SUMMARY

1. The preparation of a series of gelatins with high molecular weight is described. These were purified by partial fractionation.

2. The modulus of rigidity of the gelatin gels prepared in this way is independent of the molecular weight, as indicated by the limiting viscosity number, and of the gelatin-chain dimensions, as indicated by the *N*-terminal amino acid residues.

I am most grateful to Dr G. Stainsby for advice on the alcohol fractionation of gelatin and the interpretation of the viscosity results. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

REFERENCES

- Ames, W. M. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 5.
 Courts, A. (1954*a*). *Biochem. J.* **58**, 70.
 Courts, A. (1954*b*). *Biochem. J.* **58**, 74.

- Courts, A. (1955). *Biochem. J.* **59**, 382.
 Courts, A. (1958). *Recent Advances in Gelatin and Glue Research*, p. 145. Ed. by Stainsby, G. London: Pergamon Press Ltd.
 Courts, A. (1960). *Biochem. J.* (in the Press).
 Courts, A. & Stainsby, G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 100. Ed. by Stainsby, G. London: Pergamon Press Ltd.
 Deutsche Gelatine Fabriken (1952). Brit. Patent 682,829.
 Gallop, P. (1955). *Arch. Biochem. Biophys.* **54**, 501.
 Landucci, J. M., Pouradier, J. & Durante, M. (1958). *Recent Advances in Gelatin and Glue Research*, p. 62. Ed. by Stainsby, G. London: Pergamon Press Ltd.
 Pouradier, J. & Venet, M. (1952). *J. Chim. phys.* **49**, 85.
 Saunders, P. R. & Ward, A. G. (1953). *Proc. 2nd int. Congr. Rheology*, p. 284.
 Saunders, P. R. & Ward, A. G. (1955). *Nature, Lond.*, **176**, 26.
 Saunders, P. R. & Ward, A. G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 197. Ed. by Stainsby, G. London: Pergamon Press Ltd.
 Stainsby, G. (1954). *Disc. Faraday Soc.* **18**, 288.
 Stainsby, G., Saunders, P. R. & Ward, A. G. (1954). *J. Polym. Sci.* **12**, 325.

The Distribution of Mucoprotein in Gelatins and Fractionated Gelatins

By A. COURTS

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(Received 9 June 1959)

A method has been developed for showing the redistribution of mucoprotein brought about by fractionating gelatin, and for demonstrating the differences in mucoprotein content of gelatins inherent in the different connective tissues, species and processes used in their preparation.

The estimation of mucoprotein in gelatin was by a relatively rapid method which does not require extensive acid hydrolysis. The isolation procedure was based on Westphal (1952), in which mucopolysaccharides are separated from proteins by partition in the phenol-water system. With certain gelatins the present author found that the aqueous phase retained material giving a violet with acid diphenylamine (Dische, 1929), which shows identity from absorption curves with colours developed by serum mucoproteins and sialic acid (Werner & Odin, 1952) and urinary mucoproteins (Anderson & Maclagen, 1955).

MATERIALS AND METHODS

Monosaccharides

Commercially available products were used: D-glyceraldehyde, D-erythrose, D-galacturonic acid (L. Light and Co. Ltd.); D-glucose, D-mannose (British Drug Houses Ltd.);

L-arabinose, L-xylose, salicin (T. Kerfoot and Co.); D-glucurono-lactone (Corn Products Refining Co., New York); sedoheptulose anhydride (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.).

Collagens

Ox-bone collagen (ossein): the ossein was of the same batch as described in Courts (1958, 1959*a*). Ox-tendon collagen: tendon Achilles of Argentinian cattle was used. This is a commercially available material which has been sun-dried to about 20% moisture content. Ox-skin collagen: the skin, freshly flayed, had the hair removed by shaving but was otherwise untreated.

Gelatins

Intact gelatins: commercial gelatins were used, except where indicated; for the type of gelatin, see Table 1. Gelatins subjected to fractionation: gelatin X: this was the alkali-processed gelatin (Laboratory sample no. 127) used by Saunders & Ward (1958) and Courts & Stainsby (1958). Gelatin D: this was the acid-processed gelatin (Laboratory sample no. 111) used by Courts & Stainsby (1958).

Fractionation of gelatin

Procedure 1: this was the method of Stainsby (1954) in which a series of six fractions was prepared with ethanol as the coacervant.

Procedure 2: the method was effectively a bulk precipitation of the fractions 2-5 of procedure 1. Details are given in the preceding paper (Courts, 1959).

Estimation of mucoprotein

Partition of carbohydrate and protein. Westphal (1952) suggested that chondroitin sulphate may be isolated from cartilage by heating in a phenol-water system whereby, on cooling, the chondroitin sulphate appears in the aqueous phase and the protein in the phenolic layer. Adapting this method to gelatin, the present author found that cold aq. 50 % (w/w) phenol was a satisfactory reagent for isolating the carbohydrate moiety from a gelatin sample. The strong hydrogen-bond-breaking activity of phenol solutions liquefies and precipitates gelatin in the cold (Courts, 1958), thus obviating any heating stage and possible thermal degradation of the polysaccharides. The separation of a phenol-water-gelatin system into two phases was improved by a prior digestion of the gelatin with trypsin. The partition was carried out within the range pH 4.0-9.0.

Colorimetric estimation. The aqueous phase was examined with the acid diphenylamine reaction of Dische (1929), as used by Anderson & Maclagen (1955) for urinary mucoproteins. The colour densities were related to a mucoprotein standard, which was a preparation extracted from ox tendon by 5 % sodium dodecyl sulphate and purified by dialysis.

Reagents. (i) Gelatin: a solution of gelatin (1 g.) in water (20 ml.) was treated with 0.4 % crystalline trypsin (Armour Laboratories) (0.2 ml.) at 37° for about 2 hr. (ii) Phenol: phenol (May and Baker Ltd.) (100 g.) was dissolved in water (100 ml.) by warming and the mixture allowed to stand overnight at 20°. The upper layer was discarded. (iii) Diphenylamine: the reagent used was a solution of diphenylamine (1 g.) in acetic acid (90 ml.) and conc. H₂SO₄ (10 ml.), all of A.R. standard.

Procedure. Gelatin (20 ml.) and 20 ml. of phenol were shaken together for 10 sec. and then allowed to stand until two layers separated (1-5 hr., depending upon the gelatin). A portion (5 ml.) of the aqueous phase and 5 ml. of diphenylamine solution were mixed in a stoppered test tube and heated at 100° for 30 min.; the mixture was then allowed to cool slowly. Occasionally a white solid precipitated, which was filtrable after standing for 2-3 hr. The extinction of the violet was measured by the Spekker spectrophotometer (Hilger and Watts Ltd.) with the 604 filter (Ilford Ltd.) at 530 mμ. The colour was not affected by dissolved phenol, hexosamines, glycine, tryptophan, tyrosine, histidine, urea or thiolacetic acid.

Reproducibility is better than 5 %. The lower limit of sensitivity is about 1 part in 5000.

Absorption spectra. Extinction measurements on the diphenylamine colour complexes were made within the range 400-900 mμ in a Uvispek spectrophotometer (Hilger and Watts Ltd.). A 2 cm. cell was used.

RESULTS

The diphenylamine reagent appeared to give colour-forming reactions with two distinct classes of compounds. First, nitrates and nitrites gave a green, the absorption spectrum of which showed peaks at 405 and 800 mμ (Fig. 1). The second group,

comprising certain carbohydrates and related compounds, yielded colours for simple sugars in the range violet-blue to green, the colour depending upon the structure (see Table 3). The violet given by the reagent and showing an absorption maximum at 530 mμ appeared to be specific for mucoproteins and for sialic acid.

Fig. 1. Absorption curves of the colour complexes of diphenylamine with: —, *N*-acetylneuraminic acid; ····, acid-processed gelatin; ----, alkali-processed gelatin; - · - ·, nitric acid.

Table 1. *Mucoprotein content of different commercial gelatins and some collagens*

Source of gelatin	Pretreatment	Mucoprotein (mg./g. of gelatin)
Ox bone	Acid	15
Ox bone	Acid	19
Rabbit skin	Acid	17
Rabbit skin	Acid	17
Pig skin	Acid	13
Pig skin	Acid	9
Ox skin	Alkali	Trace
Ox skin	Alkali	Trace
Ox skin	Alkali	Trace
Ox bone	Alkali	Trace
Whale skin	Acid	1
Isinglass	Acid	1
Ox bone	Autoclaved	53
Source of collagens		
Ox bone	Demineralized	22
Ox tendon	None	5
Ox skin	None	5

A number of gelatins have been examined with the diphenylamine reagent and the mucoprotein contents are given in Table 1. Those gelatins prepared by an acid process, or by autoclaving ox bone, gave a violet. Gelatins prepared by an alkali process gave greens, generally faint in comparison with the intensities of violet.

The violet complex showed a maximum at $530\text{ m}\mu$ on the absorption curve, resembling that developed by the serum mucoproteins (Werner & Odin, 1952) and the urinary mucoproteins (Anderson & MacLagen, 1955). Gelatin D, fractionated by both the procedures 1 and 2, showed the distribution of mucoprotein given in Table 2. It was apparent that the bulk (75–80%) of the mucoprotein was concentrated into the residual fraction.

Table 2. *Distribution of mucoprotein in gelatin D fractions*

For fractionation procedures, see Courts (1959).

	Wt. of gelatin (g.)	Wt. of mucoprotein (mg.)	Concn. of mucoprotein (mg./g. of gelatin)
Unfractionated	100	1300	13
Fraction			
Procedure 1	1 3.8	49	13
	2 32.2	155	5
	3 23.3	42	2
	4 19.1	25	1
	5 15.5	25	2
	6 4.4	Not examined	
Recovery (%)	98.3	25	
Procedure 2	First 6.3	82	13
	Bulk 87.5	175	2
	Last 6.2	938	160
Recovery (%)	100	94	

The green complex developed by alkali-processed gelatins with diphenylamine gave an absorption maximum at $800\text{ m}\mu$. De-ionization of the gelatin, by the mixed-bed-resin procedure of Janus, Kenchington & Ward (1951), removed the factor able to form the green complex.

Fractionation of gelatin X (see the preceding paper, Courts, 1959) concentrated the factor into

Fig. 2. Absorption curves of the colour complexes of diphenylamine with monosaccharides. (A) General curve for C_3 – C_6 sugars; the positions and relative heights of p_1 and p_2 are given in Table 3. (B) Curve for sedoheptulose.

Table 3. *Colours and absorption maxima of the diphenylamine complex with certain compounds*

Compound	Colour of complex with diphenylamine	First peak [p_1 ($m\mu$)]	Second peak [p_2 ($m\mu$)]	Height ($p_1:p_2$)
Glyceraldehyde	Green	400	610	1:2
D-Erythrose	Green	430	620	7:1
L-Arabinose	Blue-green	490	650	1:2.4
L-Xylose	Blue-green	490	650	1:2
D-Mannose	Blue	520	650	1:1.1
D-Glucose	Blue	520	650	1:2
Salicin	Blue	520	650	
D-Glucuronic acid	Blue	500	640	1:2.4
D-Galacturonic acid	Blue	500	640	1:1.5
Glucosamine	None	—	—	
Galactosamine	None	—	—	
Sedoheptulose	Blue	—	625	
Folid acid	Green	<400	660	
Pyruvic acid	Green	<400	620	
Nitric acid	Green	405	800	
Sialic acid*	Violet	530	—	
Osseo-mucoprotein	Violet	530	—	

* The compound used was *N*-acetylneuraminic acid.

fraction 6 with the last 3% of the gelatin. Fig. 2(4) represents a general absorption curve given by carbohydrates up to C_6 . As will be seen from both Fig. 1 and Table 3, the absorption curve for the green complex showed no resemblance to a triose, tetrose, pentose, hexose or to hexuronic acid; nor did it resemble the curves produced by two other green chromophores, folic acid and pyruvic acid. The curve did, however, correspond with that given by nitric acid.

DISCUSSION

It is probable (Anderson & Maclagen, 1955; Dische, 1958) that the diphenylamine reaction for estimating mucoproteins is, in fact, dependent upon the sialic acid in association with them. The presence of sialic acid in connective tissue has not previously been reported. Other compounds are present in connective tissue, however, which could contribute to the colour density measured at $530\text{ m}\mu$, even though they do not give rise to the violet specific for sialic acid. Thus, Eastoe & Eastoe (1954) did not find free pentoses or hexoses associated with bone tissue, but consider osseo-mucoprotein to contain galactose, a small quantity of mannose, hexuronic acid, galactosamine and glucosamine. Although the hexosamines will not contribute to the colour, the hexoses and hexuronic acid will. It is difficult to obtain an assessment of the last-named compounds, and their contribution to the colour yield may be quite small. It is therefore still preferable to employ mucoprotein as the standard in the diphenylamine reaction rather than sialic acid.

Gelatins prepared by an acid process or by autoclaving retain much of the mucoprotein present in the original collagen. Alkali-processed gelatins, on the other hand, contain relatively little mucoprotein. It is likely (Courts, 1960) that the mucoprotein is almost entirely dissolved away into the alkaline-pretreatment reagents. The diphenylamine reagent therefore provides a rapid method for identifying the two types of gelatin. The chromophoric factor in the alkali-processed gelatins appears to be ionically bound nitrate (or nitrite). It is possible that the large number of free basic groups along the gelatin chains have formed ionic links with those anions made available through the tap water used in the processing. The nitrate-nitrite level in London tap water is of the order of 5 p.p.m. (Metropolitan Water Board, personal communication).

The alcohol-fractionation technique (Stainsby, 1954) has proved useful not only for fractionating gelatin by M_w , but also for removal from the bulk of the gelatin of most of the mucoprotein, inorganic nitrates, ferric salts (A. Courts, unpublished work) and probably other extraneous materials which have some solubility in the alcohol.

Ox bone is a much richer source of mucoprotein than ox tendon or ox skin, and this is most noticeable where the whole bone has been autoclaved in water. Rabbit skin appears to have a higher content of mucoprotein than ox skin.

The colours developed with diphenylamine by monosaccharides from C_3 to C_6 form an interesting family of absorption spectra in the range $400\text{--}800\text{ m}\mu$. They each show a twin-peaked curve in which position of the first peak is especially dependent on the number of carbon atoms. The absorption spectrum of a C_7 monosaccharide indicates a merger of the two peaks in a single broad peak with a shoulder.

SUMMARY

1. The carbohydrate moiety of gelatin may be separated for subsequent estimation by partition in water-phenol.
2. A fairly rapid method, this separation being used together with Dische's acid diphenylamine reagent, is described which shows marked differences between acid- and alkali-processed gelatins in their mucoprotein content.
3. The alcohol fractionation of gelatin concentrates the bulk of the non-gelatin impurities into the residual fraction.
4. The colour complexes formed by diphenylamine with monosaccharides from C_3 to C_7 show a family of absorption spectra with two peaks in the range $400\text{--}800\text{ m}\mu$. The position of the peaks is closely related to the number of carbon atoms.

I am indebted to Miss Patricia Carroll, Medical Research Council, Mill Hill, for her kind gift of *N*-acetylneuraminic acid.

REFERENCES

- Anderson, A. J. & Maclagen, N. F. (1955). *Biochem. J.* **59**, 638.
- Courts, A. (1958). *Recent Advances in Gelatin and Glue Research*, p. 145. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Courts, A. (1959). *Biochem. J.* **73**, 596.
- Courts, A. (1960). *Biochem. J.* (in the Press).
- Courts, A. & Stainsby, G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 100. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Dische, Z. (1929). *Mikrochemie*, **7**, 33.
- Dische, Z. (1958). *Chemistry and Biology of the Mucopolysaccharides*, p. 284. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
- Eastoe, J. E. & Eastoe, B. (1954). *Biochem. J.* **57**, 453.
- Janus, J. W., Kenchington, A. W. & Ward, A. G. (1951). *Research, Lond.*, **4**, 247.
- Saunders, P. R. & Ward, A. G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 197. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Stainsby, G. (1954). *Disc. Faraday Soc.* **18**, 288.
- Werner, I. & Odin, L. (1952). *Acta. Soc. Med. Upsalien*, **57**, 230.
- Westphal, O. (1952). *Z. Naturf.* **73**, 148.