SUMMARY

1. Supplements of (+)-12-methyltetradecanoic acid and (+)-14-methylhexadecanoic acid to a fat-free diet did not materially alter the fatty acid composition of the depot fats of Wistar rats.

2. The weights of (+)-12-methyltetradecanoic acid and (+)-14-methylhexadecanoic acid deposited in the carcass under identical conditions of supplementation were proportional to the amounts of supplement given, and the degree of interconversion of these acids was similar, thus affording an explanation for their occurrence in the same proportions in animal fats.

3. The metabolic turnover of (+)-*anteiso*-acids was shown to be considerably slower than that of normal fatty acids.

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The N-Terminal Amino Acid Residues of Gelatin

4. CHAIN WEIGHT AND RIGIDITY RELATIONSHIP IN FRACTIONATED GELATINS*

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The relations between the macroscopic properties (solution viscosity, gel rigidity, melting point, etc.) of the gelatin-water system and the molecular size, weight, shape and chemical structure of the gelatin molecules remains as the central problem of research in this field.

Pouradier & Venet (1952) showed that an equation of the type $\eta = KM_n^\alpha$ exists between the intrinsic viscosity η (or the equivalent limiting viscosity number, LVN) and the number-average molecular weight, M_n , determined osmotically on fractions of gelatin. Whereas K and α were each the same for two alkali-processed gelatins, they were different for an acid-processed gelatin. The evidence for multichain gelatin molecules (Courts & Stainsby, 1958) introduces a further factor into considerations of molecular structure and suggests that the 'molecular-weight' values calculated from assay of N-terminal residues (Courts, 1954a, b; 1955) should be more correctly termed 'chain weight' (C_n).

Courts & Stainsby (1958) have shown that, in accordance with a multichain molecular model, there is no direct connexion between the following pairs of quantities for fractions of an alkali-

processed gelatin: (1) Weight-average molecular weight (M_w) and C_n . Thus C_n for high-grade gelatin fractions has the approximately constant value of 70 000, whereas M_w may vary from 70 000 to 350 000. (2) C_n and LVN. This was demonstrated experimentally but also must arise as an automatic consequence of the relation between M_n and LVN and (1) above, provided that reasonable homogeneity of fractions has been secured.

The picture for acid-processed gelatin fractions is not so readily interpreted. Whereas the value of C_n at 92 000 for the higher fraction is greater than in the corresponding alkali-processed series, M_w at 194 000 is somewhat lower. Since for single chains M_w is greater than C_n unless the fractions are completely homogeneous, branching must be regarded as probable rather than proved. In addition, for the lower fractions of the acid-processed gelatin, M_w is less than C_n , suggesting that some gelatin chains occur with no free α -amino groups. This could arise through blocking with some substance which the alkali process removes. The lack of end-groups in native collagen could be regarded as supporting this view. If a similar proportion of blocked groups occurred in the larger molecules it would explain the figure C_n 92 000

* Part 3: Courts (1955).

compared with 60 000 for alkali-processed gelatin fractions. Alternatively, the blocking could arise as suggested by Landucci, Pouradier & Durante (1958), who find some of the *N*-terminal groups in gelatin to be bound to certain aldehydes. The aldehydes are said to be destroyed in alkali. The absence of a formal relationship between the rigidity of a gelatin gel and LVN was suggested by Stainsby, Saunders & Ward (1954). Detailed study of degraded gelatins by Saunders & Ward (1955, 1958) showed that the low-temperature rigidity for fractions of a single gelatin is approximately constant, if the LVN is in excess of about 40, but for fractions with LVN below this figure the rigidity decreases sharply to zero. They also showed that the value of the constant rigidity obtained above LVN 40 depended on the gelatin used, and could be varied by neutral or alkaline degradation.

In view of the lack of a unique relation between rigidity and LVN (or molecular weight) it was possible that the gel rigidity might be determined by the average chain length (C_n) of component chains of the molecules. This aspect has been studied in the present work.

Methods of gelatin preparation were adopted to give a series of gelatins with marked differences in high-molecular-weight rigidity (i.e. LVN greater than 40). A partial fractionation of the gelatins, by the alcohol coacervation procedure of Stainsby (1954), was carried out to remove the especially high- and the especially low-molecular-weight fractions from a group of gelatin samples prepared from a similarly treated collagen. The rigidity, end-group and LVN values of these were then determined.

In view of the importance often attached to the interaction of mucopolysaccharides and collagen, an examination has been carried out of the mucopolysaccharide content of certain gelatins and gelatin fractions. The results showed that these substances are present as non-active impurities playing no part in the physical properties. This work is reported in the succeeding paper.

MATERIALS AND METHODS

Laboratory-prepared gelatins

The underlying principle of making gelatins with LVN greater than 40 was to subject ossein (the bone collagen used by Courts, 1958, 1960) to mild pretreatments, followed by several extractions of short duration in water at successively higher temperatures (for extraction conditions, see Table 1).

Pretreatment with calcium hydroxide. Ossein (600 g.) was treated with a suspension of $\text{Ca}(\text{OH})_2$ (250 g.) in water (4 l.) for 16 days at 20°, was then washed in running water for 8 hr., soaked in 0.01 *N*-acetic acid overnight and finally washed to pH 7.0 with tap water.

Pretreatment with sodium hydroxide. Sodium hydroxide (0.8 *N*) may replace the $\text{Ca}(\text{OH})_2$ as pretreating agent if a swelling restrainer, e.g. Na_2SO_4 , is present (Ames, 1944; A. G. Ward, unpublished work). Soaking for 2 days was used and washing was carried out as described above.

Pretreatment with sulphuric acid. The Deutsche Gelatine Fabriken (1952) process was followed. To 20% Na_2SO_4 solution (3.5 l.) containing conc. H_2SO_4 (35 ml.) ossein (700 g.) was added and allowed to stand overnight. The mixture was then rapidly heated to 80° and maintained at that temperature for 25 min.; the ossein was then immediately strained off, cooled and washed for 1 day.

Fractionation of gelatin

The method was effectively a bulk precipitation of the fractions 2, 3, 4 and 5 of Stainsby (1954). A solution of gelatin (4%) was maintained at $40 \pm 0.25^\circ$ and treated with propan-2-ol-water (90:10, v/v) to the point of slight coacervation. The coacervate (corresponding to fraction 1) was allowed to settle overnight at 40° and the supernatant poured off. The supernatant was treated at 40° with further propan-2-ol-water to precipitate the bulk of the gelatin, which was then allowed to settle at 40° for 6 hr. The supernatant (corresponding to fraction 6) was poured off and the precipitated gelatin remaining was redissolved at 80° in water (about 10 times the weight of gelatin taken). The solution was cooled from 80° to 4°, allowed to set and the gel cut and dried in a stream of cold air. The yields of this bulk fraction were mostly 50–75% of the gelatin taken.

Measurement of gelatin properties

Modulus of rigidity. This was measured by the method of Saunders & Ward (1953). Values for the modulus at 5.65% (w/w) gelatin concentration were taken at 10° and expressed in dynes/cm². The experimental work was carried out by Mr S. C. Melnick.

Limiting viscosity number (LVN). The viscosity of a 5.65% (w/w) gelatin solution was measured at 40° in a no. 2 Ostwald-type viscometer. This value was related by a standard graph to dilute solution viscosity at 40° (gelatin

Table 1. *Conditions used for the laboratory-prepared gelatins*

Treatment	Extract	Temp.	Time (hr.)	pH
$\text{Ca}(\text{OH})_2$	1	55°	1.5	9.8
	2	65	2	10
	3	75	2.5	10
	4	85	2.5	10
	5	95	2.5	10
	6	100	1	10
	7	100	1	11.5
NaOH	1	55	1	7
	2	65	2.5	7
	3	75	2.5	7
	4	85	2	7
	5	95	2	7
	6	100	0.7	7
H_2SO_4	1	55	1.5	3
	2	65	2	3
	3	75	2	3
	4	85	2	3

concentration at 2.5 mg./ml.), and the LVN was calculated from this as in Courts & Stainsby (1958).

Chain weight (C_n). The preparation of dinitrophenyl gelatin, and the isolation and measurement of *N*-terminal residues, was carried out as in Courts (1954*a*). The total (*T*) of *N*-terminal residues/100 000 g. of gelatin was used to calculate $C_n = 100\ 000/T$.

RESULTS

The physical properties of the laboratory-prepared gelatins are given in Table 2. Most of the samples gave an increased modulus of rigidity and LVN after fractionation as a result of the elimination of impurities and degraded gelatin.

Both the alkaline pretreatments used on ossein led to a series of gelatins with a range of LVN values quite suitable for the present study. The acid pretreatment did not meet this requirement and this particular study was not pursued.

The *N*-terminal residue values, before and after fractionation, are given in Table 3 for the gelatins prepared with the sodium hydroxide pretreatment, with C_n calculated from their totals. C_n has been increased by the fractionation in all instances, as would be expected. Corresponding measurements are given in Table 3 for gelatins prepared with the calcium hydroxide pretreatment and subsequently fractionated.

Table 4 shows 13 bulk-fractionated alkali-processed gelatins in declining order of rigidity with the related C_n and LVN values. There is no correlation between any two of these columns.

DISCUSSION

The 'rigidity plateau' of Saunders & Ward (1955) describes, for a series of fractions from a single gelatin, a condition of approximately constant gel rigidity with changing LVN, holding for fractions with LVN greater than 40. It seemed plausible from Table 5 that, since C_n was also approximately constant for the series of fractions giving the 'rigidity plateau', C_n could be directly related to rigidity. The testing of a relationship of this type required the preparation of a series of gelatins embodying widely varying rigidity values, all with LVN greater than 40. A similar preparative history would be an additional requirement for the gelatins. These conditions have been met by giving a short alkaline pretreatment to ossein, followed by a series of extractions in water either at pH 7.0 or a mildly alkaline pH value.

In view of the independence of rigidity and molecular weight for high-viscosity gelatins, it was not necessary to undertake close fractionation. Instead the samples were rendered more homogeneous and free of much of the impurity by removing the top 10–20% and the bottom 20–30%. This technique is successful in removing much of the low-molecular-weight protein, as shown by the general enhancement of C_n , LVN and rigidity-modulus values by the fractionation.

It is evident that the C_n -rigidity relationship, which was suggested by a limited series of fractions of a single gelatin, all at a single value of rigidity, does not hold when tested over a wide range of

Table 2. *Properties of the laboratory-prepared gelatins*

Values based on ash-free gelatin at 15% moisture content.

Treatment	Extract	Gelatin yield (% of ossein taken)	Gelatin fraction obtained (% of gelatin yield)	$10^{-3} \times$ Rigidity (dynes/cm. ²)		LVN	
				Unfractionated	Fractionated	Unfractionated	Fractionated
Ca(OH) ₂	1	4.1	37	—	94	48	48
	2	5.4	73	77	89	40	43
	3	9.4	75	70	76	48	51
	4	18.7	56	55	71	53	54
	5	35.6	60	36	51	54	62
	6	5.9	54	33	43	75	81
	7	9.2	49	16	26	43	45
NaOH	1	5.4	60	89	99	48	52
	2	19.4	65	75	95	52	53
	3	16.4	71	74	78	64	76
	4	20.2	44	66	66	75	81
	5	26.7	55	53	65	85	68
	6	6.5	50	33	49	74	82
H ₂ SO ₄	1	23	50	89	104	44	41
	2	20	50	79	95	44	59
	3	22	54	67	86	41	56
	4	12	—	—	—	—	—

Table 3. N-terminal amino acid residues and C_n of the gelatins
Values are given as moles/100 000 g. of gelatin.

	Ca(OH) ₂ process						NaOH process					
	Fractionated gelatins						Fractionated gelatins					
	1	2	3	4	5	6	1	2	3	4	5	6
Liquors ...	...	...	...	...	...	...	...	...	...	...	...	...
Glycine	0.51	0.57	0.57	0.67	0.70	0.77	0.76	0.67	0.68	0.74	0.87	0.87
Serine	0.19	0.14	0.16	0.12	0.12	0.17	0.12	0.10	0.12	0.16	0.15	0.18
Threonine	0.17	0.12	0.12	0.15	0.20	0.12	0.12	0.12	0.10	0.17	0.11	0.14
Alanine	0.13	0.07	0.08	0.11	0.07	0.08	0.08	0.06	0.07	0.10	0.09	0.07
Aspartic acid	0.10	0.12	0.10	0.12	0.12	0.13	0.11	0.09	0.07	0.09	0.12	0.08
Glutamic acid	0.14	0.18	0.10	0.09	0.08	0.08	0.09	0.07	0.17	0.09	0.14	0.12
Others	0.15	0.09	0.12	0.11	0.08	0.08	0.07	0.07	0.17	0.10	0.13	0.04
Total	1.39	1.29	1.25	1.37	1.37	1.23	1.35	1.18	1.38	1.45	1.61	1.50
$10^{-3} C_n$	72	77	80	73	73	81	74	85	73	69	62	66

Table 4. Summary of the properties of the laboratory-prepared gelatin bulk fractions

Pretreatment of ossein	Extract	Rigidity (dynes/cm. ²)	C_n	LVN
NaOH	1	99 000	74 000	52
NaOH	2	95 000	85 000	53
Ca(OH) ₂	1	94 000	72 000	48
Ca(OH) ₂	2	89 000	77 000	43
NaOH	3	78 000	73 000	76
Ca(OH) ₂	3	76 000	80 000	51
Ca(OH) ₂	4	71 000	73 000	54
NaOH	4	66 000	69 000	81
NaOH	5	65 000	62 000	68
Ca(OH) ₂	5	51 000	73 000	62
NaOH	6	49 000	66 000	82
Ca(OH) ₂	6	43 000	81 000	80
Ca(OH) ₂	7	26 000	59 000	45

Table 5. Properties of gelatin X fractions*

Fraction	Fraction yield (%)	LVN	Rigidity (dynes/cm. ²)†	C_n
2	31.2	88	63 000	55 000
3	25.3	65.5	68 000	70 000
4	21.0	51	71 000	70 000
5	18.0	40	75 000	63 000
2/2‡	8.5	92	64 000	60 000

* From Courts & Stainsby (1958).

† Calculated from Saunders & Ward (1958).

‡ Second fraction of fraction 2 refractionated.

rigidity moduli corresponding to the different 'rigidity plateaus' of a number of gelatins.

It is also clear that the absence of relationship between C_n and LVN, as shown by Courts & Stainsby (1958), is confirmed over the wide range of LVN values between 43 and 82. The C_n measurements showed only a narrow range of values, between 59 000 and 85 000, with half the samples giving C_n 71 000 $\pm$ 4 000, i.e. within the experimental error of the mean value for the series of gelatins. This type of constancy for C_n , which is similar to the value reported for a series of gelatin fractions (Courts & Stainsby, 1958), is probably accounted for by the concept of branched-chain molecules discussed in that paper. The mean value is also consistent with the 'parent gelatin' subunit of molecular weight 70 000, proposed by Gallop (1955).

No obvious relationship occurs between the rigidity modulus and LVN for the set of gelatins, as would be expected from previous work (Saunders & Ward, 1955); in fact, two gelatins with similar LVN values (43 and 45) were shown to have rigidity moduli as divergent as 89 000 and 26 000 dynes/cm.². The modulus of rigidity of a gelatin gel, with the conditions of measurement used in this paper, is therefore independent of both the molecular weight and the individual gelatin-chain dimensions.

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.

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The Distribution of Mucoprotein in Gelatins and Fractionated Gelatins

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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.