In this work no fatty acid of chain length shorter than C_{16} was found, although lauric acid and myristic acid are present in significant amounts in the diet and, according to Swell & Treadwell (1955), are esterified with cholesterol at almost the same rates as stearic acid and palmitic acid. This raises doubt whether the saturated acids are esterified with cholesterol in the gut or in other sites such as the liver and blood. Cholesterol esterase activity does not appear to have been demonstrated in human liver although such activity has been shown in animals (Byron, Wood & Treadwell, 1953). On the other hand, cholesterol esterase activity has been demonstrated in human blood (Sperry, 1935, 1936; Tayeau & Nivet, 1956). It seems possible therefore that whereas the unsaturated acids are probably esterified with cholesterol in the gut, esterification of the saturated acids may take place in the blood. If saturated and unsaturated acids are esterified at different sites it may well be that the saturated and unsaturated esters of cholesterol have different functions.

SUMMARY

1. The cholesterol esters of two samples of human serum and two samples of human plasma have been separated from other lipids, by chromatography on silicic acid, and their component acids have been characterized by reversed-phase partition chromatography.
2. Unsaturated acids formed a high proportion of the total acids present.
3. The relationship between the pattern of the dietary fatty acids and the plasma-cholesterol ester fatty acids is discussed.

REFERENCES

Bloor, W. R., Blake, A. G. & Bullen, S. S. (1937). *J. Allergy*, **9**, 227.

Bollman, J. L. & Flock, E. V. (1951). *Amer. J. Physiol.* **164**, 480.
Byron, J. E., Wood, W. A. & Treadwell, C. R. (1953). *J. biol. Chem.* **205**, 483.
Clément, G., Clément, J. & Louedec, A. (1954). *Arch. Sci. physiol.* **8**, 233.
Crombie, M. L., Comber, R. & Boatman, S. G. (1955). *Biochem. J.* **59**, 309.
Fillerup, D. L. & Mead, J. F. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 574.
Fillerup, D. L. & Mead, J. F. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 449.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Glover, J., Green, C. & Stainer, D. W. (1959). *Biochem. J.* **72**, 82.
Holman, R. T. (1957). In *Methods of Biochemical Analysis*, vol. 4, p. 99. Ed. by Holman, R. T. New York: Interscience Publishers Inc.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Kelsey, F. E. (1939a). *J. biol. Chem.* **130**, 187.
Kelsey, F. E. (1939b). *J. biol. Chem.* **130**, 195.
Kelsey, F. E. (1939c). *J. biol. Chem.* **130**, 199.
Kelsey, F. E. & Longnecker, H. E. (1941). *J. biol. Chem.* **139**, 727.
Lambert, M. & Neish, A. C. (1950). *Canad. J. Res. B*, **28**, 83.
Lough, A. K. & Garton, G. A. (1957). *Biochem. J.* **67**, 345.
Milton, R. F. & Waters, W. A. (1955). *Methods of Quantitative Microanalysis*, p. 382. London: Edward Arnold Ltd.
Mueller, J. H. (1915). *J. biol. Chem.* **22**, 1.
Mueller, J. H. (1916). *J. biol. Chem.* **27**, 463.
Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **56**, 406.
Sperry, W. M. (1935). *J. biol. Chem.* **111**, 467.
Sperry, W. M. (1936). *J. biol. Chem.* **114**, 125.
Swell, L. & Treadwell, C. R. (1955). *J. biol. Chem.* **212**, 141.
Tayeau, F. & Nivet, R. (1956). In *Biochemical Problems of Lipids*, p. 365. Ed. by Popjak, G. & LeBreton, E. London: Butterworths.
Yasuda, M. (1931). *J. biol. Chem.* **94**, 401.

A Study of the Protein Impurities in Gelatins with Ion-Exchange Resins

By A. A. LEACH

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

(Received 8 May 1959)

From his work on mammalian collagens and gelatins, Eastoe (1955) concluded that the amino acid composition of a gelatin may be regarded as representative of a highly purified form of the collagen from which it was prepared. The amino acid composition of the gelatins derived from various mammalian sources is remarkably constant,

which suggests that the proportion of impurity proteins is small. Purification of the gelatin by ethanolic coacervation (Stainsby, 1955), followed by deionization with mixed-bed resins (Janus, Kenchington & Ward, 1951), was shown to produce only very small changes in composition (see Table 6). Certain of the amino acids present in large

amounts in the original gelatin increased slightly in percentage whereas those present in small amounts decreased. The variation of the most abundant amino acids, e.g. glycine, proline and hydroxyproline, suggests that at most only a few per cent of other proteins were present in the original gelatin.

The high purity of gelatin as a protein was disputed by Russell (1958), who reported the presence of two chemically contrasted protein components in lime-processed gelatins. He stated that about 15% of a second component could be isolated from lime-processed gelatins by use of the ion-exchange resin Amberlite IRC-50 (XE-97) and the chromatographic technique of Boardman & Partridge (1955). He also stated that the amino acid composition of this second component differed markedly from that of gelatin, being low in proline, hydroxyproline, glycine and hydroxylysine, and high in tyrosine; it broadly resembled that of the albumins.

This finding, if confirmed, would be of considerable interest owing to its bearing on the interpretation of much previous work, physical and chemical, on gelatins and collagens. It was therefore decided to attempt to repeat the work, to examine the influence of various factors on the eluted peak, and to examine quantitatively the amino acid composition of the eluted peaks.

MATERIALS

Gelatin no. 127. Lime-processed ox-hide gelatin, the same sample as ox-hide II, unpurified, in Eastoe (1955), whose amino acid analysis is given in Table 6. Amide content: 0.16 g. of N/100 g. of dry ash-free protein; *pI* 4.95.

Gelatin no. 142. Acid-processed bone (ossein) gelatin. Amide content: 0.50 g. of N/100 g. of dry ash-free protein; *pI* 7.8; produced commercially from demineralized bone by giving it a brief soak in lime water, followed by washing with water and soaking in dilute acid. The material was washed with water until it reached pH 5.0, when it was extracted with warm water. The short lime treatment produced a gelatin of *pI* value between the extremes for lime-processed and acid-processed gelatins.

Gelatin no. 149. Acid-processed pig-skin gelatin. Amide content: 0.62 g. of N/100 g. of dry ash-free protein; *pI* 9.4. Similar to the acid pig-skin in Eastoe (1955). The amide and *pI* values were determined by J. E. Eastoe, J. E. Long and A. L. D. Willan (personal communication).

Fractionated gelatin no. 127. Two fractions from a triple-fractionation procedure [kindly supplied by A. M. Kragh and P. R. Saunders, who also determined the limiting viscosity numbers (L.V.N.)], using alcoholic coacervation, were employed in the present work. These were gelatin 127/2/3/2, L.V.N. 86.5, and gelatin 127/4/3/4, L.V.N. 55.5: the parent gelatin had L.V.N. 62. From the relationship between M_w (weight av. mol.wt.) determined by light-scattering and L.V.N. for fractions of gelatin no. 127 determined by Courts & Stainsby (1958), the gelatins have M_w 320 000, 122 000 for the fractions and 220 000 for the original gelatin respectively. By comparison with related fractions Kragh & Saunders showed that 127/4/3/4 is

homogeneous and 127/2/3/2 rather less so, as far as behaviour in alcoholic fractionation and dilute solution viscosity (i.e. molecular weight) are concerned. The methods used for the determination of M_w by light-scattering and of L.V.N. values are described by Courts & Stainsby (1958).

Resins. The Amberlite resins IRC-50 (XE-97), nominal 200–400 mesh, and IRC-50 (XE-64), nominal 100–200 mesh, were treated in the manner described by Boardman & Partridge (1955), except that the former resin was allowed to settle for 1 hr. These resins will be referred to as XE-97 and XE-64 throughout this paper; XE-97 was the resin used unless specified otherwise.

Buffers. McIlvaine buffers (citric acid– Na_2HPO_4) were made up as described in Vogel (1957), final adjustments being made with 0.2M- Na_2HPO_4 or 0.1M-citric acid, usually the latter. For buffers in the region of pH 5.5 the buffer pH must be accurate to within ± 0.01 .

METHODS

Experiments with small columns

Preparation of columns. Before a chromatography column was poured, the resin was washed twice with about 20 times its own volume of the buffer to be used for elution. A column 16–17 cm. high and 1 cm. in diameter was poured and the resin equilibrated with a large volume of the McIlvaine buffer. During the equilibration the column shrank to about 14–15 cm. high. This column will be referred to as the small column throughout this paper. The larger columns were prepared in a similar manner.

Elution and fractions collected. The selected quantity of the gelatin, usually as a 0.5% solution in the same buffer, was added to the column, allowed to sink into the resin and rinsed in with more of the buffer. The column was then developed with the same buffer at a flow rate of about 4 ml./hr. Fractions of about 1.8 ml. were collected; 1 ml. was used for the protein analysis, leaving 0.8 ml. for dilution if necessary.

Fig. 1. Elution of 10 mg. of lime-processed gelatin no. 127 on a 14.5 cm. $\times$ 1 cm. diameter column of Amberlite IRC-50 (XE-97) resin, which was eluted at 40° with McIlvaine buffer, pH 5.55. The fraction size was 1.8 ml., 1 ml. of which was used in the Lowry, Rosebrough, Farr & Randall (1951) determination of protein. A 2 cm. cell was used.

Analysis of fractions. The fractions were analysed for protein by the Lowry, Rosebrough, Farr & Randall (1951) method. Gelatin was used as the reference protein for the preparation of a standard curve, which would lead to an overestimation of albumin-type proteins by a factor of about 2:1 (Lowry *et al.* 1951). The standards were prepared in the same buffer as that used with the column. A small volume of *N*-NaOH solution was added to 1 ml. of the fraction, before analysis, to bring the pH to about 7.0.

Typical experiment. A typical experiment was carried out at 40° with 10 mg. of lime-processed gelatin (no. 127), at pH 5.55, on a small column. It yielded 6.6% of eluted material, which appeared as a small peak (Fig. 1) at fraction 4, corresponding to a volume of 5.4 ml., i.e. close to the hold-up volume of the column. The peak was completely eluted at fraction 19, and continued elution up to 200 fractions did not elute any more material. On raising the pH with 0.05 *N*-NaOH, a large peak containing the bulk of the remaining material was eluted. This behaviour closely resembles that reported by Russell (1958). The first peak will be referred to as the eluted peak and is given as a percentage of the original material placed on the column.

Variation of procedure

Temperature. Elutions were carried out with 10 mg. of gelatin no. 127 on short columns at pH 5.52 at each of the temperatures 25°, 32.5° and 40°.

Column loading. Column loadings of 10, 50 and 100 mg. of gelatin no. 127 at pH 5.25, 5–135 mg. at pH 5.5 and 0.125–12.5 mg. at pH 5.75 were used on small columns. Some experiments employed large columns and the equivalent loading on a small column was then calculated.

Resin batch. In addition to the experiments described above with the XE-97 resin, two were carried out with small columns of XE-64 resin, which is known to differ from the former resin only in surface area per unit volume. Loadings of 2.5 and 5.0 mg. of gelatin no. 127 were used at pH 5.5.

pH. A constant loading of 10 mg. of gelatin no. 127 per small column was used with buffers pH 3.0–6.0.

Type of gelatin. Three gelatins (nos. 127, 142, 149; 10 mg. loads) were used on small columns, at pH values 5.5, 5.6 and 6.0 for each gelatin.

Molecular weight of gelatins. Loadings of 10 mg. of the fractions described earlier, on small columns at pH 5.5 and 5.6, were used.

Large-scale separations

Elutions of peaks were carried out on a preparative scale, with similar procedures as for the small columns, to give sufficient material for analysis. The slight differences in procedure are most conveniently given with the results from these experiments.

Hydroxyproline determinations. After acid hydrolysis for 24 hr. at 100° with 20% (w/w) HCl, the materials from the large-scale separations were examined for hydroxyproline content by the following methods:

(1) By noting the presence of hydroxyproline during the 100 cm. chromatographic analysis (Moore & Stein, 1951) for other amino acids. When using the citrate buffer, pH 3.42, with the normal procedure the hydroxyproline appears on the leading edge of the aspartic acid peak, and its presence is masked and can be detected only when

appreciable amounts are present. Separate determinations of hydroxyproline were carried out with citrate buffer, pH 3.19, and the 100 cm. column, under which conditions hydroxyproline travels between and separate from methionine sulfoxide and aspartic acid. Hydroxyproline could not be detected in fractions of the 0.36% material by these methods. The reduced column loadings which had to be used, the low colour yield of the hydroxyproline reaction with ninhydrin, and small variations of the buffer blanks from the column in this region made the methods rather insensitive to traces of hydroxyproline.

(2) By use of the Neuman & Logan (1950) technique, as modified by Leach (1959). The values quoted in Table 5 (columns *A*, *B*, *C*) and Table 6 (columns *C*, *D*, *E*) were determined by this method.

Amino acids. The hydrolysates were examined for amino acids by the Moore & Stein (1951) technique as described by Eastoe (1955).

Hexosamines. The hexosamines were determined on the 15 cm. Dowex columns during the analyses for basic amino acids (Eastoe, 1954).

RESULTS

It was found necessary to control the pH of the buffers used in the region of pH 5.5–6.0, at least to within ± 0.01 pH unit, in order to give reasonably reproducible yields in the first peak. The shape of the curve for percentage of material eluted/pH (at constant loading; Fig. 3) shows why this is required. The consequent alteration of the buffer ionic strength during the adjustment of the pH, as will be seen later, has its own effect. For all elutions carried out in the present investigation the position of the first eluted peak remained unchanged, i.e. close to the fraction corresponding to the hold-up volume of the column. This implies negligible adsorption of the component constituting the first peak.

Once the conditions for reproducibility had been established it became possible to examine the remaining variables.

Temperature. Elutions carried out at a constant load and pH 5.52 on the small column in three separate experiments at 25°, 32.5° and 40° gave eluted peaks of 3.6, 3.3 and 3.4% respectively. Most of the subsequent experiments were carried out at 40° to avoid possible gelation taking place in the drop-counting mechanism of the fraction-collector.

Column loading. The results obtained at pH 5.25, 5.5 and 5.75 with various loadings are given in Table 1 and Fig. 2. Variation in loadings of 10–100 mg. on the small column at pH 5.25 does not produce any marked effect on the proportion of material eluted. At higher pH values, with gelatin no. 127, the effect of increased column loading becomes apparent. At pH 5.5 a marked increase in the percentage eluted occurs above a loading of 12 mg./small column. This behaviour is confirmed

by experiments carried out on large columns of 3 cm. diameter at pH 5.5. Thus columns 13.1, 13.7 and 11.9 cm. high, with loadings of 80, 400 and 1000 mg. respectively, equivalent to small column loadings of 12, 47 and 135 mg., gave proportions

Table 1. *Variation of the proportion of gelatin eluted with column loading*

Columns were 14.0–15.0 cm. high $\times$ 1 cm. diameter.

pH	Load (mg. of air-dried material)	Concn. of gelatin solution added to column (mg./ml.)	Percentage eluted
5.25	10	5	1.4
	50	5	0.7
	100	5	0.6
5.50	5	5	1.1
	5	5	0.9
	10	5	1.1
	20	5	2.0
	20	10	2.7
	40	10	14.6
5.75	0.125 (A)*	1.25	18
	0.25 (A)	2.5	35
	0.5 (B)	2.5	39
	1.0 (C)	2.5	42
	1.0 (A)	2.5	50
	2.5 (C)	2.5	64
	2.5 (A)	2.5	70
	5 (A)	2.5	80
	8 (B)	2.5	73
	12.5 (A)	2.5	92

* Experiments with the same batch of buffer have the same letter.

Fig. 2. Proportion of gelatin no. 127 eluted from small columns of Amberlite IRC-50 (XE-97), with McIlvaine buffers, pH 5.5, as a function of column loading (mg.). $\circ$, Small columns; $\bullet$, with large column calculated to give equivalent small column load.

eluted of 1.0, 15.0 and 46.7 %. At pH 5.75 a column loading as small as 0.125 mg. still gives an increased proportion eluted, and thus the adsorption capacity of the column at this pH is very limited. The marked decrease in capacity of the resin on changing the pH from 5.25 to 5.50 to 5.75 may arise from changes both in ionization of the resin and of the gelatin in this region. The reproducibility of the results at pH 5.57 is low when different batches of buffer are used. It will be noted that the points representing experiments carried out with a single batch of buffer (A, Table 1) would fall on a smooth curve.

Resin batch. Results with small columns of XE-64 instead of XE-97 are shown in Table 2. At 3.1 % of material eluted the ratio of loadings XE-97:XE-64 was 8.8, and at 12.6 % eluted the ratio was 8.4. If the differences between the resins are only a function of surface area, then the mean-particle radii for the two resin batches would be in the ratio $\sqrt{(8.6:1)}$, approx. 3:1. In view of the nominal mesh sizes of the resins (XE-97, 200–400 mesh; XE-64, 100–200 mesh) this difference in behaviour can be explained in terms of surface area/unit volume of resin.

pH. The effect of pH variation at a constant loading of 10 mg. of gelatin no. 127, with a small column, is shown in Fig. 3. In the range pH 3.0–5.0, 0.4–0.5 % of the original material was eluted. At pH 5.5 there is a small increase in the percentage eluted, and after pH 5.5 there is a more rapid increase followed by a sharp rise in the region of pH 5.55. The slight scatter of results on the steep portion of the curve arises from buffer variations.

Type of gelatin. The results with three gelatins differing in amide N and pI are given in Table 3. The pattern of behaviour of gelatins nos. 142 and 149, which have high pI values and high amide contents, is similar to that of no. 127 but takes place in a higher pH range. Although the pI of gelatin no. 149 is pH 9.4, it is probable that in the buffer used it is isoelectric in the region pH 5.0–6.0 (Veis, Anesey & Cohen, 1958; Jackson & Neuberger, 1957). This may explain why the pH for a given percentage eluted is not raised for gelatin no. 149 to a much greater extent.

Table 2. *Comparison of the behaviour of resins XE-97 and XE-64*

Load (mg.) on XE-64 (Y)	Percentage eluted from small column (E)	Load to produce (E) with small column of XE-97* (X)	(X)/(Y)
2.5	3.1	22	8.8
5.0	12.6	42	8.4

* Calculated from Fig. 2.

Molecular weight of gelatins. The behaviour of gelatins of differing molecular weights is shown in Table 4. The eluate from the high-molecular-weight fraction at pH 5.5 is significantly smaller than the amount with the unfractionated gelatin. The

difference is probably too large to be ascribed solely to purification of the gelatin during fractionation. At pH 5.6 the low-molecular-weight fraction is much less firmly held, although poorer reproducibility at this pH limits the accuracy of the figures. Differences in molecular weight are not sufficient to account for the differences in behaviour shown by the three gelatins reported in Table 3.

Large-scale separations

Separation (a). Gelatin 127 was used on a column of XE-97 27 cm. high and 6 cm. diameter, at pH 5.0 and at 40°. The column was equilibrated with buffer containing a trace of phenylmercuric acetate as a preservative and the elution was carried out as described for small columns. A portion (720 ml.) of a 1% solution of the gelatin in the buffer was placed on the column; this is equivalent to 100 mg. on the small column. Fractions of about 200 ml. were collected and two 1 ml. portions from each were used for colorimetric determinations of protein. As judged by the method of Lowry *et al.* (1951), 0.5% of the original gelatin was eluted, i.e. 36 mg. from 7.2 g. of original gelatin. This was present in approx. 2 l. of buffer. The fractions containing the protein were dialysed in cellophan tubes for 3 days against tap water, and for a further 3 days against distilled water containing thymol, to remove buffer salts. The solution from the dialysis tubes was evaporated by boiling. As the volume of the solution reached about 200 ml. it became turbid and a white precipitate was formed. Evaporation was continued to

Fig. 3. Proportion of gelatin no. 127 eluted from small columns of Amberlite IRC-50 (XE-97), at a constant column load of 10 mg. of gelatin no. 127, as a function of the pH of the McIlvaine buffer used for elution.

Table 3. *Effect of type of gelatin on the proportion eluted*

Column loading: 10 mg. added as 0.5% solution.

Gelatin no.	pI	Amide N (g./100 g. of protein)	Percentage eluted		
			pH 5.5	pH 5.6	pH 6.0
127	4.95	0.16	1.1	19.4	93.0
142	7.8	0.50	1.3	2.2	64.0
149	9.4	0.62	0.9	1.1	30.7

Table 4. *Influence of gelatin molecular weight on percentage of material eluted*

Load: 10 mg. per small column.

Gelatin no.	M_w (mol.wt.)*	L.V.N.	Percentage eluted	
			pH 5.5	pH 5.6
127/2/3/2	350 000	86.5	0.2	18.3 18.9
127	220 000†	64.0	1.0	15.7 18.6 24.0
127/4/3/4	120 000	55.5	1.5	45 56 60 79

* From L.V.N. values, as described under Materials.

† Heterodisperse with respect to molecular weight.

50 ml. and the turbid solution was centrifuged. A white precipitate and a clear solution were obtained and these were analysed for amino acid composition separately.

The white precipitate was hydrolysed with 20% (w/w) HCl for 24 hr. at 100° in a sealed tube. The hydrolysate contained an appreciable amount of humin, admixed with what appeared to be extremely fine resin. The hydrolysate was filtered and diluted, and the acid was removed from portions in a vacuum desiccator over P_2O_5 and NaOH and analysed for amino acids.

The clear solution obtained above was made 20% (w/w) with respect to HCl and hydrolysed for 24 hr. in a sealed tube. Humin was again present, but in a much smaller quantity than that obtained above. A small amount of residue, probably resin, was also present. The analysis of amino acids was carried out as before.

The approximately 0.5% fraction, separated at pH 5.0, gave 10.36 mg. of amino acid residues (i.e. amino acids less 1 molecule of water) associated with the white precipitate, and 15.02 mg. in the clear solution. This corresponds to a total of 25.38 mg., i.e. 0.36% of the original gelatin, compared with 0.50% as judged by the method of

Lowry *et al.* (1951). This difference arises since gelatin was used as the reference standard in the Lowry method. No difficulty was experienced for the larger percentages eluted since for these, as will be seen later, gelatin made up the bulk of the eluate protein. Results of the amino acid analyses are given in Table 5.

Before studying the amino acid composition of the larger amounts eluted, reported in Table 6, it is advisable to consider the limitations of the amino acid analyses. In the absence of either sample weight or figure for N, owing to lack of sufficient material, the sum of the residue weights was used to give the weight of material placed on the Dowex columns (Moore & Stein, 1951). This procedure means that any errors in one amino acid are also spread over all the amino acids and will be most apparent in the amino acids present in large quantities. Little importance can be attached to the figure for ammonia, as this has arisen in part from decomposition of mucoproteins and mucopolysaccharides and from other degradation reactions resulting from their presence. Some of the ammonia may have accumulated during isolation procedures. There were large amounts of mucopolysaccharides-proteins in the two fractions of

Table 5. *Amino acid composition of non-gelatin components*

A, Material from 0.36% fraction (associated with resin). *B*, Material from 0.36% fraction (free from resin). *C*, 0.36% fraction (combined analysis). *D*, Impurity prepared by Maron (1958). Approximately 0.3% in original gelatin: g. of amino acid/16 g. of N determined by quantitative paper chromatography. *E*, Amino acids plus hexosamines from 'osseomucoid' (Eastoe & Eastoe, 1954): g./115 g. of amino acid + hexosamines recovered.

Amino acid	<div><div><i>A</i> <i>B</i> <i>C</i></div><div>(g. of amino acid/100 g. of dry ash-free protein)</div></div>			<i>D</i>	<i>E</i>
Alanine	12.0	10.1	10.9	9.4	5.2
Glycine	5.9	9.1	7.9	13.1	3.8
Valine	6.9	7.0	6.9	6.6	6.4
Leucine	12.3	7.7	9.6	12.3	10.3
Isoleucine	5.2	3.7	4.3		5.1
Proline	4.4	5.1	4.9		6.0
Phenylalanine	5.5	4.7	5.0	3.5	4.1
Tyrosine	2.2	2.6	2.4	4.4	2.8
Tryptophan	Not determined				
Serine*	5.3	5.2	5.2	2.6	5.1
Threonine*	6.0	5.3	5.6	2.6	5.8
Cystine	Not determined			6.5	1.6
Methionine†	3.9	Trace	1.6	Trace	1.2
Arginine	8.0	6.4	7.1	7.8	5.5
Histidine	2.2	1.6	1.9	3.0	3.6
Lysine	6.1	6.1	6.1	5.2	6.0
Aspartic acid	10.5	13.6	12.3	10.5	13.7
Glutamic acid	14.3	16.1	15.3	15.9	16.5
Hydroxyproline	0.2	1.2	0.8	1.2	0
Hydroxylysine	0.8 (?)	Trace	0.3	—	0
Hexosamines	2.4	5.3	4.1	—	12.6
Amide‡	2.5	4.9	3.9	—	1.5

* Corrected for decomposition during hydrolysis.

† Sum of methionine and methionine sulphoxide peaks.

‡ Corrected for serine and threonine decomposition.

Table 6. *Amino acid composition of gelatin no. 127 and fraction*

A, Gelatin no. 127 (Eastoe, 1955). B, Purified gelatin no. 127 (Eastoe, 1955). C, 6.6% fraction from gelatin no. 127. D, 6.6% fraction corrected for 0.36% fraction. E, 6.6% fraction corrected for 0.36% fraction on basis of relative hexosamines content.

Amino acid	Concn. (g./100 g. dry, ash-free protein)				
	A	B	C	D	E
Alanine	11.1	11.3	11.4	11.4	11.4
Glycine	27.2	27.6	23.0	23.9	25.3
Valine	2.82	2.63	3.0	2.8	2.0
Leucine	3.41	3.36	4.7	4.4	3.3
Isoleucine	1.68	1.70	2.1	2.0	1.5
Proline	16.3	16.5	14.2	14.7	15.7
Phenylalanine	2.24	2.23	2.8	2.7	2.4
Tyrosine	0.29	0.18	0.8	0.7	0.5
Tyrtptophan	Not determined				
Serine*	4.11	4.15	3.6	3.6	3.3
Threonine*	2.23	2.27	2.6	3.6	3.3
Cystine	Not determined				
Methionine†	0.81	0.82	0.3	0.2	0.1
Arginine	9.1	8.9	7.5	7.5	7.6
Histidine	0.74	0.76	0.6	0.5	0.4
Lysine	4.70	4.70	6.0	6.0	5.9
Ornithine	Trace	Trace	Trace	Trace	Trace
Aspartic acid	6.6	6.6	6.5	6.0	5.5
Glutamic acid	11.1	11.4	12.7	12.6	12.2
Hydroxyproline	14.4	14.4	13.5	14.2	15.4
Hydroxylysine	1.07	1.04	1.0	1.0	1.0
Hexosamines	—	—	0.6	—	—
Amide‡	0.16	0.08	2.2	—	—

* Corrected for decomposition during hydrolysis.

† Sum of methionine and methionine sulphoxide peaks.

‡ Corrected for serine and threonine decomposition.

the 0.36% material, eluted at pH 5.0, indicated by the humin formed and the hexosamines present.

The white precipitate obtained during the pre-hydrolysis evaporation stage and the humin-contaminated precipitate resulting from hydrolysis both had the appearance of resin. This must have arisen from a true solution, or from very finely dispersed colloidal solution, of resin in the eluting buffers, since the fractions obtained from the columns appeared perfectly clear. It has been found that, even after double pretreatments of the resin with acid and alkali, traces of the resin are still leached into the solution. These coagulate only after evaporation of the solution to a small volume or during acid hydrolysis of the solution.

Separation (b). The second large-scale experiment was carried out at pH 5.55 (the increased amount eluted being due to the increased pH) and 40° on a column of XE-97, 9.9 cm. high and 6 cm. in diameter. Gelatin no. 127 (50 ml. of 0.5% solution) was used, equivalent to a load of 10 mg. on a small column. The experiment was carried out in the normal way and 6.6% of the original material was eluted. In this experiment a precipitate did not form until the solution was hydrolysed. The hydrolysate contained a little humin together with a large amount of white precipitate, which was filtered off. The hydrolysate was analysed for

amino acids as described earlier; the results are given in Table 6.

Separation (c). In the third experiment (the increased amount eluted was due to increased loading) a 400 mg. load of gelatin no. 127 was used on a column of XE-97, 13.7 cm. high and 9.9 cm. in diameter, at pH 5.5 and 40°; this is equivalent to a load of 47 mg. on a small column. Of the original material, 15% was eluted. The fractions containing this material were dialysed, concentrated by evaporation and the protein was precipitated with a large excess of ethanol. It was then hydrolysed with 20% (w/w) HCl and analysed for total N and hydroxyproline. The small amount of white precipitate appearing during hydrolysis was filtered off. The hydroxyproline N as a percentage of the total N was 7.3%, compared with 8.5% on the original gelatin. The decrease may be due either to the presence of non-collagenous proteins or to the preferential elution of gelatin molecules containing less hydroxyproline than the remainder. If it is assumed that the effect is due entirely to non-collagenous proteins and that the hydroxyproline content of the original high-grade gelatin represents approx. 100% purity, then the eluted material represents 86% of gelatin and 14% of other proteins. That is, about 2% of the original gelatin was eluted as other protein. This figure may be too high,

as some low-molecular-weight gelatin will have been lost during the ethanolic precipitation.

Separation (d). G. Russell (personal communication) has suggested that ionic strength is an important factor in the behaviour of gelatin on the Amberlite columns. This is to be expected from the extremely critical effect of pH on behaviour demonstrated in earlier experiments. The original McIlvaine buffers had an ionic strength in terms of citric acid molarity of 0.043M for the pH 5.5 buffer; this was increased to 0.055M at the same pH.

Gelatin no. 127 (83 mg.) was used on a column of XE-97, 13.4 cm. high and 3.0 cm. in diameter. This is equivalent to a loading of 10 mg. on a small column; 24.2% of the gelatin was eluted. The fractions were dialysed, evaporated and hydrolysed, and the hydroxyproline N:total N ratio (as a percentage) was determined. This ratio was 7.3%, which is approximately equivalent to 86% of gelatin and 14% of non-gelatin protein in the eluate. This is equivalent to 3.4% of non-gelatin protein in the original gelatin. The inaccuracies in the various measurements suggest that this figure should be regarded only as indicative of a probable 2–4% of non-gelatin proteins.

DISCUSSION

The amino acid composition of the peaks obtained in separations (a) to (d) reveal the main chemical features of the separation which the XE-97 resin can effect. The small peak (0.36%) eluted from the lime-processed gelatin no. 127 has composition *C* (Table 5), made up from columns *A* and *B*. The hydroxyproline content of the eluted material indicates that not more than 5.5% of gelatin was present. The formation of humin during hydrolysis, together with the presence of a substantial quantity of hexosamine, shows that the protein is probably present as mucoproteins. These are possibly partially degraded during treatment with alkali and originate from mucoproteins of the skin.

These mucoproteins are very similar in composition and behaviour to the mucoproteins ('osseomucoid') isolated from bone collagen by Eastoe & Eastoe (1954), and to the non-collagenous proteins obtained from calf skin by Bowes, Elliott & Moss (1958). The 'osseomucoid' was extracted by Eastoe & Eastoe from bone collagen with lime water. The composition after acid hydrolysis is shown in Table 5, column *E*. The three non-collagen-type protein fractions of Bowes *et al.* obtained from calf skin were: (a) citrate-soluble protein not precipitated by sodium chloride; (b) acetic acid-soluble protein not precipitated by sodium chloride; (c) dilute sodium hydroxide-soluble protein. The extracts showed the presence of hexosamines with

the Elson & Morgan method (1933), thus indicating that they are either associated with polysaccharides or are in fact present as mucoproteins.

The 0.36% material also shows a marked resemblance to the 0.30% of protein isolated by Maron (1958) from a lime-processed gelatin by adsorption on active charcoal. The amino acid composition determined by him by quantitative paper chromatography is given in Table 5, column *D*. Maron does not record any investigation of hexosamines or mention humin formation during hydrolysis. However, the method of purification by precipitation at pH 4.0 which he describes indicates a similarity to the behaviour of mucoproteins in glues and some gelatins, which can be removed by centrifuging dilute aqueous solutions at pH 4.0 (author's unpublished observations).

The 0.36% material isolated may also be identical with the heat-coagulable protein found in gelatins by Sheppard, Hudson & Houck (1931), for the mucoproteins isolated from glues and some gelatins, as described above, are denatured very rapidly by heat and then coagulate once the protective action of the gelatin is removed (author's unpublished observations).

Separations (b), (c) and (d), in which increased proportions were eluted by increasing the buffer pH (b), by increasing the column loading (c) and by increasing the buffer ionic strength (d), show that the increased proportion eluted from the columns under these conditions can be accounted for in terms of gelatin in addition to the small amount of non-gelatin proteins eluted in the lower pH ranges. Experiment (b) indicates that 0.36% of the original gelatin has been eluted as non-gelatin protein along with about 6% of gelatin. Experiments (c) and (d) indicate the level of non-gelatin protein in the original gelatin to be 2.0–3.5%. These proportions are consistent with the work of Eastoe (1955), in which purification of gelatin by alcoholic coacervation indicates a level of impurity of this order.

The amino acid analysis of the 6.6% fraction eluted at pH 5.55 is given in Table 6, column *C* and (i) corrected for the 0.36% fraction, in column *D*, (ii) corrected for the 0.36% fraction, the ratio of hexosamine contents being used as an index of contamination, in column *E*. The material corresponding to columns *C*, *D* and *E* of Table 6 closely resembles gelatin in composition, i.e. it shows high glycine, hydroxylysine, proline and hydroxyproline contents. It is not identical in composition with the parent gelatin (Table 6, column *A*) even after the above-mentioned corrections. There are two main reasons for this: (i) the limitations of the amino acid analyses already described; (ii) it has been shown that both the *pI* and the molecular

weight of the gelatin play a part in the adsorption on the resin; materials of lowest pI and lowest molecular weight are the least strongly adsorbed. If the separations are based on either of these factors it is possible that they may be associated with regions of the collagen chain differing slightly in composition from the remainder. There must come a stage, as the collagen chain is subdivided, where local differences in composition are sufficient to affect separation procedures.

The physicochemical experiments show that none of the variables examined shifts the position of the peak in relation to the volume of eluate, which occurs close to the hold-up volume of the column. The system corresponds to a single-plate column. The pattern of behaviour of acid-processed gelatins with change of pH is similar to that of lime-processed gelatins except that it takes place in a higher pH range.

The experiments discussed do not confirm the claim of Russell (1958) that 10–19% of a second, very different, component can be isolated from lime-processed gelatins with Amberlite IRC-50, XE-97 resins. It is not possible from the results described here to suggest the origin of the discrepancy.

SUMMARY

1. The behaviour of gelatins on columns of Amberlite resin IRC-50, XE-97 and XE-64 grades has been studied.

2. The proportion of gelatin eluted has been shown to depend on column loading, buffer pH and ionic strength, surface area of resin, type of gelatin and gelatin molecular weight.

3. A small proportion (0.36%) of a protein-polysaccharide complex, probably derived from mucoproteins, has been isolated from a lime-processed gelatin under certain conditions. When the proportion of the original material eluted is increased by raising the buffer pH , the ionic strength or the column load, the increase arises from gelatin passing through the column in addition to the small amount of other protein.

4. Acid-processed gelatins, which have higher amide contents and pI values than lime-processed gelatins, behave similarly to them but in a higher pH range.

5. The non-gelatin protein isolated is similar in amino acid composition to that already reported by Maron (1958).

6. The view that lime-processed gelatins contain 10–19% of a second, very different, component is not confirmed.

The author is grateful to Mr J. Angus and Mr A. P. Williams of The British Gelatine and Glue Research Association for setting up fraction-collectors, and to the Director and staff of the Association for helpful discussions. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

REFERENCES

- Boardman, N. K. & Partridge, S. M. (1955). *Biochem. J.* **59**, 543.
- Bowes, J. H., Elliott, R. G. & Moss, J. A. (1958). *Recent Advances in Gelatin and Glue Research*, p. 71. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Courts, A. & Stainsby, G. (1958). *Recent Advances in Gelatin and Glue Research*, p. 71. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Eastoe, J. E. (1954). *Nature, Lond.*, **173**, 540.
- Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
- Eastoe, J. E. & Eastoe, B. (1954). *Biochem. J.* **57**, 453.
- Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
- Jackson, D. S. & Neuberger, A. (1957). *Biochim. biophys. Acta*, **26**, 638.
- Janus, J. W., Kenchington, A. W. & Ward, A. G. (1951). *Research, Lond.*, **4**, 247.
- Leach, A. A. (1959). *Biochem. J.* **74**, 70.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Maron, N. (1958). *Recent Advances in Gelatin and Glue Research*, p. 221. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
- Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
- Russell, G. (1958). *Nature, Lond.*, **181**, 102.
- Sheppard, S. E., Hudson, J. H. & Houck, R. C. (1931). *J. Amer. chem. Soc.* **75**, 5528.
- Stainsby, G. (1955). *Disc. Faraday Soc.* **11**, 288.
- Veis, A., Anesey, J. & Cohen, J. (1958). *Recent Advances in Gelatin and Glue Research*, p. 155. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Vogel, A. I. (1957). *Quantitative Inorganic Analysis*, p. 869. London: Longmans, Green and Co.