APPENDIX

The Carbohydrate of Collagen

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The presence of up to 1% of sugars in collagen has been suggested by several workers, notably Grassmann & Schleich (1935) and Beek (1941), but none of these workers definitely established their identity. More recently, Glegg, Eidinger & Leblond (1953) have reported the identification by paper chromatography of glucose, galactose, mannose, fucose and a trace of ribose in reticulin and tendon collagen, whilst Consden, Glynn & Stanier (1953), using a combination of paper electrophoresis and paper chromatography, have found glucose, mannose, galactose and glucosamine, the last two predominating, in subcutaneous tissue.

Preliminary attempts to detect sugars in purified skin collagen by these methods were unsuccessful. This failure was attributed to the very low concentrations present and to considerable interference from the high amino acid and peptide content of the hydrolysates.

A number of methods used for the determination of sugars have been applied to collagen. Some estimates made of the interference due to protein hydrolytic products, which are of general interest in connexion with the determination of sugars in other proteins, have been recorded.

EXPERIMENTAL

Materials. The collagenous materials used were similar to those described in the main part of this paper.

Hydrolysis. The protein was hydrolysed with 2N-HCl for 16 hr. in a sealed tube at 105°.

Reducing sugars. The method of Somogyi (1945) and the method of Hagedorn & Jensen (1923) as modified by Lampitt, Fuller, Goldenberg & Green (1947) were used. A modification of the anthrone colorimetric method (see Scott & Melvin, 1953) was also used. To each of several tubes 6 ml. of 0.15% (w/v) anthrone in 87% (v/v) H₂SO₄ were added. After cooling in ice for 5 min., 1 ml. of unknown or standard containing up to 200 µg. of glucose or its equivalent was added slowly to form a top layer. After cooling for a further few min., the contents of the tubes were mixed by inversion. The stoppered tubes were then transferred to a water bath at 80° for 15 min. After cooling, the absorption was measured on a Hilger 'Uvispek' spectrophotometer at 625 mµ. Scott & Melvin (1953) report that the anthrone colour is increased in the presence of chloride

ions which may be present in hydrolysates. In the present work it was found that this increase was virtually independent of chloride concentration above 0.1N and was of the order of 9%.

Hexosamine. This was determined by the method of Elson & Morgan (1933) as modified by Blix (1948). Following the suggestion made by Schloss (1951), readings were also made at 515 mµ. after 20 hr. incubation at 30° using a Hilger 'Uvispek' spectrophotometer. When the determinations were made on the 1 ml. fractions eluted from a chromatographic column the reaction was carried out in the tubes used for the collection of the samples with appropriate adjustments to the volumes of reagents added.

Paper chromatography. Two-dimensional chromatograms were run on Whatman no. 2 paper, 24 in. square. The systems phenol-water and *n*-butanol-water were found to be a suitable combination of solvents for satisfactory separation of glucose, galactose and mannose when each was run the full length of the paper. The papers were sprayed with aniline hydrogen phthalate reagent (Partridge, 1949), and, after heating for 5 min., examined in ultra-violet light.

Chromatograph technique. Hexosamines were separated from other material present in the hydrolysates, on a column of Dowex-50 1 × 23 cm. high, similar to that used by Moore & Stein (1951) for the separation of basic amino acids (see also Eastoe, 1954). Under these conditions glucosamine emerged after 84 ml. Determination by ninhydrin and by the modified Elson & Morgan (1933) procedure gave the same values, indicating that the glucosamine emerged free from peptides and amino acids. Thus, either method may be used for the determination. Using a larger column (2 cm. diam. × 20 cm.) of Dowex-50 equilibrated with 0.05N-HCl and the same acid as developer, it was found that hexoses and uronic acids were eluted together and free from amino acids, etc., after about 20 ml. of acid had passed through the column (cf. Gardell, 1953).

RESULTS AND DISCUSSION

Attempts to determine reducing sugars directly on a collagen hydrolysate gave results depending on the method used (Table 1, columns 1 and 2). Trials with a synthetic hydrolysate containing the correct proportions of all the amino acids, with the exception of hydroxylysine, which have been identified in collagen, indicated that the interference due to these was considerable with the Hagedorn & Jensen (1923) method, but was relatively slight in

Table 1. *Reducing sugars in collagen and citrate-soluble collagen (CS-collagen)*

The results are expressed as mg. glucose/100 mg. protein.

Method	Uncorrected		Synthetic hydrolysate (3)	Corrected for the contribution of amino acids (col. 3)		Neutral sugars + uronic acids after separation on Dowex-50	
	Collagen (1)	CS-collagen (2)		Collagen (4)	CS-collagen (5)	Collagen (6)	CS-collagen (7)
Hagedorn & Jensen (1923)	2.98	2.40	2.31	0.67	—	0.27	—
Somogyi (1945)	0.32	0.19	0.04	0.28	0.15	0.27	—
Anthrone	0.33	0.43	0.04	0.29	0.39	0.26	0.01-0.3

Table 2. *Hexosamine content of collagens*

The results are expressed as mg. glucosamine/100 mg. protein.

	Collagen	Citrate-soluble collagen	Synthetic hydrolysate
Uncorrected values	0.30	0.15	0.07
Glucosamine equivalent of unacetylated blanks	0.11	0.10	0.02
Values corrected for blanks	0.19	0.05	0.05
After separation on Dowex-50	0.05	0.01	—

the Somogyi (1945) and anthrone methods (Table 1, column 3). The amino acids chiefly responsible for the high values in the Hagedorn & Jensen method were tyrosine, methionine, proline and hydroxyproline, which gave 76, 42, 3 and 2%, respectively, of the values obtained for the same weight of glucose. After correction for the contribution of the amino acids (Table 1, columns 4 and 5) the Hagedorn & Jensen method still gives higher values for collagen than the other two methods. The correction is not valid for the citrate-soluble collagen owing to its much lower tyrosine content; if, however, allowance is made for this, a value of the same order as that found by the Somogyi method is obtained. The determination of hexosamine is also subject to interference by protein hydrolytic products. Colour contributed by hydroxyproline under the present conditions was 0.1% of that of an equal weight of glucosamine. In collagen this contribution would represent about 0.015% of glucosamine. Colour may also be contributed by amino acid-sugar reactions (Vasseur & Immers, 1949; Stary, Yenson, Lisie & Bilen, 1951; Horowitz, Ikawa & Fling, 1950) and is particularly important if small amounts of glucosamine are to be determined.

It has been suggested (Anastassiadis & Common, 1953) that allowance can be made for this interference by the inclusion of blanks of the hydrolysate treated exactly as the test solution except that sodium carbonate containing no acetylacetone is used. With both collagen and citrate-soluble

collagen the blanks obtained in this way were relatively large (see Table 2). This does not, however, give an entirely satisfactory blank since the values obtained for the synthetic hydrolysate showed that acetylation increased the contribution due to amino acids, and Vasseur & Immers (1949) state that the same is also true of the amino acid-sugar reaction products. Development of the colour for 24 hr. as recommended by Schloss (1951) gave similar results.

As it appears from the above that direct determinations of reducing sugars and hexosamines in collagen hydrolysates give misleading results, separation of these substances from other protein hydrolytic products using Dowex-50 was investigated (see 'Methods').

Hydrolysate equivalent to up to 1 g. protein was put on the larger-diameter column and developed with 0.05N-HCl, 1 ml. fractions being collected. Hexoses were determined by the anthrone method on 0.2 ml. portions of the fractions. Those containing anthrone positive material were then bulked and the reducing power towards the Somogyi (1945) and Hagedorn & Jensen (1923) reagents determined (Table 1, column 6). The values obtained by the three methods agree. Since galacturonic acid gives 5% of the colour yield of an equal weight of glucose with the anthrone reagent, 14% of the reducing power towards the Hagedorn & Jensen reagent and 90% towards the Somogyi reagent, it may be concluded that the amount of uronic acid present is very small. The remaining eluate was evaporated to dryness and paper chromatograms were run. Glucose and galactose in approximately equivalent quantities and a smaller amount of mannose were found.

A similar procedure in the case of citrate-soluble collagen showed the presence of glucose, galactose and mannose, the spots being all of approximately the same size and intensity on the paper chromatogram. In neither material was uronic acid detected.

For the separation of hexosamine, samples of hydrolysate equivalent to about 100 mg. protein were put on the smaller column. 1 ml. fractions were collected and hexosamine was determined by the modified Elson & Morgan procedure. The results

are given in Table 2. The amount of hexosamine found in the ox-hide collagen by this method was even smaller than the corrected value obtained directly on the hydrolysate, and the amount found in the citrate-soluble collagen was negligible.

The sugars and sugar derivatives which have been identified in ox-hide collagen are the same as those found in other collagenous tissues, e.g. tendon, but the relative proportions are different and their total amount is less, especially in the citrate-soluble collagen. The negligible amount of hexosamine and the very small and variable amounts of hexoses found in the soluble protein suggest that these may be present as impurities. This is contrary to the views of Kuntzel (1954) and of Grassmann, Endres & Steber (1954) who have found appreciable amounts of hexoses and amino sugars, respectively, which they consider to be an integral part of the citrate-soluble collagen molecule.

SUMMARY

1. Some methods used for the determination of sugars have been examined. Their application to complete protein hydrolysates is discussed.

2. The carbohydrate content of ox-hide collagen and citrate-soluble collagen has been determined.

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Cyanide Compounds of Ferropoxidase and Myoglobin and their Reversible Photodissociation

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The inhibition of peroxidatic activity of tissue extracts by cyanide was already known to Linossier (1898), who was the first to ascribe this activity to special oxidizing enzymes which he named peroxidases. The formation of a reversible and well-defined compound between horse-radish peroxidase and cyanide was noted in the course of spectroscopic studies of purified preparations during which the haematin nature of peroxidase was definitely demonstrated (Keilin & Mann, 1937). The absorption spectrum of this peroxidase-cyanide was found to be of the same pattern as those of the cyanide compounds of methaemoglobin and catalase (Keilin & Hartree, 1951). Since cyanide

combines reversibly not only with the trivalent iron of these three haemoproteins but also with the bivalent iron of haem, haemoglobin and myoglobin, a property which cyanide shares only with nitric oxide (J. Keilin, 1955), it appeared interesting to investigate the possibility of a reaction between cyanide and ferropoxidase.

The objects of this paper are: (1) to show that ferropoxidase, like haemoglobin and myoglobin, forms a reversible compound with cyanide, (2) to define the conditions under which these compounds are formed, (3) to compare their properties with those of other haem compounds, and (4) to demonstrate their reversible dissociation in light. The