Biochem. J. (1968) **108**, 587
Printed in Great Britain

Carbohydrate Content of Bovine Collagen Preparations

By M. E. GRANT and D. S. JACKSON
Department of Medical Biochemistry, Lapworth Laboratories, University of Manchester

(*Received 2 February* 1968)

Collagen preparations from bovine tissues were analysed for their carbohydrate content. Crude preparations of tropocollagen and polymeric collagen were found to be contaminated with considerable amounts of mannose, fucose and hexosamine, sugars known to be present in the mucoprotein of the interfibrillar material with which collagen is associated *in vivo*. A pure preparation of tropocollagen obtained by ethanol precipitation procedures contained only galactose and glucose in the approximate ratio of 7:3 residues/3000 amino acid residues. Purification of crude polymeric collagen by EDTA extraction or by crude bacterial amylase extraction considerably decreased the mucoprotein contamination, particularly in the enzymic treatment, which yielded a preparation containing predominantly galactose and glucose in the ratio of 4:2 residues/3000 amino acid residues. The results confirm previous work that demonstrated the purity of these collagen preparations as inferred by amino acid analysis. The results also indicate the suitability of the pure tropocollagen and the amylase-extracted polymeric collagen for studies on the role of the carbohydrate residues in intramolecular and intermolecular cross-linking in collagen.

The isolation of collagen free from the interfibrillar material with which it is associated *in vivo* has in the past proved difficult, particularly with insoluble polymeric collagen. Comparison of the amino acid composition of the isolated material with that of the purified gelatin obtained by Eastoe (1955) has frequently been used as a criterion of purity, though the absence of hexosamine has also been considered as an index of purity (Gross, Dumsha & Glazer, 1958; Jackson, Leach & Jacobs, 1958; Kühn, Grassmann & Hofmann, 1959). Exhaustive extraction of non-collagenous material has been found to decrease the hexosamine content of collagen preparations to a very low level (0·1–0·2%, w/w), but complete removal has often proved difficult (Bowes, Elliott & Moss, 1955; Drake, Davison, Bump & Schmitt, 1966).

Recent papers from this Laboratory (Steven, 1967; Steven & Jackson, 1967) have described the purification and amino acid composition of monomeric and polymeric collagens. The present paper reports the determination and composition of carbohydrates associated with these collagen preparations and confirms their high degree of purity.

METHODS

Materials. Hexamethyldisilazane and trifluoroacetic acid were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Dowex 50 was purchased from V. A. Howe and Co. Ltd., London, W.11. Activated charcoal was purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex.

All other chemicals were supplied by British Drug Houses Ltd., Poole, Dorset, and were of AnalaR grade whenever available.

Anhydrous pyridine was prepared by redistillation at 114–116° of pyridine previously dried over NaOH pellets.

Preparation of monomeric and polymeric collagens. A crude preparation of acetic acid-soluble tropocollagen was obtained from calf skin (Steven & Tristram, 1962) and purified by the ethanol precipitation procedure described by Steven & Jackson (1967) to yield pure tropocollagen.

A crude preparation of polymeric collagen was obtained from ox tendon by the method described by Drake *et al.* (1966) for the extraction of 'insoluble collagen'. EDTA-extracted polymeric collagen was prepared from ox tendon by the EDTA extraction procedure of Steven (1967). Amylase-extracted polymeric collagen was also prepared from ox tendon by using crude bacterial α-amylase (Steven, 1964) and purified by salt precipitation to remove physically associated non-collagenous materials (Steven & Jackson, 1967).

Hydrolysis of collagen. About 70–100 mg. of dried material was refluxed in a 50 ml. flask fitted with an air condenser (100 cm. in length) with 10 ml. of 33% (w/v) Dowex 50 (H^+ form; 4% cross-linking; 200–400 mesh) suspension in 0·05 N-HCl. The resin was prepared just before use by the procedure described by Moore & Stein (1951). After hydrolysis the flask and contents were cooled and the suspension was quantitatively transferred to a column (40 cm. × 1·2 cm. diam.) with a sintered-glass disk at the base. The procedure described by Anastassiadis & Common (1958) for the separation of neutral sugars from the amino acids and amino sugars was followed but with double their volumes of

distilled water and HCl (2N). Hence the neutral sugars were eluted in 50ml. of distilled water (eluate I) and the amino acids and amino sugars eluted in 50ml. of HCl solution (eluate II).

The optimum time for hydrolysis of collagen was 18hr., when release of hydroxyproline, neutral sugars and hexosamine reached a maximum (Fig. 1).

Colorimetric analyses. The carbohydrate content of eluate I was determined by a modification of Devor's method (Fuller & Northcote, 1956). To 2ml. samples (in triplicate) of eluate I in tubes cooled in an ice–water bath was added 1-naphthol reagent (4ml.) prepared by dissolving 2g. of 1-naphthol in 500ml. of conc. H_2SO_4 and keeping the solution overnight in the dark. The tubes were shaken vigorously, heated at 100° for 10min. and cooled, and the E_{555} values were measured. Results are expressed in terms of moles of galactose, which was run as a standard.

The hydroxyproline present in eluate II was determined by the method of Woessner (1961). A 1ml. portion of eluate II was diluted to 50ml. with distilled water and samples (2ml.) of the diluted solution were used in the Woessner procedure.

The hexosamine content of eluate II was determined after initial concentration of the solution and removal of HCl. A 40ml. portion of eluate II was evaporated to dryness on a rotary evaporator at 30° and final traces of HCl were removed by leaving overnight over NaOH pellets. The residue was dissolved in distilled water (5ml.) and a 2ml. portion (in duplicate) put through the procedure of Johansen, Marshall & Neuberger (1960). Results are expressed in terms of moles of glucosamine, which was run as a standard.

When a standard solution of hydroxyproline (5mg.), galactose (0·5mg.) and glucosamine hydrochloride (0·18mg.) was put through the hydrolysis procedure, separated and analysed as described above, the mean percentage recovery ±S.D. of each was: hydroxyproline, 96·3±3·4 (10); galactose, 94·7±6·4 (12); glucosamine hydrochloride, 91·4±3·2 (9). Numbers of determinations are given in parentheses.

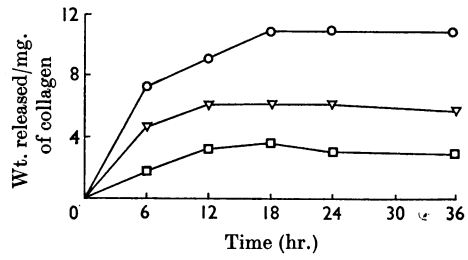

Fig. 1. Release of hydroxyproline, neutral sugars and hexosamine from α-amylase-extracted polymeric collagen by resin hydrolysis. A 100mg. sample of dry amylase-extracted polymeric collagen was refluxed with 10ml. of 33% (w/v) Dowex 50 (H^+ form) suspension in 0·05N-HCl. The hydrolysate was separated into a neutral fraction (eluate I) and an acidic fraction (eluate II) as described in the text, and the release of hydroxyproline (○, 10μg. of hydroxyproline/mg.), neutral sugar (□, 1·0μg. of hexose/mg.) and hexosamine (△, 0·1μg. of hexosamine/mg.) determined.

Chromatography. The sugars present in eluate I were evaporated to dryness on a rotary evaporator at 30°. Then 1ml. of distilled water was added and the solution passed through a column (10cm.×0·6cm. diam.) of 0·2g. of activated charcoal prepared by the method of Thomson (1958), to remove traces of a water-soluble pigment arising from the resin hydrolysis. The sugars were eluted with distilled water (5ml.) and further concentrated on the rotary evaporator before chromatography.

Standard sugars and the unknown solutions were applied to Whatman no. 1 chromatography paper (45cm. long) and two successive descending runs of 20hr. and 18hr. respectively were made with the organic phase of the solvent system benzene–butan-1-ol–pyridine–water (1:5:3:3, by vol.) (Gaillard, 1953). Sugars were detected by spraying with aniline hydrogen phthalate solution (Partridge, 1949). The $R_{glucose}$ values were 0·87 for galactose, 1·20 for mannose, 1·42 for xylose and 1·46 for fucose.

Confirmation of the identity of the sugars present and their quantitative determination were carried out by gas–liquid chromatography. A portion of the concentrated sugar solution obtained above was evaporated to dryness and the trimethylsilyl derivatives were prepared by the method of Brobst & Lott (1966). Anhydrous pyridine (0·05ml.) was added to the samples in stoppered flasks to dissolve the sugars. Hexamethyldisilazane (0·045ml.) and trifluoroacetic acid (0·005ml.) were added, the samples were shaken vigorously for 30sec. and chromatographed after standing for 15min. Analyses were performed on a Perkin–Elmer 800 machine with dual SE–30 columns [1·5% silicone gum rubber E.301 on hexamethyldisilazane-treated Chromosorb W (80–100 mesh) in 6ft.×$\frac{1}{8}$in. outer-diameter stainless-steel tubes]. The separations obtained in this work were achieved under the following conditions: an injection-block temperature of 200°, column temperature programming from 140° to 170° at 1·7°/min. and a nitrogen carrier gas flow rate of 30ml./min. In order that the retention times of the trimethylsilyl-sugars and the size of the response recorded on the chart could be evaluated, a known weight of internal standard (0·4mg. of sorbitol) was added to all samples before silylation. The areas under the peaks were measured by using an Integrator–Calculator (Technicon Instruments Co. Ltd., Chertsey, Surrey) and evaluated by comparison with the area under the peak of the added standard. Duplicate chromatographic anaylses were performed and the average of these values was used. Because complete resolution of all the peaks was not achieved on the SE–30 column it was necessary to measure the area under the α-anomers of galactose and mannose and the

Table 1. *Retention times of trimethylsilyl derivatives of sugars relative to trimethylsilyl-sorbitol on gas–liquid chromatograms*

Conditions were as described in the Methods section.

Sugar	$R_{sorbitol}$	Sugar	$R_{sorbitol}$
α-Fucose	0·37	α-Glucose	0·81
β-Fucose	0·42	β-Glucose	1·14
γ-Fucose	0·34	α-Mannose	0·62
α-Galactose	0·75	β-Mannose	0·87
β-Galactose	0·87	α-Xylose	0·38
γ-Galactose	0·72	β-Xylose	0·48

β-anomer of glucose. Retention times of the trimethylsilyl derivatives of fucose, galactose, glucose, mannose and xylose relative to trimethylsilyl-sorbitol, under the gas–liquid-chromatographic conditions described above, are shown in Table 1.

RESULTS

Colorimetric analyses of neutral sugars and hexosamines. Determination of the neutral sugars as total carbohydrate by the 1-naphthol method indicated that the number of hexose units associated with the collagen preparations were as shown in Table 2. These results are expressed in terms of a galactose standard, since initial chromatographic evidence had shown galactose to be the major component of eluate I of amylase-extracted polymeric collagen. The values were calculated in terms of the hydroxyproline present in eluate II, assuming 94 residues of hydroxyproline/1000 amino acid residues (Bornstein & Piez, 1964; Steven & Jackson, 1967). Since the individual sugars do not all give exactly the same colour value with the 1-naphthol reagent (Grant, 1966), interpretation of colorimetric analyses of a multi-sugar solution must be viewed cautiously. However, in conjunction with evidence obtained by gas–liquid chromatography, it is possible to state that associated with crude tropocollagen are approx. 20 hexose units/3000 amino acid residues. Further purification of the crude tropocollagen by ethanol precipitation decreased this value to approx. 10 hexose units/3000 amino acid residues in pure tropocollagen.

The crude polymeric collagen preparation contained approx. 23 hexose units/3000 amino acid residues, whereas extraction by EDTA decreased this value by 50% to approx. 12 hexose units/3000 acid residues. Polymeric collagen prepared by the α-amylase extraction procedure contained only 6–7 hexose units/3000 amino acid residues.

When these collagen preparations were hydrolysed under nitrogen with 6N-hydrochloric acid in a sealed tube at 100° for 24 hr. (Eastoe, 1955) and subsequently analysed on a Technicon Auto-analyser, no hexosamine could be detected on the column chromatograms. The elution system used (Hamilton, 1963) clearly resolves glucosamine from neighbouring amino acid peaks, but galactosamine and valine chromatograph as partially resolved but distinguishable peaks. However, concentration of eluate II and application of the method of Johansen *et al.* (1960) to avoid interference by amino acids revealed the presence of hexosamine in all five preparations (Table 2). The hexosamine values are expressed in terms of a glucosamine standard, but preliminary experiments with the method of Cessi & Serafini-Cessi (1963) to determine galactosamine in the presence of glucosamine suggest that the major hexosamine present in these preparations is galactosamine.

Crude tropocollagen has approx. 4 hexosamine units/3000 amino acid residues, but removal of non-collagenous materials by the ethanol purification procedure decreases the hexosamine content to the very low value of 0·33 hexosamine unit/3000 amino acid residues. Crude polymeric collagen has approx. 7 hexosamine units/3000 amino acid residues, but this value is decreased when the polymeric collagen is extracted with EDTA. However, greatest purification of polymeric collagen is achieved in amylase-extracted polymeric collagen, where the hexosamine content is decreased to less than 1 hexosamine unit/3000 amino acid residues.

Chromatographic analyses. The identity and relative concentrations of sugars found to be associated with the collagen preparations, as demonstrated by paper chromatography, are shown in Table 3. Galactose and glucose were present in all five preparations and mannose was detected in all except the pure tropocollagen. Traces of a fourth sugar were found with crude tropocollagen, crude polymeric collagen and EDTA-extracted polymeric collagen, and this sugar was tentatively identified as xylose since it had $R_{glucose}$ 1·43 and gave a faint

Table 2. *Carbohydrates associated with collagen preparations*

Hexosamine values are expressed in terms of a glucosamine standard and hexose values in terms of a galactose standard, both as means ± S.D. for the numbers of determinations given in parentheses. Molar proportions of sugars were determined by gas–liquid chromatography.

	Hexosamine (units/3000 residues*)	Hexose (units/3000 residues*)	Molar proportion of sugars Gal : Glc : Man : Fuc
Crude tropocollagen	4·26 ± 0·11 (5)	19·89 ± 1·10 (5)	11·8 : 7·0 : 1·7 : Trace
Pure tropocollagen	0·33 ± 0·02 (6)	9·84 ± 0·48 (6)	6·7 : 3·0 : 0·1 : 0
Crude polymeric collagen	6·64 ± 0·17 (6)	22·78 ± 0·78 (6)	11·1 : 6·0 : 4·6 : 1·9
EDTA-extracted polymeric collagen	3·87 ± 0·18 (6)	11·75 ± 0·36 (6)	4·0 : 5·6 : 1·6 : Trace
Amylase-extracted polymeric collagen	0·85 ± 0·02 (6)	6·37 ± 0·25 (6)	4·0 : 2·0 : 0·38 : Trace

*Calculated assuming 94 hydroxyproline residues/1000 residues (Steven & Jackson, 1967).

Table 3. *Identification of neutral sugars associated with collagen preparations by paper chromatography*

+ indicates the relative intensity of the spots on the chromatogram after spraying with aniline hydrogen phthalate solution.

	Galactose	Glucose	Mannose	Fucose	Uronic acid
Crude tropocollagen	++++	++	+	Trace	
Pure tropocollagen	++++	++			
Crude polymeric collagen	++++	+++	++	+	+?
EDTA-extracted polymeric collagen	+++	+++	+	Trace	
Amylase-extracted polymeric collagen	++++	++	Trace		

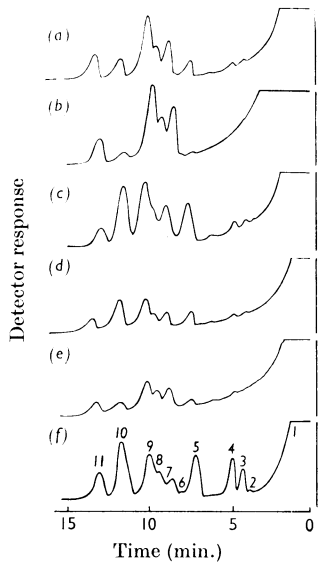

Fig. 2. Traces of gas–liquid chromatograms of trimethylsilyl-sugars from collagen preparations. (*a*) Crude tropocollagen; (*b*) pure tropocollagen; (*c*) crude polymeric collagen; (*d*) EDTA-extracted polymeric collagen; (*e*) amylase-extracted polymeric collagen; (*f*) standard; mixture of sugars. Identity of peaks: *1*, pyridine; *2*, γ-fucose; *3*, α-fucose; *4*, β-fucose; *5*, α-mannose; *6*, γ-galactose; *7*, α-galactose; *8*, α-glucose; *9*, β-galactose+β-mannose; *10*, sorbitol; *11*, β-glucose.

reddish-brown spot on spraying with aniline hydrogen phthalate solution. Subsequent analysis by gas–liquid chromatography demonstrated that this fourth sugar was in fact fucose. The possible presence of uronic acid was indicated in sugars from crude polymeric collagen only, where a brown spot on the chromatogram with $R_{glucose}$ 0·38 was detected after spraying with aniline hydrogen phthalate solution.

Further identification of the sugars was carried out by gas–liquid chromatography with sorbitol as internal standard as described in the Methods section. The traces (Fig. 2) correlate well with the results of paper chromatography, and the increased sensitivity of the gas–liquid-chromatographic method permitted detection of a trace of mannose in the pure tropocollagen. The fourth sugar associated with crude tropocollagen gave peaks with retention times of 0·36 and 0·41, from crude polymeric collagen retention times of 0·36 and 0·42, and from EDTA-extracted polymeric collagen retention times of 0·37 and 0·43. These values compare well with the retention times of authentic fucose (see Table 1) and rule out the presence of xylose.

Determination of the galactose:glucose:mannose:fucose molar proportions was achieved by measuring the areas under the appropriate peaks of the gas–liquid-chromatographic traces. These molar proportions were employed in calculating the molar proportions of the sugars corresponding to the total carbohydrate content obtained by colorimetric analysis (Table 2).

DISCUSSION

The presence of D-glucose and D-galactose in acid hydrolyses of a variety of collagen preparations has been established by a number of workers (Grassmann, Hörmann & Hafter, 1957; Gross *et al.* 1958; Kühn *et al.* 1959), and evidence has been presented suggesting that these hexoses are covalently linked to hydroxylysine of collagen both as disaccharide units and as single residues (Blumenfeld, Paz, Gallop & Seifter, 1963; Butler & Cunningham, 1966; Cunningham, Ford & Segrest, 1967). The precise role of these hexose residues remains to be determined, but they have been considered as possible participants in the formation of intramolecular and intermolecular cross-links (Gallop, Seifter & Meilman, 1957; Hörmann, 1962; Butler & Cunningham, 1966) and may play an important role in the aggregation or polymerization of collagen.

The preparation of pure monomeric and polymeric collagen is an obvious prerequisite for studies on such a role. Since only small amounts of carbohydrate are found to be associated with pure collagen preparations of vertebrate origin, the application of sensitive techniques such as gas–liquid chromatography to carbohydrate analysis can prove most valuable in detecting the presence of mannose,

fucose, hexosamine etc. in contaminating mucoprotein (Neuberger, Marshall & Gottschalk, 1966).

Our studies have demonstrated that the crude tropocollagen prepared by acetic acid extraction is contaminated with sugars of the interfibrillar material. However, by the ethanol precipitation procedures of Steven & Jackson (1967), physically associated non-collagenous materials can be eliminated, yielding a highly purified preparation of tropocollagen virtually free from hexosamine (<0·02%, w/w), mannose (<0·006%, w/w) and fucose.

Gas–liquid-chromatographic studies on the crude polymeric collagen prepared by the method of Drake *et al.* (1966) demonstrated a high degree of mucoprotein contamination as inferred by the presence of considerable amounts of hexosamine, mannose and fucose. The EDTA extraction procedure yields a cleaner preparation, but greater purification is achieved by using crude α-amylase, which markedly decreases the hexosamine (to <0·05%, w/w), mannose (to <0·03%, w/w) and fucose contents to give a highly purified polymeric collagen.

The importance of the decrease in the number of galactose and glucose units present in amylase-extracted polymeric collagen as compared with those in pure tropocollagen cannot be assessed until the mode of enzyme action has been determined. Work is proceeding on this problem, but initial analyses of the carbohydrate content of amylase-extracted polymeric collagen from other bovine tissues suggest that the amount of galactose and glucose varies with different tissues, and the suggestion that these sugars might play a role in the polymerization and differentiation of collagen in structural tissues seems a real possibility.

Bowes *et al.* (1955) suggested that insoluble collagen prepared by simple extraction procedures was a complex of collagen and mucoprotein. A model structure for the insoluble fibre has been proposed on the basis of this and other evidence (Jackson & Bentley, 1960). The results reported here and the marked effect of removal of the mucoprotein on the dispersibility of collagen (Steven, 1964) would support this view.

The authors thank Dr F. S. Steven for providing the collagen preparations and also acknowledge the valuable technical assistance of Miss E. Beddall and Miss S. Eltherington. We also thank Dr G. N. Smith and Dr T. J. Langley for their advice and guidance in the techniques of gas–liquid chromatography.

REFERENCES

Anastassiadis, R. A. & Common, R. H. (1958). *Canad. J. Biochem.* **36**, 413.
Blumenfeld, O. O., Paz, M. A., Gallop, P. M. & Seifter, S. (1963). *J. biol. Chem.* **238**, 3835.
Bornstein, P. & Piez, K. A. (1964). *J. clin. Invest.* **43**, 1813.
Bowes, J. H., Elliott, R. G. & Moss, J. A. (1955). *Biochem. J.* **61**, 143.
Brobst, K. M. & Lott, C. E. (1966). *Cereal Chem.* **43**, 35.
Butler, W. T. & Cunningham, L. W. (1966). *J. biol. Chem.* **241**, 3882.
Cessi, C. & Serafini-Cessi, F. (1963). *Biochem. J.* **88**, 132.
Cunningham, L. W., Ford, J. D. & Segrest, J. P. (1967). *J. biol. Chem.* **242**, 2570.
Drake, M. P., Davison, P. F., Bump, S. & Schmitt, F. O. (1966). *Biochemistry*, **5**, 301.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Fuller, K. W. & Northcote, D. H. (1956). *Biochem. J.* **64**, 657.
Gaillard, B. D. E. (1953). *Nature, Lond.*, **171**, 1160.
Gallop, P. M., Seifter, S. & Meilman, E. (1957). *J. biol. Chem.* **227**, 891.
Grant, M. E. (1966). D.Phil. Thesis: University of Oxford.
Grassmann, W., Hörmann, H. & Hafter, R. (1957). *Hoppe-Seyl. Z.* **307**, 87.
Gross, J., Dumsha, B. & Glazer, N. (1958). *Biochim. biophys. Acta*, **30**, 293.
Hamilton, P. B. (1963). *Analyt. Chem.* **35**, 2055.
Hörmann, H. (1962). *Leder*, **13**, 179.
Jackson, D. S. & Bentley, J. P. (1960). *J. biophys. biochem. Cytol.* **7**, 37.
Jackson, D. S., Leach, A. A. & Jacobs, S. (1958). *Biochim. biophys. Acta*, **27**, 418.
Johansen, P. G., Marshall, R. & Neuberger, A. (1960). *Biochem. J.* **77**, 239.
Kühn, K., Grassmann, W. & Hofmann, V. (1959). *Z. Naturf.* **14**b, 436.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Neuberger, A., Marshall, R. D. & Gottschalk, A. (1966). In *Glycoproteins*, vol. 5, p. 158. Ed. by Gottschalk, A. Amsterdam: Elsevier Publishing Co.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Steven, F. S. (1964). *Ann. rheum. Dis.* **23**, 300.
Steven, F. S. (1967). *Biochim. biophys. Acta*, **140**, 522.
Steven, F. S. & Jackson, D. S. (1967). *Biochem. J.* **104**, 534.
Steven, F. S. & Tristram, G. R. (1962). *Biochem. J.* **83**, 240.
Thomson, R. Y. (1958). In *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 242. Ed. by Smith, I. London: W. Heinemann (Medical Books) Ltd.
Woessner, J. F. (1961). *Arch. Biochem. Biophys.* **93**, 440.