Substrates for Hyaluronidase

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The substrate used in most of the quantitative work on the enzyme complex known as 'hyaluronidase' has been the hyaluronic acid obtained from vitreous humour, synovial fluid or umbilical cord (for example see Meyer, Smyth & Dawson, 1939; Chain & Duthie, 1940; Madinaveitia & Quibell, 1940; McClean & Hale, 1941). Hyaluronic acid occurs in many animal tissues and in some bacteria (for literature see Duran-Reynals, 1942) as a mucopolysaccharide, the carbohydrate residue of which is built up of *N*-acetyl glucosamine units and glucuronic acid units (Meyer, 1938). The action of hyaluronidase preparations is not sharply specific and is not limited to the breakdown of hyaluronic acid only. Thus, Meyer and collaborators (Meyer & Chaffee, 1940*a*, *b*; 1941) found that some preparations could hydrolyze a sulphated mucopolysaccharide from the cornea and also a sulphated polysaccharide from skin. In the latter case a liberation of sulphate residues was detected, while with both substrates hydrolysis proceeded much more slowly than with hyaluronic acid.

RESULTS

In the present investigation a number of polysaccharides, containing no detectable bound protein material and containing known constituents and in some cases known linkages, have been used as possible substrates for a preparation of hyaluronidase obtained from bovine testicle. This preparation was essentially free from β -glucosaminidase (East, Madinaveitia & Todd, 1941). Since hyaluronidase appears to have the three effects mentioned later and since two of these may eventually be correlated with polysaccharide structure, it was our object to determine whether the enzyme had (*a*) a depolymerase action on the protein-free polysaccharides, and (*b*) whether there was any hydrolytic breakdown of known linkages. Only the results of the investigation (*a*) are reported in this paper, and they are given in Table 1.

It has been found that a sulphated polysaccharide, chondroitin sulphate, from the cartilage of bovine nasal septa, is also attacked by this hyaluronidase, albeit at a slower rate than is hyaluronic acid (Table 1). In addition to its possessing sulphate residues, the cartilage polysaccharide differs from

hyaluronic acid in respect of its amino-sugar constituent, which is *N*-acetyl chondrosamine. It has been suggested (Lythgoe & Madinaveitia, 1943) that hyaluronidase plays a role in making available for nutritional purposes the glucosamine present in hyaluronic acid. The above findings indicate that it can also make available the chondrosamine of some polysaccharides. In this connexion it is of interest to note that chondrosamine can be deaminated and oxidized by an enzyme system, discovered by Lutwak-Mann (1941), which attacks glucosamine.

Not every polysaccharide constituted of amino-sugars and uronic acid residues is attacked by hyaluronidase. This is exemplified in the cases of heparin and the Type I *Pneumococcus* specific polysaccharide, which are quite unaffected by the enzyme (Table 1). Of the other substrates examined, hyaluronidase attacked relatively rapidly a polysaccharide prepared from bovine placenta and more slowly an acid polysaccharide from ovarian tumour mucin, both substances being constituted of *N*-acetyl glucosamine and glucuronic acid residues and both having nitrogen content and specific rotation of the same order as those of hyaluronic acid.

A structural examination of carbohydrates of the hyaluronic acid type has not yet been carried out although some progress has been made in determining the configuration of a derivative of chondroitin sulphate (Bray, Gregory & Stacey, 1944). This material, termed 'degraded chondroitin', possesses a complicated branched chain structure having one-half of its glucuronic acid residues as 'end-groups' attached by glucosidic linkages to the rest of the molecule. These high polymers appear to possess complex structures in which several types of linkage exist and this fact is in accordance with the mode of action of hyaluronidase. We and others (Duran-Reynals, 1942) have recognized that *in vitro* three distinct phases in the breakdown of hyaluronic acid can be demonstrated. Initially there is a destruction of the power of the viscous mucopolysaccharide to form a mucin clot and we regard this as indicating that the carbohydrate has become dissociated from its combination with protein, a combination which may be to some extent ionic. Secondly, there is a dramatic decrease in viscosity of aqueous solutions of the residual complex without

Table 1. Properties of substrates for hyaluronidase

Source of polysaccharide substrate

No. of specimen	Umbilical cord (hyaluronic acid)	Umbilical cord (hyaluronic acid)	Umbilical cord (degraded polysaccharide)	Cartilage (bovine trachea)	Cartilage (bovine nasal septa)	Bovine placenta	Ovarian tumour	Pancreas (galactan)	Gastric mucin	Commercial peptone	Ovomucoid	Dextran from <i>Leuconostoc dextranum</i>	<i>Rhizobium radicicolum</i>	Luteose from <i>Penicillium luteum</i> Zukal	<i>Azobacter chroococcum</i>	Levan from <i>Bacillus megatherrum</i>	Type I <i>Pneumococcus</i>	Heparin	1	2	3	4	5	6	7	8	9
Amino-sugar content (%)	35	38	35	*30	*30	34	35	15	35	35	70	—	—	—	—	—	—	—	16	17	18	25-35	—	—	—	—	—
Uronic acid content (%)	45	46	45	55	55	40	37	—	—	—	—	—	20	—	5	—	52.5	42.5	42.5	—	—	—	—	—	—	—	—
Other sugars	—	—	—	*Chon-dros-amine	*Chon-dros-amine	—	—	Galac-tose	Man-nose Galac-tose	Man-nose Galac-tose	Man-nose Galac-tose	Glucose	Glucose	Glucose	Glucose	Fructose	—	—	—	—	—	—	—	—	—	—	—
Concentration (mg./ml.)	29.7	23.1	32.7	26.6	24.9	22.5	46.9	21.3	61.0	34.7	46.7	9.0	5.6	14.8	8.7	20.1	14.1	20.5	—	—	—	—	—	—	—	—	—
Specific viscosity	1.59	1.60	0.29	0.16	0.67	1.00	0.82	0.33	0.33	0.12	0.20	0.87	1.63	0.13	1.87	0.10	5.4	1.10	—	—	—	—	—	—	—	—	—
Time of incubation (min.)	—	—	0.16	0.19	—	—	0.85	0.44	0.30	0.14	0.19	0.95	—	0.11	—	0.11	—	—	—	—	—	—	—	—	—	—	—
2	0.77	1.32	—	—	0.66	0.98	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	0.12	0.18	—	—	—	—	0.28	0.13	0.20	—	1.73	0.08	1.73	0.11	—	—	—	—	—	—	—	—	—	—	—
5	0.52	—	—	—	0.62	—	—	0.42	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	0.11	0.18	—	0.89	0.80	—	0.28	0.13	0.20	0.93	—	0.08	1.66	0.11	—	—	—	—	—	—	—	—	—	—	—
8	0.41	1.00	—	—	0.60	—	—	0.36	—	—	—	—	1.63	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	—	—	0.94	—	0.08	—	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—	0.36	0.27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	—	0.92	—	—	—	0.83	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	0.57	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	—	0.82	—	—	0.55	—	—	—	0.27	—	—	0.95	1.61	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	0.76	—	—	—	—	—	—	1.62	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	—	0.74	—	—	—	—	—	—	0.27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
23	—	0.67	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
28	—	—	—	—	0.52	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31	—	—	—	—	—	—	0.70	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
32	+	+	+	—	+	+	+	—	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Action of enzyme	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Without diffusing factor	1.37	1.51	0.25	0.16	0.63	0.87	0.79	0.31	0.31	0.11	0.16	0.80	1.61	0.05	1.50	0.17	5.4	1.11	—	—	—	—	—	—	—	—	—
With diffusing factor	0.08	0.09	0.06	0.15	0.07	0.42	0.16	0.32	0.22	0.11	0.16	0.85	1.56	0.08	1.56	0.11	5.25	1.08	—	—	—	—	—	—	—	—	—

liberation of reducing sugars and this means that the enzyme is exerting a powerful 'polysaccharide depolymerase' action. Finally, there is a slower progressive liberation of reducing sugars, namely glucuronic acid and *N*-acetyl glucosamine, which indicates a hydrolytic breakdown of the polysaccharide chain.

Polysaccharides which were not attacked by the preparation were the following: (a) the blood group A polysaccharides from both gastric mucin and from pepsin, the latter containing units of *l*-fucose, mannose, galactose and *N*-acetyl glucosamine (Stacey, 1943); (b) a galactan from the pancreas (Stacey, 1944); (c) ovomucoid, containing units of *N*-acetyl glucosamine, mannose and galactose united in a complicated branched chain structure (Stacey & Woolley, 1940); (d) dextran from *L. dextranicum* constituted of long chains of glucose units (Peat, Schlüchterer & Stacey, 1939) linked through the 1- and 6-positions; (e) the *Rhizobium radicicolum* polysaccharide (Cooper, Daker & Stacey, 1938) containing units of glucuronic acid and glucose linked in a manner generally similar to the Type III *Pneumococcus* polysaccharide; (f) luteose from *P. luteum* Zukal (Anderson, Haworth, Raistrick & Stacey, 1939), a ' β -dextran' constituted solely of units of glucose linked through the 1- and 6-positions; (g) an *Azotobacter* polysaccharide containing units of glucuronic acid and glucose (Cooper *et al.* 1938); (h) the *B. megatherium* levan which is built up from units of fructose linked through the 2- and 6-positions (Cooper & Preston, 1935; Lyne, Peat & Stacey, 1940).

A method is described in the experimental section for preparing hyaluronic acid free from detectable protein.

EXPERIMENTAL

Determination of hyaluronidase activity

Aqueous solutions of the polysaccharides having the concentration indicated in § 6 of the table were mixed with 0.2 vol. of a mixture of equal amounts of *m*-NaCl and *m*-citrate buffer pH 4.5 and were filtered through glass-wool. In Table 1 (§ 7) the specific viscosity of each of these solutions (at 25°) diluted with 0.2 vol. of water is recorded.

The enzyme used was a solution of a preparation of hyaluronidase from bovine testicle purified by lead acetate and ammonium sulphate precipitation (Madinaveitia & Quibell, 1940; East *et al.* 1941). Buffered polysaccharide solution (1 vol.) and enzyme (0.2 vol.) were mixed at 25° and variation of the viscosity with time at this temperature followed, as described by Madinaveitia & Quibell (1940). Control solutions without enzyme and the solutions with enzyme were placed for 18 hr. in a thermostat at 37°. Toluene was added as a preservative. In some solutions a slight precipitate was formed; any precipitate was removed in the centrifuge and the specific viscosity determined at 25°. The prolonged incubation at 37° permits the establishment of the significance of small variations of viscosity observed during the shorter incubation at 25°.

Preparation of substrates

In the preparation of the polysaccharides, emphasis has been laid on the removal from them (as far as present methods permit) of detectable 'bound' protein constituents and on subsequent isolation of an essentially homogeneous polysaccharide fraction as far as can be shown by fractionation methods. It is realized that some degradation of carbohydrate structure may have occurred and that the macromolecular state may not be the optimum obtainable for each polysaccharide, for it has been emphasized by one of us (Stacey, 1943) that the nature of a carbohydrate prepared from a polysaccharide-protein complex varies sharply with the mode of isolation. Where an effect of the enzyme has been observed we consider that it is an effect on the polysaccharide structure. Tests for proteins were carried out on 2% solutions of the polysaccharides, using, for example, the following reagents at various concentrations: lead acetate, sulphosalicylic acid, trichloroacetic acid, Esbach's picric acid reagent, Millon's solution, glycoylic acid, and phosphotungstic acid. The following tests were also negative: biuret, Sakaguchi.

The amino sugars were estimated by the method of Elson & Morgan (1933) and the uronic acid content by gravimetric estimation of CO₂ evolved on heating the polysaccharides with 12% HCl saturated with NaCl.

(1) *Hyaluronic acid* (cf. McClean & Hale, 1941). Umbilical cords were collected and stored under 1% aqueous phenol, in which they could be kept for several months. They were freed from blood-clots by dissection, washed with a rapid stream of water, and minced coarsely. This material (500 g.) was stirred briskly into boiling water (1 l.) and immediately filtered through a fine-meshed sieve. Four or five extractions were made in this way, the last extract being relatively non-viscous. The combined extracts were cooled and acidified with 5*N*-acetic acid, when a mucin clot separated. The mucin clot was collected on a cloth and was dissolved in a small amount of *N*-NaOH. The solution was diluted to 300 ml. and the pH adjusted to 8.5 by addition of acetic acid. BaCO₃ (20 g.) was added and the liquid boiled briskly for a few minutes until a little of the cleared supernatant failed to give a precipitate with acetic acid. BaCO₃, with coagulated protein material, was removed and washed on the centrifuge, and hyaluronic acid (Ba salt) obtained from the clear supernatant by addition of 2 vol. of ethanol. The product, a fibrous precipitate, was washed with ethanol and ether and dried (5 g.). It was purified by dissolving it in water, centrifuging and precipitating with ethanol and by dialysis followed by ethanolic precipitation, until all the fractions showed $[\alpha]_D^{20} - 60^\circ$ in water. Yield, 3-4 g. (found: N, 5.0; amino sugar, 35.4; uronic acid, 45%). No tests for proteins were given by, for example, 2% solutions of this and other polysaccharides described below or by the products obtained from them on acid hydrolysis. The product gives highly viscous solutions in water and does not give a precipitate on addition of acetic acid. We regard this fact as a further indication of the absence of protein since the power to give a precipitate can be restored by addition of a serum (0.1 ml.) to a 1% aqueous solution (10 ml.) of hyaluronic acid or its salts. The ability to give a precipitate with acetic acid appears to be retained even when the hyaluronic acid has been degraded by alkali treatment, but with degraded hyaluronic acid the precipitate obtained on acidification is not a true mucin clot. When hyaluronic acid is combined with protein it is found that

the protein-hyaluronic acid complex becomes completely insoluble in water on intensive drying at 100° or on being kept over P_2O_5 for some weeks. In this respect the complex differs obviously from our preparation of hyaluronic acid.

(2) Prepared from sample (1) by shaking a dilute aqueous solution of it with carbon tetrachloride to remove possible traces of extraneous protein, followed by centrifuging and ethanolic precipitation. It showed $[\alpha]_D^{20} - 58^\circ$ in water (found: N, 5.0; amino sugar, 38.0; uronic acid, 46%).

(3) Prepared from sample (2) by heating its aqueous solution at pH 8.5 for 2 hr. and then isolating the product by ethanolic precipitation. It showed $[\alpha]_D^{20} - 40^\circ$ in water (found: N, 4.8; amino sugar, 35; uronic acid, 45%).

(4) This specimen was prepared from bovine tracheal cartilage by essentially the method described by Bray *et al.* (1944) and was carefully fractionated by precipitation from aqueous solution by ethanol. The main fraction showed $[\alpha]_D^{17} - 26^\circ$ in water (found: N, 3.0; S, 2.3; amino sugar, 30.0; uronic acid, 55.2; K (K-salt), 18.5; acetyl, 8.4%) and could not be further separated into fractions having different properties. Neither the polysaccharide nor its acid hydrolysis products gave any of the usual tests for amino acids or proteins.

(5) This sample was prepared from bovine nasal septa by the same method as specimen (4). The main product free from detectable protein had $[\alpha]_D^{17} - 25^\circ$ in water (found: N, 4.3; S, 2.5; amino sugar, 30.0; uronic acid, 55.0; K (K salt), 18.4; acetyl, 8.3%). This chondroitin sulphate gave aqueous solutions which were more viscous than those obtained from the tracheal preparations, but evidence has not yet been provided to show that chondroitin sulphates from all types of cartilage are identical.

(6) Bovine placental tissue was separated by dissection, and dissolved rapidly in warm dilute NaOH. The solution was immediately neutralized with acetic acid and stirred at 70° with $BaCO_3$ for 10 min. The filtered solution was poured into ethanol and the precipitate carefully purified by fractional precipitation from aqueous solution by ethanol. A product (50% of the crude product) was obtained; it was free from protein and could not be separated into fractions showing different properties. After dialysis through cellophan the polysaccharide had $[\alpha]_D^{20} - 45^\circ$ in water (found: N, 4.5; amino sugar, 34.2; uronic acid, 40.0%).

(7) Part of a large non-malignant ovarian tumour was minced coarsely and the cystic fluid obtained by passing the mince through cloth. The fluid was diluted with an equal volume of 2% sodium acetate and the solution rapidly raised to the boiling-point in the presence of $BaCO_3$. The solution, filtered whilst hot, was cooled and poured into excess ethanol and the copious precipitate of polysaccharide material collected (centrifuge). It was separated by ethanol precipitation into four main fractions:

F₁: yield, 10%; $[\alpha]_D^{20} - 50^\circ$; ash, 25%.

F₂: yield, 50%; $[\alpha]_D^{20} - 40^\circ$; ash, 20%.

F₃: yield, 25%; $[\alpha]_D^{20} - 11^\circ$; ash, 40%.

F₄: yield, 10%; $[\alpha]_D^{20} - 12^\circ$; ash, 35%.

Fraction F₂ was the only one further examined. It was dialyzed for 12 hr. in parchment and then isolated by concentration of the solution followed by precipitation with acetone. Extraneous protein material was removed by shaking a solution of the polysaccharide with small quantities of carbon tetrachloride until no more insoluble material was thrown out of solution.

The polysaccharide was now precipitated by fractional addition of ethanol and the fractionation continued until an essentially homogeneous fraction (7% yield of crude product) was obtained. This had the following properties (Ba salt): $[\alpha]_D^{17} - 40^\circ$ in water (found: N, 4.5; amino sugar, 34.5; uronic acid, 37.4%).

(8) Commercial trypsin was dissolved in water (5% solution); 10% trichloroacetic acid was added until no further precipitate was formed. This precipitate was removed (centrifuge) and discarded. The supernatant liquid was dialyzed through cellophan for 12 hr., concentrated *in vacuo* and a precipitate thrown out in three fractions by adding ethanol. The middle fraction (yield, 40%; $[\alpha]_D^{17} - 30^\circ$) was dialyzed in cellophan for 24 hr., the solution concentrated *in vacuo* and a polysaccharide precipitated by adding acetone (3 vol.). The polysaccharide was further fractioned until an essentially homogeneous product (15% yield) was obtained. This showed $[\alpha]_D^{17} - 35^\circ$ in water (found: N, 2.8; amino sugar, 15.5; galactose (estimated by isolation of mucic acid), c. 50%).

(9) Method of Landsteiner & Harte (1940). It showed $[\alpha]_D^{20} - 10^\circ$ in water (found: N, 4.8; amino sugar, 35.0%). This product still contained amino-acid residues in agreement with Landsteiner & Harte (1940).

(10) Method of Landsteiner & Harte (1940). It showed $[\alpha]_D^{21} + 8^\circ$ in water (found: N, 5.0; amino sugar, 35.0%) and still contained amino-acid residues.

Samples (11)–(16) inclusive are adequately described in the literature.

(11) Method of Stacey & Woolley (1940). It showed $[\alpha]_D^{21} - 57^\circ$ in water.

(12) Method described by Stacey & Youd (1938). It showed $[\alpha]_D^{20} + 180^\circ$ in water.

(13) Method of Cooper *et al.* (1938). It showed $[\alpha]_D^{21} - 20^\circ$ in water.

(14) Method of Anderson *et al.* (1939). It showed $[\alpha]_D^{20} - 38^\circ$ in water.

(15) Method of Cooper *et al.* (1938). It showed $[\alpha]_D^{20} - 10^\circ$ in water.

(16) Method of Cooper & Preston (1935). It showed $[\alpha]_D^{20} - 43^\circ$ in water.

(17) *Type I Pneumococcus polysaccharide*. This product was prepared under the personal direction of Dr M. Heidelberger, and was freed from protein by Sevag's method (1934). It had $[\alpha]_D^{20} + 235^\circ$, uronic acid 52.5, N 5.2, and acetyl 4.5%. Amino-sugar determinations were unsatisfactory (25–35%).

(18) *Heparin*. A sample of heparin (British Drug Houses Ltd., 500 units/mg.) (0.5 g.) was purified by Sevag's method (1934) followed by dialysis and ethanolic fractionation methods. The product used (0.35 g.) had $[\alpha]_D^{20} - 42^\circ$ in water (found: S, 9.5; N, 2.5; uronic acid, 42.5; amino sugar, 16%) and contained no detectable protein constituents.

SUMMARY

1. The action of hyaluronidase in destroying the viscosity of aqueous solutions of various polysaccharides has been investigated.

2. In addition to being active against hyaluronic acid and closely related polysaccharides from placenta and tumour mucin, it lowers the viscosity of chondroitin sulphate from nasal septa.

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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*-carbobenzyloxy-cysteyl-*O*-benzoyltyrosine on treatment with alkali in the cold gave *S*-benzyl-*N*-carbobenzyloxy-cysteyltyro-

sine 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 tyrosylcysteine; 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 tyrosylcysteine 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