Isolation and Hydroxylysine Glycoside Content of some Cyanogen Bromide-Cleaved Fragments of Collagen from Bovine Corneal Stroma

By NOORJAHAN A. PANJWANI* and JOHN J. HARDING†

Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, U.K.

(Received 13 October 1977)

Six CNBr-cleaved fragments of insoluble collagen from bovine cornea were isolated, characterized and examined for hydroxylysine glycosides. Thus the general distribution of most of the glycoside along the collagen molecule was determined. Collagen from bovine stroma is almost entirely type I. This work forms a basis for the pinpointing of glycoside-attachment sites along the collagen molecules of bovine cornea.

There are at least four different types of collagen in which are found five genetically distinct α -chains (Miller, 1976). Of these, type IV, found in basement membranes, appears to be highly glycosylated; type II, from cartilage, is less extensively glycosylated, and type I and type III are generally poorly glycosylated.

Lathyrin chick corneal collagen and rabbit corneal collagen contain $\alpha 2$ -chains and are largely type I (Trelstad & Kang, 1974; Cannon & Cintron, 1975). Collagen from corneal stroma is of particular interest, because it is more heavily glycosylated than type-I collagen from other tissues (Spiro, 1969; Grant *et al.*, 1969) and because it is found as thin uniform fibres in a regular array essential for transparency.

There appears to be an inverse relationship between the diameter of collagen fibres and their glycoside content (Spiro, 1969; Grant *et al.*, 1969). Morgan *et al.* (1970) have suggested that the glycoside moiety of some relatively highly glycosylated collagens may be in the overlap region of the fibre unable to fit into the axial holes and may therefore interfere with fibre formation beyond a certain limiting diameter. The present paper reports the isolation and glycoside analysis of CNBr-cleaved fragments of collagen from the bovine corneal stroma as the first stage in pin-pointing the glycoside sites. The following paper (Panjwani & Harding, 1978) describes the final pin-pointing of two glycoside sites in fragment $\alpha 1$ -CB3.

Materials and Methods

Preparation of insoluble collagen from bovine corneal stroma

Bovine eyes were obtained as soon as possible after death from A. G. Hedges (Abingdon, Berks., U.K.).

* Present address: Harvard Medical School, Elliot P. Joslin Research Laboratory, Boston, MA, U.S.A.

† To whom reprint requests should be addressed.

The corneas were removed and stored at -20°C until use. The corneas were thawed and epithelium and endothelium removed by gentle scraping. Each cornea was then pinned on a cork, with Descemet's membrane facing upward. Descemet's membrane was then peeled off with the help of curved forceps and a Beaver mini-blade no. 64 (Macarthy's Ltd., Dagenham RM8 1QD, Essex, U.K.). The stroma was diced with a scalpel blade into 1–2 mm squares. Proteoglycans and other non-collagenous components of the cornea were extracted from the cubes in 15 vol. of 4.0 M-guanidinium chloride/50 mM-Tris, adjusted to pH 7.5 with 3 M-HCl, with continuous shaking at 20°C for 18 h (Miller & Lunde, 1973). The solution was changed five or six times during the extraction. After extraction, the insoluble material was washed with distilled water at 4°C until free of guanidinium chloride and then freeze-dried. The amino acid composition of this stromal collagen (Table 1) was similar to published compositions of bovine corneal collagen (Freeman *et al.*, 1968; Lian *et al.*, 1973) and indicated that it was reasonably pure. It contained no cystine.

Cleavage with CNBr

The CNBr-cleavage method of Epstein *et al.* (1971) was used, but at 40°C instead of 30°C because at the lower temperature some methionine remained in the digest; at 40°C methionine was not detectable even when the amino acid analyser was grossly overloaded.

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the CNBr-cleaved peptides of corneal collagen was carried out on 7.5% (w/v) polyacrylamide gels by the method of Furthmayr & Timpl (1971). Solutions of peptides (0.25%) in sample buffer, 10 mM-sodium phosphate buffer, pH 7.2, containing 0.2% sodium dodecyl sulphate and 2 M-

urea, were heated at 50°C for 30 min. For each gel 5–10 μ l (12–25 μ g) of sample was mixed with 1 drop of glycerol, 10 μ l of 0.05% Bromophenol Blue (BDH Chemicals, Poole, Dorset, U.K.) in sample buffer and 50 μ l of sample buffer (Weber *et al.*, 1972). After electrophoresis (Furthmayr & Timpl, 1971) the centre of the dye band was marked with a hypodermic needle dipped in waterproof drawing ink before staining with Coomassie Brilliant Blue [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] and destaining as described by Weber *et al.* (1972).

Chromatography on CM-cellulose

Samples of CNBr digests (400 mg) were suspended in 6 ml of starting buffer (20 mM-sodium citrate, pH 3.6), centrifuged (20000g-min) and the supernatant was applied to a column (12.5 cm $\times$ 2.3 cm) of CM-cellulose (CM-52 microgranular form, pre-swollen; from Whatman Biochemicals, Maidstone, Kent, U.K.). The column was eluted at 40°C with a linear gradient (from 0 to 140 mM over a volume of 2 litres) of NaCl in 20 mM-sodium citrate, pH 3.6 (Volpin & Veis, 1973).

Final purification of fragment α 1-CB7 was achieved by chromatography on the same column at pH 4.8 with a concave gradient (Butler *et al.*, 1967) or on a smaller column (6 cm $\times$ 1.9 cm) of CM-cellulose with a linear gradient (Volpin & Veis, 1973).

Amino acid analysis

To determine amino acid compositions, collagen (1 mg) and its peptides (40–400 nmol) were hydrolysed in 500 μ l of 6 M-HCl (Aristar), in evacuated sealed tubes, at 108°C for 18 h. After hydrolysis the contents of the tube were dried by rotary evaporation and analysed on a Locarte amino acid analyser by a single-column method.

Hydroxylysine glycoside analysis

To determine glucosylgalactosylhydroxylysine and galactosylhydroxylysine content, the corneal collagen (5–20 mg/ml), its peptides (0.25–1.0 mg/200 μ l) and lens capsules (5–20 mg/ml) were hydrolysed in 2 M-NaOH, at 108°C for 24 h, in tightly capped polypropylene tubes essentially as described by Spiro (1967). The hydrolysate was acidified to below pH 2.5 by 2 M-HCl and analysed by using a Locarte amino acid analyser and/or by high-voltage electrophoresis.

A method was developed for the quantitative determination of glucosylgalactosylhydroxylysine and galactosylhydroxylysine, without prior desalting, with stepwise elution with the following three buffers, which are similar to those described by Spiro (1972): 1, pH 5.2, sodium citrate buffer (0.1 M- Na^+); 2, pH 5.0, sodium citrate buffer (0.18 M- Na^+); 3, pH 6.0, sodium citrate buffer (0.35 M- Na^+). All three buffers contained 1 g of phenol/litre. The column

was re-equilibrated with the pH 3.25 buffer used for amino acid analysis. The pH 5.2, 5.0 and 6.0 buffers were pumped for 75, 35 and 150 min respectively at 30 ml/h.

Authentic galactosylhydroxylysine and glucosylgalactosylhydroxylysine were isolated from alkaline hydrolysates of bovine corneal collagen by gel chromatography (Spiro, 1967) and glucosylgalactosylhydroxylysine from alkaline hydrolysates of bovine lens capsule by high-voltage electrophoresis at pH 3.8 (Moczar & Moczar, 1970); these preparations were used to identify the glycoside peaks on the amino acid-analysis charts. The times of elution of phenylalanine, glucosylgalactosylhydroxylysine and galactosylhydroxylysine were 112, 124 and 210 min respectively. The positions were confirmed by showing that the glucosylgalactosylhydroxylysine peak was smaller and the galactosylhydroxylysine peak was larger after weak acid hydrolysis as described by Spiro (1967).

Determination of N-terminal amino acids

The N-terminal amino acid of fragment α 1-CB7 was identified by the method of Gray (1972), the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) amino acids being separated on polyamide layers by the system of Woods & Wang (1967) with the modification described by Croft (1972). A short (6 h) acid hydrolysis was used because dansylhydroxyproline is presumably unstable like dansylproline (Gray, 1972). Hydroxyproline was dansylated by the method of Gray (1972).

Results and Discussion

Collagen type in corneal stroma

Collagens from rabbit cornea and from lathyrus chick contain α 1 (I) and α 2 chains and therefore these collagens are largely of type I (Cannon & Cintron, 1975; Trelstad & Kang, 1974). However, these authors did not remove corneal basement membranes, so their collagen preparations presumably contained a mixture of types with type I predominating (Trelstad & Kang, 1974). As sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of CNBr digests of the different types of collagen give characteristic patterns (Eyre & Muir, 1975; Scott & Veis, 1976a,b; Weber *et al.*, 1977), it was used in the present work for the first time to find out if corneal collagen is entirely type I and also to confirm that the CNBr reaction had proceeded successfully. The gel scan pattern obtained for the CNBr digest of collagen from bovine corneal stroma (Fig. 1) is similar to that obtained for insoluble type-I collagen from bovine skin (Scott & Veis, 1976b). The absence of type-II and -III collagen is indicated by the absence of peaks in the positions marked by arrows Y and Z in Fig. 1,

Fig. 1. Staining profile of gel obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of CNBr-cleaved peptides of bovine corneal collagen

Arrow Y indicates the position where the marker peptide $\alpha 1(\text{II})$ -CB10 of type-II collagen appears (Eyre & Muir, 1975) and arrow Z indicates the position where the marker peptide $\alpha 1(\text{III})$ -CB8 of type-III collagen appears (Scott & Veis, 1976b).

where marker peptides for these collagens would appear. Absence of type IV was demonstrated by the absence of cystine in the acid hydrolysate of the stromal collagen.

The individual peptide bands (Fig. 1) were identified by comparison with gel scans for digests of bovine skin collagen. A linear relationship between their relative mobilities and the logarithm of their molecular weights was obtained. The presence of peptide $\alpha 2$ -CB(3-5) (relative mobility 0.31) after our relatively harsh CNBr treatment supports the view that this region of the bovine $\alpha 2$ -chain lacks the methionine found in other species (Fietzek *et al.*, 1970) rather than the contrary view that the methionine is there but resists CNBr (Volpin & Veis, 1973).

It appears that the CNBr digestion has proceeded successfully and that the collagen of corneal stroma is essentially all type I.

Glycoside analysis of the bovine corneal collagen (four determinations on two preparations) showed the presence of 3.1 ± 0.05 residues of glucosylgalactosylhydroxylysine and 1.9 ± 0.10 residues of galactosylhydroxylysine per thousand amino acid residues, results close to published values (Spiro, 1969).

CNBr-cleaved peptides of bovine corneal collagen

Initial fractionation of the CNBr digest was on CM-cellulose (CM-52 microgranular, preswollen; from Whatman Biochemicals) as shown in Fig. 2. The chromatogram is similar to that obtained by

Volpin & Veis (1973) for insoluble collagen from bovine skin and supports the view that collagen of corneal stroma is type I. This chromatographic step was performed successfully ten times during this work; corresponding components from the last seven separations were pooled to give larger amounts of CNBr-cleaved fragments for detailed study.

The known CNBr-cleaved peptides $\alpha 1$ -CB2, $\alpha 1$ -CB3 and $\alpha 1$ -CB7 were isolated from fractions 2, 4 and 7 respectively. Gel chromatography of fraction 5 yielded peptides $\alpha 1$ -CB4 and $\alpha 1$ -CB6, and a fraction that was largely peptide $\alpha 2$ -CB(3-5) was isolated from fraction 10. Identifications by amino acid composition were made by comparison with CNBr-cleaved fragments of insoluble bovine skin collagen (Volpin & Veis, 1973).

Peptide $\alpha 1$ -CB2. Component 2 (Fig. 2) fractionated on a column (67cm $\times$ 1.9cm) of Sephadex G-25 eluted with 100mM-acetic acid, pH3.0, yielded one fraction 2A, which was identified as peptide $\alpha 1$ -CB2 by comparison with the amino acid composition (Table 1) of peptide $\alpha 1$ -CB2 from bovine skin collagen (Volpin & Veis, 1973). This peptide lacks lysine and hydroxylysine and therefore lacks hydroxylysine-bound glycoside.

Peptide $\alpha 1$ -CB3. Component 4 (Fig. 2) was desalted on a column (70cm $\times$ 1.9cm) of Sephadex G-10 by elution with 100mM-acetic acid, pH3.0, and then chromatographed on a column of Sephadex G-50 (superfine grade) giving two components 4A and 4B (Fig. 3), which were desalted on Sephadex G-10 and freeze-dried. Component 4A was identified as peptide $\alpha 1$ -CB3 by its amino acid composition (Table 1), by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and from its elution position on CM-cellulose.

The peptide $\alpha 1$ -CB3 from bovine corneal collagen has 0.9 out of 5 residues of lysine hydroxylated compared with only 0.3 residue in peptide $\alpha 1$ -CB3 from bovine skin (Table 1). Glycoside analysis showed the presence of 0.54 residue of glucosylgalactosylhydroxylysine and 0.36 residue of galactosylhydroxylysine per peptide. The total value of glucosylgalactosylhydroxylysine plus galactosylhydroxylysine plus hydroxylysine of 1.2 residues per peptide in the alkaline hydrolysates was close to 0.9 residue of hydroxylysine per chain found in the acid hydrolysate.

Chymotryptic and tryptic peptides of corneal peptide $\alpha 1$ -CB3 were prepared to compare its covalent structure with that of peptide $\alpha 1$ -CB3 from skin and to pinpoint the sites of glycoside attachment (Panjwani & Harding, 1978).

Peptide $\alpha 1$ -CB4. Component 5 (Fig. 2) was fractionated on a column (67cm $\times$ 1.9cm) of Sephadex G-25 in 100mM-acetic acid, pH3.0, giving two peptide fractions 5A and 5B followed by the citrate fraction (Fig. 4). Component 5B was identified as peptide

Fig. 2. CM-cellulose chromatogram of CNBr-cleaved peptides of bovine corneal collagen (410 mg). Elution was at 40°C with a linear gradient (0 to 140 mM in a total volume of 2 litre) of NaCl in 20 mM-sodium citrate, pH 3.6. The flow rate was 120 ml/h; 8 ml fractions were collected; column size 12.5 cm × 2.3 cm. The bars indicate the fractions pooled.

α 1-CB4 by amino acid analysis (Table 1) and from its elution position on CM-cellulose and Sephadex G-25. Its composition is similar to peptide α 1-CB4 from bovine skin (Volpin & Veis, 1973) and to that of peptide α 1-CB4 from calf skin collagen, which has been sequenced (Fietzek & Kühn, 1975). As corneal peptide α 1-CB4 lacks hydroxylysine, it was not analysed for glycosides. Presumably it lacks hydroxylysine because its two lysine residues are in the X position in the triplet Gly-X-Y as are those of calf skin collagen (Fietzek & Kühn, 1975).

Peptide α 1-CB6. Component 5A (Fig. 4) was shown to be homogeneous by chromatography on agarose (Sephacrose 6B; Pharmacia, London W5 5SS, U.K.),

Fig. 3. Gel chromatography of fraction 4 (Fig. 2) pooled from seven separations on a column (137 cm × 2 cm) of Sephadex-G50 (superfine grade).

Elution was achieved at 35°C with 30 mM-sodium acetate, pH 4.8. The flow rate was 18 ml/h and 3 ml fractions were collected. The bars indicate the fractions pooled.

Table 1. *Amino acid compositions of bovine corneal collagen and its CNBr-cleaved fragments*
Values are expressed as residues per peptide, except those for collagen which are expressed as residues per 1000 residues. Homoserine values for corneal CNBr-cleaved fragments are not corrected for incomplete conversion of the lactone into homoserine. Compositions of bovine skin CNBr-cleaved fragments are from Volpin & Veis (1973).

Amino acid	Corneal collagen	$\alpha 1$ -CB2		$\alpha 1$ -CB3		$\alpha 1$ -CB4		$\alpha 1$ -CB6		$\alpha 1$ -CB7		$\alpha 2$ -CB(3-5)	
		Cornea*	Skin	Cornea*	Skin	Cornea†	Skin	Cornea	Skin	Cornea†	Skin	Cornea‡§	Skin
Hydroxyproline	82	6.0	5.6	13	14	5.3	5.6	20.5	20.7	24.9	23	60	49
Aspartic acid	48	0	0	6.3	6.5	2.8	2.7	8.8	8.7	12.1	12	29	33
Threonine	18	0	0	0.3	0	1.0	0.9	3.6	3.8	4.8	5.0	11	11
Serine	33	1.8	1.8	3.0	2.8	0.36	0	6.3	7.4	7.4	7.4	20	22
Glutamic acid	77	4.0	3.8	16.1	16	3.1	3.0	13.5	13	16.8	17	41	44
Proline	112	6.0	6.0	15.8	16	5.8	5.8	26.7	26	36.6	37	72	71
Glycine	334	11.1	12	51.7	51	15.3	16	65	64	86.7	90	215	210
Alanine	115	2.6	2.2	22	22	3.2	3.0	20.1	22	35.5	34	60	64
Valine	22	0	0	3.6	3.8	0	0	3.5	3.2	5.0	4.7	18	17
Isoleucine	12	0	0	0.4	0	0	0	2.2	2.1	2.5	2.7	9.5	8.8
Leucine	30	1.0	1.0	3.1	3.0	1.8	1.8	4.4	4.0	3.8	3.9	17	18
Tyrosine	5.0	0	0	0	0	0	0	0	0	0	0	1.6	2.2
Phenylalanine	14	1.0	1.0	2.8	3.0	0	0	2.2	1.9	3.2	2.9	8.4	6.9
Hydroxylysine	8.3	0	0	0.9	0.3	0	0	2.3	1.1	1.5	0.9	4.7	6.9
Histidine	4.4	0	0	0	0	0	0	Trace	0.7	0	0	5.7	6.0
Lysine	29	0	0	4.2	4.8	2.1	1.8	4.9	5.5	8.6	8.8	15	12
Arginine	52	1.0	1.0	6.2	6.0	3.8	3.9	11.1	11	13	13	29	34
Homoserine	4.4‡	0.5	1.0	0.4	0.9	0.4	0.8	0	0	0.6	1.0	0	1.0‡
Total	1000	35	35	150	150	45	45	195	195	263	263	617	617

* Mean composition of three preparations.
† Mean composition of two preparations.
‡ Methionine.
§ Not homogeneous (see Fig. 5).

Fig. 4. Gel chromatography, on a column (67 cm $\times$ 1.9 cm) of Sephadex G-25, of fraction 5 (Fig. 2). Elution was achieved with 100 mM-acetic acid, pH 3.0. The bars indicate the fractions pooled.

eluted with 1 M-CaCl₂/50 mM-Tris/HCl, pH 7.5. Its composition (Table 1) was similar to that of peptide α 1-CB6 from bovine insoluble skin collagen (Volpin & Veis, 1973); no homoserine was detected even when four times the usual amount of hydrolysate was analysed. Thus component 5A was identified as peptide α 1-CB6, the C-terminal peptide of the α 1-chain, by its amino acid composition (Table 1) and from its elution position on CM-cellulose. The lack of tyrosine in corneal peptide α 1-CB6 indicates that it has lost most of the extrahelical C-terminal region, probably by the action of proteolytic enzymes *in vivo* or during extraction. Loss of this region from other collagens during extraction (Kang & Gross, 1970; Rauterberg *et al.*, 1972; Stoltz *et al.*, 1972), is usually prevented by guanidine, which we have used. The

lysine of corneal peptide α 1-CB6 is more heavily hydroxylated (2.3 residues per chain, see Table 1) than peptide α 1-CB6 from bovine skin (1.1 residues per chain, see Volpin & Veis, 1973). The alkaline hydrolysate of corneal peptide α 1-CB6 contained 1.15 residues of glucosylgalactosylhydroxylysine, 0.51 residue of galactosylhydroxylysine and 0.81 residue of hydroxylysine per chain, a total of 2.5 residues per chain compared with 2.3 residues of hydroxylysine per chain in the acid hydrolysate.

In the α 1-chain of calf skin collagen, residues 927–935 (numbering from the beginning of the helical region at the N-terminus of the α 1-chain), which occur in peptide α 1-CB6, have the same sequence as residues 87–95 (Wendt *et al.*, 1972; Fietzek & Kühn, 1975), the region where glucosylgalactosylhydroxylysine is attached to several collagens (Butler *et al.*, 1967; Butler, 1970; Morgan *et al.*, 1970). Further, residues 925–930 of calf skin collagen (Wendt *et al.*, 1972) have the same sequence as the glycopeptide Gly-Ile-Hyl-(Gal-Glc)-Gly-His-Arg isolated from carp swim bladder (Morgan *et al.*, 1970). Thus it seems likely that one residue of glucosylgalactosylhydroxylysine found in peptide α 1-CB6 of bovine corneal collagen is situated at residue 927.

Peptide α 1-CB7. Component 7 (Fig. 2) was dialysed to remove citrate, and freeze-dried. Electrophoresis on sodium dodecyl sulphate/polyacrylamide gels showed the major component to be peptide α 1-CB7 or α 1-CB8 or α 2-CB4 with two minor components, one of which was peptide α 1-CB6. Isolation of peptide α 1-CB7 from component 7 was by ion-exchange chromatography on CM-cellulose at pH 4.8 with either a concave gradient (Butler *et al.*, 1967) or a linear gradient (Volpin & Veis, 1973). Both methods separated three components, but the linear gradient was simpler, quicker and gave superior resolution. The major component was identified as peptide α 1-CB7 by amino acid analysis (Table 1), by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and by determination of its N-terminal amino acid, which is hydroxyproline; no other CNBr-cleaved fragment from type-I collagen has N-terminal hydroxyproline. The dansylated amino acid coincided with dansylhydroxyproline when it was run on the back of the polyamide plate, and did not coincide with any of the common dansyl amino acids when they were run on the back of a second sheet.

In corneal peptide α 1-CB7, 1.5 residues of lysine per chain are hydroxylated compared with 0.9 residue in peptide α 1-CB7 from bovine insoluble skin collagen (Table 1). Of the hydroxylysine 0.5 residue are present as glucosylgalactosylhydroxylysine and 0.6 residue as galactosylhydroxylysine.

Peptide α 2-CB(3–5). This peptide, the largest CNBr-cleaved fragment of bovine type-I collagen, contains about 620 amino acid residues. It was isolated from the whole CNBr digest as well as from

fraction 10 by gel chromatography on agarose (Fig. 2). The amino acid compositions of both preparations were close to that of peptide $\alpha 2$ -CB(3-5) from bovine skin (Table 1), but electrophoresis on sodium dodecyl sulphate/polyacrylamide gels showed that, although peptide $\alpha 2$ -CB(3-5) was the major component, there were smaller amounts of material of similar molecular weight (Fig. 5).

Alkaline hydrolysates of these preparations of peptide $\alpha 2$ -CB(3-5) contained 1.8 residues of glucosylgalactosylhydroxylysine, 2.0 residues of galactosylhydroxylysine and 4.6 residues of free hydroxylysine per thousand amino acid residues. The total (8.4 residues) is close to the 7.5 residues of hydroxylysine per thousand amino acids found in the acid hydrolysate.

Peptides $\alpha 1$ -CB5 and $\alpha 2$ -CB4. These peptides

are the major glycoside-containing peptides of calf skin collagen, but could not be isolated from corneal collagen in the present work. They would be expected to form part of components 5 and 9 (Fig. 2). However, when component 5 was chromatographed on Sephadex G-25 only the two peptides, identified as $\alpha 1$ -CB6 and $\alpha 1$ -CB4, were eluted (Fig. 4). No amino acids were present in hydrolysates of later fractions. The methionine between peptides $\alpha 1$ -CB5 and $\alpha 1$ -CB8 is resistant to cleavage by CNBr (Scott & Veis, 1976a), and this could explain why peptide $\alpha 1$ -CB5 was not found in this work especially as peptide $\alpha 1$ -CB8 was also not found. However, this explanation is open to question, as no methionine was detected when the acid hydrolysate of as much as 400 μ g of the soluble fraction of the CNBr digest was loaded on the amino acid analyser. In calf skin colla-

Fig. 5. Absorption profile of the gel obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a preparation of peptide $\alpha 2$ -CB(3-5). The gel contained 7.5% polyacrylamide and was stained with Coomassie Brilliant Blue.

Table 2. *Glucosylgalactosylhydroxylysine, galactosylhydroxylysine and free hydroxylysine contents of CNBr fragments of bovine corneal collagen*

Values are expressed as residues per peptide.

Peptide	Glc-Gal-Hyl	Gal-Hyl	Free Hyl
$\alpha 1$ -CB3	0.54	0.36	0.32
$\alpha 1$ -CB6	1.15	0.51	0.81
$\alpha 1$ -CB7	0.52	0.61	0.45
Total	2.21	1.48	1.58
$\alpha 2$ -CB(3-5)	1.12	1.24	2.85

gen (Becker *et al.*, 1975) and in calf bone collagen (Eyre & Glimcher, 1973) the $\alpha 1$ -CB5 region has been found cross-linked to the $\alpha 1$ -CB6 region. Thus it is possible that in bovine corneal collagen too, this peptide is cross-linked and would therefore be eluted in a different position on the CM-cellulose column.

Similarly fragment $\alpha 2$ -CB4 is joined to peptide $\alpha 1$ -CB6 in dentin collagen (Scott *et al.*, 1976), giving a cross-link peptide that appears on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with a relative mobility identical with that of the component with a mobility of 0.33 shown in Fig. 1. It seems possible that our failure to isolate peptides $\alpha 1$ -CB5 and $\alpha 2$ -CB4 is because both are present as cross-link peptides.

Distribution of glycosides in bovine corneal collagen

Of the CNBr-cleaved fragments, peptides $\alpha 1$ -CB2 and $\alpha 1$ -CB4 contain no glycoside. The glycoside compositions of the others are shown in Table 2; the $\alpha 2$ -CB(3-5) fragment is separated from the others because it is not homogeneous. Bovine corneal collagen contains 9.3 residues of glucosylgalactosylhydroxylysine and 5.7 residues of galactosylhydroxylysine per three-chain collagen molecule. Of this, 4.4 residues of glucosylgalactosylhydroxylysine and 3.0 residues of galactosylhydroxylysine were found in the peptides isolated from the two $\alpha 1$ -chains (Table 2), accounting for about half of the total. The glycoside present in the $\alpha 2$ -CB(3-5) preparation would account for a further 1.1 residues of glucosylgalactosylhydroxylysine and 1.2 residues of galactosylhydroxylysine. In all other collagens studied one residue of glucosylgalactosylhydroxylysine is in the $\alpha 1$ -CB5 region (Butler *et al.*, 1967; Morgan *et al.*, 1970; Kang *et al.*, 1975), so this glycoside is probably also present in corneal collagen. Including the glycoside of peptide $\alpha 1$ -CB5 we have accounted for 7.5 out of the 9.3 residues of glucosylgalactosylhydroxylysine and 4.2 out of the 5.7 residues of galactosylhydroxylysine per molecule of corneal collagen and attributed it to known CNBr-cleaved peptides. To understand the role of the hydroxylysine-bound glycosides of collagen, it is

necessary to pinpoint their exact location along the molecule. This we have done for the glycoside of peptide $\alpha 1$ -CB3 from corneal collagen by investigation of its tryptic and chymotryptic peptides (Panjwani & Harding, 1978).

We thank the Royal National Institute for the Blind for financial support for N. A. P. and the Medical Research Council for the amino acid analysers. This work forms part of a thesis submitted to the University of Oxford by N. A. P.

References

- Becker, U., Furthmayr, H. & Timpl, R. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 21-32
- Butler, W. T. (1970) *Biochemistry* **9**, 44-50
- Butler, W. T., Piez, K. A. & Bornstein, P. (1967) *Biochemistry* **6**, 3771-3780
- Cannon, D. J. & Cintron, C. (1975) *Biochim. Biophys. Acta* **412**, 18-25
- Croft, L. R. (1972) *Biochem. J.* **128**, 961-970
- Epstein, E. H., Scott, R. D., Miller, E. J. & Piez, K. A. (1971) *J. Biol. Chem.* **246**, 1718-1724
- Eyre, D. R. & Glimcher, M. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 663-671
- Eyre, D. R. & Muir, H. (1975) *Biochem. J.* **151**, 595-602
- Fietzek, P. P. & Kühn, K. (1975) *Eur. J. Biochem.* **52**, 77-82
- Fietzek, P. P., Munch, M., Breitreutz, D. & Kühn, K. (1970) *FEBS Lett.* **9**, 229-231
- Freeman, I. L., Steven, F. S. & Jackson, D. S. (1968) *Biochim. Biophys. Acta* **154**, 252-254
- Furthmayr, H. & Timpl, R. (1971) *Anal. Biochem.* **41**, 510-516
- Grant, M. E., Freeman, I. L., Schofield, J. D. & Jackson, D. S. (1969) *Biochim. Biophys. Acta* **177**, 682-685
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121-138
- Kang, A. H. & Gross, J. (1970) *Biochemistry* **9**, 796-804
- Kang, A. H., Dixit, S. N., Corbett, C. & Gross, J. (1975) *J. Biol. Chem.* **250**, 7428-7434
- Lian, J. B., Morris, S., Faris, B., Albright, J. & Franzblau, C. (1973) *Biochim. Biophys. Acta* **328**, 193-204
- Miller, E. J. (1976) *Mol. Cell. Biochem.* **13**, 165-192
- Miller, E. J. & Lunde, L. G. (1973) *Biochemistry* **12**, 3153-3159
- Moczar, E. & Moczar, M. (1970) *J. Chromatogr.* **51**, 277-282
- Morgan, P. H., Jacobs, H. G., Segrest, J. P. & Cunningham, L. W. (1970) *J. Biol. Chem.* **245**, 5042-5048
- Panjwani, N. A. & Harding, J. J. (1978) *Biochem. J.* **171**, 697-703
- Rauterberg, J., Fietzek, P., Rexrodt, F., Becker, U., Stark, M. & Kühn, K. (1972) *FEBS Lett.* **21**, 75-79
- Scott, P. G. & Veis, A. (1976a) *Connect. Tissue Res.* **4**, 107-116
- Scott, P. G. & Veis, A. (1976b) *Connect. Tissue Res.* **4**, 117-129
- Scott, A. G., Veis, A. & Mechanic, G. (1976) *Biochemistry* **15**, 3191-3198
- Spiro, R. G. (1967) *J. Biol. Chem.* **242**, 4813-4823
- Spiro, R. G. (1969) *J. Biol. Chem.* **244**, 602-612
- Spiro, R. G. (1972) *Methods Enzymol.* **28B**, 3-43

- Stoltz, M., Timpl, R. & Kühn, K. (1972) *FEBS Lett.* **26**, 61–65
- Trelstad, R. L. & Kang, A. H. (1974) *Exp. Eye Res.* **18**, 395–406
- Volpin, D. & Veis, A. (1973) *Biochemistry* **12**, 1452–1464
- Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26C**, 3–27
- Weber, L., Meigel, W. N. & Rauterberg, J. (1977) *Arch. Derm. Res.* **258**, 251–257
- Wendt, P., von der Mark, K., Rexrodt, F. & Kühn, K. (1972) *Eur. J. Biochem.* **30**, 169–183
- Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370