %. $C_{20}H_{23}O_4NS$ requires N, 3·8; S, 8·6 %.)

S-Benzyl-N-carbobenzyloxycysteyl hydrazide. The above ester (25·5 g.) was boiled under reflux for 3·5 hr. with ethanol (60 ml.) and hydrazine hydrate (6 ml.). On cooling, a first crop of 15·3 g. separated. A further crop was obtained by concentrating the mother liquor and leaving in an unevacuated desiccator overnight with sulphuric acid to remove excess of hydrazine. The total yield was 19·7 g. (80 %). The hydrazide crystallized from ethanol in large prisms, m.p. 133–134°. (Found: N, 11·8 %. $C_{18}H_{21}O_3N_3S$ requires N, 11·7 %.)

O-Benzoyl-N-carbobenzyloxytyrosine ethyl ester. *N*-Carbobenzyloxytyrosine ethyl ester (11·5 g.) was dissolved in pyridine (50 ml.) and treated with benzoyl chloride (8 ml.). The solution was warmed for 15 min. on the boiling water-bath, cooled and poured into 250 ml. of 3N-hydrochloric acid; the oil which precipitated rapidly hardened. After keeping overnight in the ice-chest, the product was collected, triturated with dilute hydrochloric acid, filtered off, washed with water and dried. It was crystallized from a mixture of benzene and ligroin from which it separated in prisms, m.p. 92°. The yield was 14 g. (94 %). (Found: N, 3·1 %. $C_{26}H_{25}O_6N$ requires N, 3·1 %.)

O-Benzoyltyrosine ethyl ester hydrochloride. The above ester (6·9 g.) was dissolved in methanol (80 ml.) and hydrochloric acid (1·25 ml. of 6·85N) and shaken in an atmosphere of hydrogen with palladium black (1·08 g.); evolution of carbon dioxide was complete in 1¾ hr. The filtered solution was evaporated in a vacuum, the evaporation being repeated after addition of ethanol; the residue was taken up in hot ethanol and the solution mixed with dry ether. The total yield (two successive crops) was 4·56 g. (85 %).

The compound crystallized from ethanol-ether in rectangular prisms, m.p. 225° (decomp.). (Found: N, 4·0; Cl, 10·15%. $C_{18}H_{20}O_4NCl$ requires N, 4·0; Cl, 10·15%.)

S-Benzyl-N-carbobenzyloxycysteyl-O-benzoyltyrosine ethyl ester. *S*-Benzyl-*N*-carbobenzyloxycysteyl hydrazide (6·3 g.) was dissolved in a mixture of water (79 ml.), acetic acid (79 ml.) and 3N-hydrochloric acid (11·7 ml.); the cooled solution was treated gradually, with stirring, with sodium nitrite (1·23 g.) in water (13 ml.). The azide which separated was extracted with ether (200 ml.) and the extract, after rapid drying over sodium sulphate, was added immediately to a solution of *O*-benzoyltyrosine ester in ether; the latter solution was prepared by shaking a chilled suspension of *O*-benzoyltyrosine ester hydrochloride (8·4 g.) in ether (200 ml.) with N-sodium hydroxide (24 ml.), separating the ether and drying over sodium sulphate. Crystallization of the reaction product began immediately after mixing of the ethereal solutions; after 40 hr. in the ice-chest the crystals were collected, washed with cold ether and dried. The yield was 7·96 g. (71% calculated on the hydrazide) of a product crystallizing from ethanol in spear-shaped needles, m.p. 149°. (Found: N, 4·4; S, 4·8%. $C_{36}H_{36}O_7N_2S$ requires N, 4·4; S, 5·0%.)

S-Benzyl-N-carbobenzyloxycysteyltyrosine. The above ester (12 g.) was dissolved in dioxan (80 ml.) and shaken mechanically for 1 hr. with 5N-sodium hydroxide (11·3 ml.); the mixture was diluted with water, acidified and the product collected, dried and extracted with boiling ligroin to remove benzoic acid. It was crystallized from ethanol diluted with a little water and separated in sheaves of fine needles, m.p. 198–200°. The yield was 8·7 g. (92%). (Found: N, 5·5%. $C_{27}H_{28}O_6N_2S$ requires N, 5·5%.)

Cysteyltyrosine. *S*-Benzyl-*N*-carbobenzyloxycysteyltyrosine (5·4 g.) was dissolved in liquid ammonia (75 ml.); sodium in small pieces was added to the stirred solution until a blue colour was obtained, 1·3 g. being required. Ammonium sulphate (3·75 g.) was introduced to discharge the blue colour and the ammonia was evaporated as rapidly as possible; removal of ammonia was completed by warming at 40° in a vacuum followed by 3½ hr. in a vacuum desiccator over sulphuric acid. The residue was taken up in 0·5N-sulphuric acid (80 ml.) and filtered from dibenzyl; it was then treated with mercuric sulphate (Hopkins's reagent as modified by Kendall, McKenzie & Mason, 1929) until precipitation was complete, 16·5 ml. being needed. Next day the precipitate was collected; it was washed five times by grinding with water and refiltration on a Buchner funnel and was then decomposed by suspension in water (80 ml.) and saturation with hydrogen sulphide; the mixture was filtered and the precipitate again suspended in water, saturated with hydrogen sulphide and filtered. The combined filtrates were concentrated to a low bulk under diminished pressure (bath temperature not >50°), transferred to a dish and adjusted to pH about 4·8 by addition of concentrated ammonia. Crystals were obtained by adding ethanol cautiously to a small portion of the solution and on inoculation of the main bulk crystallization rapidly set in. After keeping overnight the precipitate was collected and dried (1·6 g.). A further crop (0·41 g.) was obtained by concentrating the mother liquor and again adjusting the pH by addition of sodium acetate, the total yield thus being 70%.

The dipeptide crystallized from water, in which it is readily soluble at the boiling-point, in prisms having m.p. >300° (decomp.). It had $[\alpha]_D^{25°}+15·2°$ ($c=5$ in N-hydrochloric acid). (Found: C, 50·2; H, 5·7; N, 10·0; S, 11·3%. $C_{12}H_{16}O_4N_2S$ requires C, 50·7; H, 5·6; N, 9·9; S, 11·3%.)

In 50% ethanol containing N-hydrochloric acid 28·4 mg. required 9·95 ml. 0·01N-iodine (calc. 10·0 ml.) (cf. Lucas & King, 1932).

Cystyltyrosine. Cysteyltyrosine (2 g.) was suspended in water (50 ml.) and cold saturated baryta was added until all was in solution and the pH was about 8·5; a trace of ferrous sulphate was added and a current of air was drawn through the solution. When the nitroprusside reaction had become negative the solution was freed from barium with sulphuric acid and concentrated under diminished pressure, being finally evaporated to complete dryness in a desiccator over sulphuric acid. The residue was taken up in a little water and left in a partly evacuated desiccator over a dish of ethanol; after 2 days the peptide had separated in a hard crust which was slightly yellow in tint and was not crystalline; it was collected, washed with ethanol and dried; the yield was 1·65 g. (80%). The solution of the peptide in N-hydrochloric acid at about 5% concentration slowly deposited crystals; addition of an equal volume of concentrated hydrochloric acid and chilling gave a good yield of the *hydrochloride*; bunches of prismatic needles, m.p. 242–243° (decomp.). The hydrochloride was dissolved in water and treated with 1 equiv. of sodium hydroxide; the solution was evaporated to a low bulk in a vacuum desiccator and the partly crystalline residue collected. On solution in a small amount of warm water with the minimum hydrochloric acid and neutralization to about pH 4·0 with sodium acetate the peptide was obtained in the form of rosettes of spear-shaped needles, m.p. 294° (decomp.). It gave no nitroprusside reaction and reduced no iodine in acid solution. It had $[\alpha]_D^{25°}$ $-50·8°$ ($c=5$ in N-hydrochloric acid). (Found: N, 9·9; S, 10·9%. $C_{24}H_{30}O_8N_4S_2$ requires N, 9·9; S, 11·3%.)

N-Carbobenzyloxycystyltyrosine. Cystyltyrosine (0·564 g.) was dissolved in water (5 ml.) with sodium carbonate (0·52 ml. of 3·86N; 1 equiv.); the solution was chilled and shaken with benzyl chloroformate (0·35 ml.); a second equal quantity of sodium carbonate was added and shaking continued until the reaction was over. During the reaction the sparingly soluble sodium salt of the product separated. The mixture was acidified with hydrochloric acid (3 ml. of 3N) and extracted with ethyl acetate; the extract was washed with dilute hydrochloric acid and then three times with brine; it was dried over sodium sulphate and evaporated under diminished pressure to a low volume. The product was isolated by precipitation from the concentrated ethyl acetate solution with light petroleum, but could not be obtained crystalline. It had m.p. 158°. (Found: N, 6·8; S, 7·4%. $C_{40}H_{42}O_{12}N_4S_2$ requires N, 6·7; S, 7·7%.)

N-Carbobenzyloxycysteyltyrosine. N-Carbobenzyloxycystyltyrosine (0·4 g.) was dissolved in ethanol (20 ml.); 2·1N-sulphuric acid (10 ml.) was added to the warmed solution followed by zinc dust in excess; intermittent warming was continued until vigorous reaction ceased (about 35 min.); the solution was then filtered, diluted with water and freed from ethanol by distillation under diminished pressure. The residue was extracted with ethyl acetate and the latter was washed with dilute hydrochloric acid and water and dried; the product was isolated by evaporation of the ethyl acetate and precipitation with light petroleum; it was crystallized from aqueous ethanol, from which it separated in prisms having m.p. 160–162°. The yield was 0·27 g. (68%). (Found: N, 6·8; S, 7·4%. $C_{20}H_{22}O_6N_2S$ requires N, 6·7; S, 7·7%.) In 50% ethanol containing N-hydrochloric acid 41·8 mg. required 5·1 ml. 0·01N-iodine (calc. 5·0 ml.).

B. *Synthesis of tyrosylcysteine*

N-Carbobenzyloxytyrosyl hydrazide. N-Carbobenzyloxytyrosine ethyl ester (8 g.) was dissolved in ethanol (12 ml.) and treated with hydrazine hydrate (1·75 ml.); the mixture was boiled under reflux for 75 min., when the contents of the flask set to a semi-solid mass. After chilling, the solid was collected with the aid of more ethanol and dried. A further small crop was obtained by concentrating the mother liquor. The total yield was 5·73 g. (75%). For analysis the hydrazide was recrystallized from ethanol, in which it is sparingly soluble; it formed needles having m.p. 220–221°. (Found: N, 12·7%. $C_{17}H_{19}O_4N_3$ requires N, 12·8%.)

N-Carbobenzyloxytyrosyl-S-benzylcysteine ethyl ester. This compound was prepared as in the case of *S*-benzyl-*N*-carbobenzyloxycysteyl-*O*-benzoyltyrosine ester (see above); the azide was prepared by addition of sodium nitrite (1·85 g.) in water (18 ml.) to the above hydrazide (8·65 g.) dissolved in acetic acid (64 ml.), 3N-hydrochloric acid (45 ml.) and water (170 ml.), and the free ester was obtained from *S*-benzylcysteine ethyl ester hydrochloride (9·6 g.). Crystallization of the product started 10 min. after the ethereal solutions of azide and ester were mixed; after keeping for 40 hr. in the ice-chest the crystals were collected, washed with ether and dried. The yield was 10·82 g. (81% calculated on the hydrazide); two-thirds of the excess *S*-benzylcysteine ester employed could be recovered as hydrochloride by saturating the ethereal mother liquor with dry hydrogen chloride.

The product of the reaction crystallized from 75% ethanol in needles having m.p. 142–145°. (Found: N, 5·2; S, 6·2%. $C_{29}H_{32}O_6N_2S$ requires N, 5·2; S, 6·0%.)

N-Carbobenzyloxytyrosyl-S-benzylcysteine. The above ester (6·05 g.) was stirred with a mixture of N-sodium hydroxide (22·6 ml.) and dioxan (23 ml.) until dissolved. After 30 min. at room temperature the solution was diluted with water and made acid to Congo red with dilute hydrochloric acid; an oil separated which soon solidified. After keeping some time in the ice-chest the solid was collected and dried. The yield was quantitative. The acid crystallized from aqueous methanol in fine needles having m.p. 166°. (Found: N, 5·3%. $C_{27}H_{28}O_6N_2S$ requires N, 5·5%.)

Tyrosylcysteine. The reduction of *N*-carbobenzyloxytyrosyl-*S*-benzylcysteine and the isolation of the dipeptide were carried out exactly as described above for cysteyltyrosine. From 13·05 g. of starting material there were obtained 4·83 g. (66%) of tyrosylcysteine.

The dipeptide crystallized from water, in which its solubility is similar to that of cysteyltyrosine, in large prisms melting above 300° with decomposition. It had $[\alpha]_D^{23°} + 22·6°$ ($c = 5$ in N-hydrochloric acid). (Found: C, 50·9; H, 5·5; N, 10·1; S, 11·4%. $C_{12}H_{16}O_4N_2S$ requires C, 50·7; H, 5·6; N, 9·9; S, 11·3%.)

In 50% ethanol containing N-hydrochloric acid 28·4 mg. reduced 10·1 ml. 0·01N-iodine (calc. 10·0 ml.).

Tyrosylcystine. Tyrosylcysteine (2 g.) was suspended in water (50 ml.) and brought into solution by addition of baryta (27·5 ml. of 0·35N). The solution was aerated until the nitroprusside reaction was negative (several hours). Barium was removed exactly with sulphuric acid and the filtrate was concentrated under diminished pressure, the final stage being carried out in a vacuum desiccator over sulphuric acid. Crystalline material amounting to 1 g. separated, and a further crop of 0·48 g. of non-crystalline but almost pure product was obtained by concentrating the mother liquor and precipitating with ethanol.

The dipeptide crystallized from water in prisms, m.p. 292° (decomp.). It had $[\alpha]_D^{23°} - 70·8°$ ($c = 5$ in N-hydrochloric acid). (Found: N, 9·9; S, 11·0%. $C_{24}H_{30}O_8N_4S_2$ requires N, 9·9; S, 11·3%.)

N-Carbobenzyloxytyrosylcystine. Tyrosylcystine (0·42 g.) was dissolved in water (5 ml.) with sodium carbonate (0·75 ml. of 2N) and the solution was shaken at 0° with benzylchloroformate (0·3 ml.); a further equal quantity of sodium carbonate was added and shaking continued until the reaction was over. The sodium salt which had separated was collected, dissolved in water (40 ml.) and acidified with dilute hydrochloric acid. The precipitate was collected, washed and dried. The yield was 0·45 g. (72%).

For analysis the compound was purified by dissolving in ethyl acetate, washing the solution with dilute hydrochloric acid and water, drying and precipitating with light petroleum after concentration; it was crystallized from dilute ethanol from which it separated in prisms having m.p. 150° (decomp.). (Found: N, 6·7%. $C_{40}H_{42}O_{12}N_4S_2$ requires N, 6·7%.)

N-Carbobenzyloxytyrosylcysteine. N-Carbobenzyloxytyrosylcystine (0·4 g.) was dissolved in ethanol (20 ml.) and warmed to 50°; sulphuric acid (10 ml. of 2·1N) was added followed by excess of zinc dust; intermittent warming was maintained for about 35 min., by which time the vigorous reaction was over. The filtered solution was diluted with water and freed from ethanol by distillation under diminished pressure; it was then extracted with ethyl acetate. The extract was washed with dilute hydrochloric acid and water, dried and concentrated; addition of light petroleum precipitated the product, which could not however be obtained crystalline. The yield was 0·22 g. (54%) of a substance having m.p. 120° (decomp.). (Found: N, 6·8; S, 7·3%. $C_{20}H_{22}O_6N_2S$ requires N, 6·7; S, 7·7%.)

In 50% ethanol containing N-hydrochloric acid 41·8 mg. required 4·7 ml. of 0·01N-iodine (calc. 5·0 ml.); the compound was thus 94% reduced.

Enzymic experiments

The crystalline pepsin used was prepared from 1:10,000 pepsin (Parke Davis Ltd.) by the method of Northrop (1939) and was twice crystallized. Its activity was determined by the haemoglobin method described by Northrop.

The course of the enzymic splitting of the peptides was followed by amino-acid determinations by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941), this being the only method applicable to cysteine and cystine. The conditions for satisfactory quantitative determinations of both cysteine (cystine) and tyrosine were found to be 8 min. heating at pH 1·0 with 0·1 g. of ninhydrin for a 2 ml. sample and these conditions were adhered to throughout.

The actual experiments were carried out in the reaction vessels for the ninhydrin amino-acid determinations. In the various series of experiments the contents of the vessels were as follows.

Acylated peptides at pH 4·0. 0·04mM. of the peptide in 0·4ml. 0·1N-sodium hydroxide was mixed with 1·0 ml. 0·2M-acetate buffer of pH 4·0 followed by 0·1 ml. 0·4N-hydrochloric acid and 0·5 ml. 4% pepsin solution.

Non-acylated peptides at pH 4·0. As above except that 0·02mM. of peptide in 0·4 ml. 0·05N-sodium hydroxide followed by 0·1 ml. 0·2N-hydrochloric acid were used.

Acylated peptides at pH 1·8. 0·04mM. peptide in 0·4 ml. 0·1N-sodium hydroxide was mixed with 1·0ml. 0·08N-hydrochloric acid followed by 0·1 ml. 0·4N-hydrochloric acid and 0·5 ml. 4% pepsin solution.

Non-acylated peptides at pH 1·8. As above except that 0·02mM. peptide in 0·4 ml. 0·05N-sodium hydroxide, 1·0 ml. 0·08N-hydrochloric acid and 0·1 ml. 0·2N-hydrochloric acid were used.

Blank determinations. The control experiments carried out by Fruton & Bergmann (1939) consisted in incubation of the substrates with pepsin which had been inactivated by bringing to pH 8·0. We have ourselves done controls of this nature but since, particularly at pH 1·8, there is the possibility of partial autolysis of the pepsin with resultant increase in the blank, it has seemed better to us to use the figures obtained by incubating active pepsin under the conditions of the experiment but in the absence of substrate; in separate determinations it was found that the substrates themselves, in absence of pepsin, gave negligible blank figures both at pH 4·0 and 1·8. The pepsin blanks determined in this way are larger than those measured by the method of Fruton & Bergmann so that their use in the calculations gives a more conservative estimate of the true hydrolysis of the substrates.

The remote possibility that the apparently greater extent of hydrolysis of the —SH substrates might be due to activation of the autolysis of pepsin was excluded by an experiment in which pepsin was incubated at pH 4·0 in presence of (*a*) 0·01M-cysteine and (*b*) 0·005M-cystine; the pepsin blanks in this experiment were indistinguishable from one another and from the blank determined in the ordinary way without substrate.

Finally the use of acetate buffer in the concentrations employed was shown in separate experiments to be without effect on the ninhydrin amino-acid determination.

SUMMARY

1. The syntheses are described of cysteyl- and cystyltyrosine, of tyrosylcysteine and -cystine and of the *N*-carbobenzyloxy derivatives of these peptides.

2. It is shown that both the *N*-carbobenzyloxy derivatives and to a smaller but significant extent the free peptides are hydrolyzed by crystalline pepsin; the hydrolysis is more rapid at pH 4·0 than at pH 1·8. The bearing of these results on Bergmann's theory of the conditions determining peptic action is discussed.

3. The cysteine derivatives are more susceptible to peptic hydrolysis than the corresponding compounds containing cystine; this observation is considered in relation to the fact that pepsin attacks proteins more rapidly after denaturation which is itself accompanied by the appearance of —SH groups.

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Riboflavin Content of Canteen Meals

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At a time when diet is restricted and when the public is encouraged to supplement home supplies by eating at canteens, the dietary value of the meals served is important, especially in regard to the content of essential nutrients possibly in short supply. The estimations of riboflavin reported here formed part of a survey of meals served in canteens in the Leatherhead district.

METHODS

Riboflavin was estimated microbiologically on all meals and on three the values were checked by the rat growth method.

Preparation of samples. A complete meal was collected in the canteens as served, care being taken to see that the portions of food represented average servings. The whole meal was air-dried at 70–80°, then thoroughly mixed and finely ground. Samples of the dry powder were autoclaved with dilute HCl for 15 min. at a pressure of 15 lb.; the extracts adjusted to pH 6·8 with KOH, made to volume and filtered. Usually 2 g. of powder hydrolyzed with 25 ml. 0·01 N-HCl and diluted after neutralization to 100 ml. gave an extract of suitable strength. Extraction with 0·25 N-HCl (Barton-Wright & Booth, 1943) or water also gave satisfactory extraction, but the presence of starch in the water extracts complicated subsequent estimation of riboflavin (see below); the more dilute acid was preferred for extraction, as a larger volume, and better mixing with the sample, could be used without increase in the salt content of the extract. KOH was used to adjust the pH; it is recommended by Snell & Strong (1939) for neutralization of the growth medium. In several cases where fat was removed from the dry powder by extraction with petrol ether, riboflavin determinations on the defatted powder were not significantly different from those on the unextracted powder. For the rat tests the dry food powder was fed as a supplement to the basal ration. In these experiments nine meals were bulked before drying and grinding to obtain sufficient quantities of food powder.

Microbiological method. The organism used was a strain of *Lactobacillus helveticus*, kindly given to us by Dr C. H. Gray and obtained by him originally from the National Collection of Type Cultures. Fresh inocula were prepared from the stock agar culture for each series of estimations as recommended by Barton-Wright & Booth (1943). The medium used was that described by Snell & Strong (1939). Xylose, nicotinic and pantothenic acids (Barton-Wright & Booth, 1943) or the liver extract used in the rat-growth experiments, which contained all B vitamins except riboflavin (Chattaway, Happold & Sandford, 1943), added to the medium, caused no increase in acid production. Starch added to the medium (0·4–1·0%) caused additional acid production in control tubes and at all concentrations of riboflavin, but did not alter the relationship between acid production and riboflavin content, i.e. the slope of the curve obtained by plotting acid produced against riboflavin content was not altered by the presence of starch. Similarly, alteration of the pH of the medium (pH 6·4–7·0) affected the amount of acid produced, but not the response to added riboflavin. As a routine extracts and media were adjusted to pH 6·8.

Fermentations were carried out in McCartney culture bottles having tops with a central hole and rubber liner (A. Gallenkamp and Co. Ltd., London). Inoculations were made by injecting through the rubber liner one drop of the saline suspension of the organism from a sterile syringe. Bottles were incubated for 24 hr. only because this shorter time was found to give a more nearly linear relationship between acid production and riboflavin content. Acid production was estimated by titrating directly into the bottle