amide are discussed. It could best be explained by a kind of catalytic action of tryptophan on the nicotinamide synthesis

prove, as is claimed by these authors, that 'the conversion proceeds in the same fashion in the rat as in *Neurospora*'.

NOTE ADDED 7 APRIL 1949

Since this paper was submitted, Heidelberg, Abraham & Lepkowsky (1948) have reported feeding DL-tryptophan labelled in the β -position of the side chain with ^{14}C to rats; nicotinamide methochloride eliminated after ingestion contained a labelled Catom in the carboxylic acid group. This does not, however,

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Some Observations on the Amino-acid Distribution of Collagen, Elastin and Reticular Tissue from Different Sources

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By histological methods three types of connective tissue can be shown to be present in skins; collagen, elastic fibres and reticular tissue. Collagen, in the form of white bundles of interweaving fibres, makes up the greater part of the skin; the elastic fibres are pale yellow in colour and occur mainly in the grain layer; reticular tissue occurs around fat deposits and possibly as sheaths round the collagen fibre bundles (Dempsey, 1946). It is extremely difficult to isolate these three different tissues from skins. Tissues which stain similarly can be isolated fairly readily from other parts of the animal body: elastic fibres from the ligamentum nuchae (Vandegrift & Gies, 1901) and reticular tissue from lymph nodes (Bate-Smith, 1947) and from the large fat deposits (Maximow & Bloom, 1935; Dempsey, 1946), and some studies have been made on them. Before these tissues can be assumed to be identical with those occurring in skin, however, more conclusive evidence than staining reactions is required.

The aim of the present investigation was to determine what major differences in composition existed between the three main types of connective tissue, and to obtain information as to whether similar tissue preparations from different sources had the same composition.

EXPERIMENTAL

Analytical methods

Total N, amide N, amino N and titration curves were determined as described by Bowes & Kenten (1948a).

Preparation of samples

Ox-hide and alkali-treated sheepskin collagen were prepared as previously described (Bowes & Kenten, 1948a, b). Reticular tissue was prepared from lymph nodes and from the adipose tissue of ox. The wet lymph nodes were sliced, extracted with 5% (w/v) NaCl, washed and macerated with many changes of 30% ethanol in water. The fibrous mass was

dehydrated in 98% ethanol, extracted with light petroleum at 35° and air dried. Sheets of tissue from the fat deposit of an ox were mechanically freed from fat, extracted with light petroleum at 35°, washed with water and ethanol and dried in air.

The total N of the reticular tissues was rather lower than that of the collagen or elastin. The amide N was higher than that of elastin or of alkali-treated sheepskin collagen, and of the same order as ox-hide collagen (Table 1).

Table 1. *Analyses of connective tissues*

(Moisture and ash-free basis.)

Preparation	Total N (%)	Amide N (mmol./g.)	Amino N (mmol./g.)
Collagen (ox-hide)	18.6	0.66	0.46
Collagen (sheepskin, alkali-treated)	17.3	0.35	0.35
Elastin (ligamentum nuchae)	17.0	0.20	0.07
Reticular tissue (adipose tissue)	16.1	0.50	—
Reticular tissue (lymph nodes)	16.1	0.58	—

Elastin was prepared from the ligamentum nuchae of a freshly slaughtered ox. The tissue was freed from adhering matter, cut into approximately 0.5 cm. cubes, extracted with 10% (w/v) NaCl, washed free from salt, dehydrated with acetone and extracted with light petroleum (b.p. 40–60°) in a Soxhlet for 12 hr. The air-dried material (180 g.) was a pale yellow colour (N content 16.3%, moisture- and ash-free basis). To remove collagen, 168 g. of this material were autoclaved with 500 ml. water for 2 hr. at 120°. The supernatant liquor and the hot-water washings were combined and the total N extracted determined (see Table 2). (Increasing

Table 2. *Soluble nitrogen extracted from ligamentum nuchae*

Extractions	Nitrogen extracted	
	(% dry tissue)	(% total N)
During autoclaving	2.00	14.92
Subsequent extractions with water:		
First four	0.81	6.05
Fifth	0.066	0.49
Sixth	0.035	0.26
Seventh	0.008	0.06
Eighth	0.010	0.08
Total	4.921	21.86

the time of autoclaving to 3 hr. caused no appreciable increase in the N extracted.) The autoclaved material was given eight further extractions of 1 hr. duration with 350 ml. portions of boiling water, and the total N removed in these extractions determined (Table 2). The elastin was dehydrated with acetone, again extracted with light petroleum and air dried (132 g.). Assuming that the residual material is elastin, the ligamentum nuchae after extraction with salt and removal of fat contained about 78% elastin and 22% collagen. This is in agreement with the value of 81% elastin found by Vandegrift & Gies (1901) and later by Lowry, Gilligan & Katersky (1941) for elephant ligamentum nuchae.

The value for the total N of elastin (Table 1) is in agreement with previously determined values (Zoja (1897), 16.96; Richards & Gies (1902), 16.87; Stein & Miller (1938), 17.1%). The amide N, however, is appreciably lower than the two other values reported in the literature (Horbaczewski (1882), 0.41; Stein & Miller (1938), 0.38 mmol./g.); this is probably due to the fact that under the conditions used in earlier methods N, other than amide N, was returned as NH₃.

RESULTS

Titration curve of elastin

The titration curve of elastin with hydrochloric acid in the presence of 0.5 M-sodium chloride is given in Fig. 1, together with the corresponding curve for ox-hide collagen. The elastin shows an isoelectric point in the neighbourhood of pH 6. The curve shows

Fig. 1. Titration curves of elastin and ox-hide collagen in the presence of 0.5 M-NaCl.

a tendency to flatten between pH 2.0 and 1.5, but as the pH decreases further the amount of acid bound begins to increase again. This may be due to titration of fairly strong acid groups or of some weakly basic groups. The soluble nitrogen of the final solutions at pH values below 2.0 was only of the order of 0.5 mg./g. elastin, indicating that there was little solubilization of the protein. The curve indicates the presence of at least 0.2 mmol. basic groups per g. elastin.

Paper partition chromatography of samples

The technique described by Consden, Gordon & Martin (1944) was followed. The air-dry tissue (10 ± 1 mg.) was hydrolysed with 0.5–1.0 ml. 6N-HCl at 100° for 24–30 hr. The hydrolysate was concentrated three times *in vacuo*, dissolved in 0.2 ml. water, and 10 μl. applied to a large sheet of Whatman no. 1 filter paper. The chromatogram was run in one direction with *s*-collidine in an atmosphere of diethylamine and HCN, and in the other direction with phenol in an atmosphere of NH₃ and coal gas. The chromatograms were examined immediately after spraying with ninhydrin, and

an estimation of the amino-acids present made from the size and colour intensity of the spots (Table 3). The strongest spots were arbitrarily assigned the value 9 and those just visible in a good light 1. Proline and hydroxyproline, which give yellow and orange colours respectively, were assessed as strong (S), medium (M) and weak (W).

Qualitative analyses by two-dimensional paper chromatographs of the reticular membrane from fat deposits and of elastin from ligamentum nuchae have been reported (Jordan Lloyd, 1946). In general the results agree, except that Jordan Lloyd reports the presence of hydroxyproline in elastin; this may be due to incomplete removal of the collagen. This author also reports that reticular tissue from fat deposits contains no tyrosine or hydroxylysine, whereas these were present in small amounts in our preparations. Consideration of the figures in Table 3 shows that elastin differs from collagen and both

reticular tissue is converted to gelatin and a loose powdery substance which he terms 'reticulin' and which only slowly passes into solution. Both tissues lost approximately 50% in weight, but while the chromatogram of the lymph-node tissue was not appreciably changed, that of the reticular tissue from the fat deposits showed a definite decrease in hydroxyproline. This suggests that this reticular tissue contained some collagen, and, in confirmation, the aqueous extract obtained on autoclaving formed a gel, while a similar extract from lymph nodes did not (see also Bate-Smith, 1947). On autoclaving for 18 hr. at 105°, 73 mg. of the air-dry reticular tissue from the fat deposits gave a residue of only 6 mg. This residue resistant to autoclaving might be expected to be elastin, but the paper chromatogram showed that this was unlikely since it contained appreciably more of the di-

Table 3. *The amino-acid distribution in some preparations of connective tissues as indicated by the relative intensities of spots on paper chromatograms*

Material ... Source ... Amino-acids:	Collagen			Reticular tissues					Collagen residues	
	Ox-hide	Alkali-treated sheep-skin	Elastin Ligamentum nuchae	Lymph nodes		Fat deposits			Ox-hide	Alkali-treated sheep-skin
				Macerated	Auto-claved 4 hr.	Extracted with light petroleum	Auto-claved 3 hr.	Auto-claved 18 hr.		
Alanine	7	7	9	8	8	9	8	8	6	9
Glycine	9	9	9	9	8	9	9	8	6	9
Leucine, etc.*	5	6	9	9	9	7	7	9	9	8
Phenylalanine	2	2	3	3	3	2	2	2	3	3
Valine	5	5	8	5	5	6	6	7	7	7
Cysteic acid	1?	1?	1	1	1	1	1	1	?	1
Serine	3	3	2	5	4	5	4	4	4	4
Threonine	2	2	2	4	4	3	3	4	3	3
Tyrosine	1	1	2	3	3	1	2	1	2	2
Arginine	5	5	1	6	6	4	6	3	4	3
Histidine	2	2	—	2	2	1	1?	—	2	1
Hydroxylysine	2	2	—	1	1	2	2	—	—	—
Lysine	5	5	1	6	7	4	5	5	5	3
Aspartic acid	5	6	3	7	7	5	6	5	7	4
Glutamic acid	7	7	4	8	8	7	9	6	8	6
Proline	S	S	S	M	M	M	M	M	M	M
Hydroxyproline	S	S	—	M	M	M	W	Trace	—	—

* Leucine, etc., refers to a composite spot which may contain leucine, isoleucine, and methionine.

types of reticular tissue in containing less arginine, lysine, hydroxyproline and glutamic acid, rather less aspartic acid, and rather more valine. In addition, it differs from the reticular tissues in containing rather less serine and rather more proline. Collagen differs from the reticular tissues in containing more proline and hydroxyproline. The reticular tissue from the fat deposits contains rather less of the dicarboxylic and basic amino-acids than the lymph node tissue.

It was considered possible that the reticular tissues were contaminated with collagen. The tissues were autoclaved for 3 hr. at 105°. This should remove collagen; but according to Siegfried (1902)

carboxylic and basic amino-acids than elastin from ligamentum nuchae. It is possible that it is identical with Siegfried's 'reticulin'. Similarly, the residues (11 mg.) obtained on twice autoclaving ox-hide (2 g.) for 6 hr. at 105° followed by boiling for 1–2 hr., and the residue (30 mg.) obtained by boiling 4.76 g. limed sheepskin with 100 ml. 0.01 N-sodium hydroxide for 15 min., followed by repeated extractions with boiling water, gave paper chromatograms distinguishable from that of elastin by the presence of histidine and larger amounts of the dicarboxylic and basic amino-acids. The two residues differed slightly from one another, particularly in respect to the dicarboxylic acids, and from lymph-node reticular

tissue in containing no hydroxyproline. The absence of this amino-acid indicated the complete removal of collagen.

DISCUSSION

It is of interest to compare values given in the literature for the amino-acid composition of elastin

Table 4. *Composition of elastin*

	Amino-acid		Relative intensities of spots on paper chromatograms
	(g./100 g.)	(N as % protein N)	
Glycine	29.4 (a)	32.3	9
	25.75 (b)	28.3	
Alanine	6.85 (b)	6.3	9
Leucine fraction	30.0 (a)	18.8	9*
	21.38 (b)	13.4	
	10.7 (h)	6.7	
Valine	13.5 (a)	9.5	8
	13.8 (h)	9.7	
Phenylalanine	3.89 (b)	1.9	3
	3.34 (c)	1.7	
	4.8 (h)	2.4	
Tryptophan	0.0 (a)	—	—
Serine	—	—	2
Threonine	2.7 (g)	1.9	2
	1.1 (h)	0.8	
Cystine	0.23 (a)	0.2	(Cysteic acid=1)
	0.6 (h)	0.4	
Methionine	0.38 (a)	0.2	—
	0.03 (h)	—	
Proline	15.2 (a)	10.9	5
	15.6 (h)	11.2	
Hydroxyproline	2.0 (a)	1.3	0
Arginine	1.0 (a)	1.9	1
	0.3 (f)	0.6	
	1.1 (h)	2.1	
Histidine	0.0 (a)	0.0	0
	0.04 (h)	0.1	
Lysine	0.0 (a)	0.0	1
	0.5 (h)	0.6	
Hydroxylysine	—	—	0
Aspartic acid	0.0 (a)	0.0	3
	0.6 (b)	0.4	
Glutamic acid	2.7 (b)	1.5	4
	3.3 (h)	1.8	
Tyrosine	1.6 (b)	0.7	2
	0.25 (d)	0.1	
	0.34 (e)	0.2	
	1.4 (h)	0.6	

* Isoleucine is present.

(a) Stein & Miller (1938). (b) Abderhalden & Schittenhelm (1904). (c) Kapeller-Adler (1932). (d) Horbaczewski (1882). (e) Schwarz (1893). (f) Kossel & Kutscher (1898). (g) Brand & Kassel (1942). (h) Graham *et al.* (1949).

with the results of paper partition chromatographic examination of the present preparation of elastin (Table 4). The chromatogram indicates that the values reported for aspartic acid and alanine are

low, and that serine is present in approximately the same amount as threonine. The low figures for leucine and isoleucine obtained by Graham, Waitkoff & Hier (1949), using microbiological methods, suggest that the 'leucine fraction' of Table 4 may contain amino-acids other than leucine and isoleucine in comparatively large amounts. No hydroxyproline was detected in the chromatogram of the present preparation, and although it may not be entirely absent, the value of 2% given by Stein & Miller (1938) is possibly too high and may be due to incomplete removal of collagen. The low value for the amino nitrogen is in agreement with the small amount of lysine detected by analysis and by paper chromatography. The titration curve indicates that at least 0.2 mmol./g. of basic groups must be present in elastin; the amino nitrogen (0.07 mmol./g.), together with 0.06 mmol./g. of arginine (Stein & Miller, 1938), accounts for 0.13 mmol./g., leaving 0.07 mmol. of base to be accounted for. Histidine was not detected by paper chromatography, and consequently is present, if at all, in very small amount; it seems likely, therefore, that the discrepancy between the titration curve and analysis is due to a low arginine value.

Taking the highest figure for the 'leucine fraction' (30%) it is possible to account for approximately 90% of the total nitrogen of elastin. Judging from the chromatogram and the titration curve, about half this deficiency is due to the presence of more serine, arginine and aspartic acid than the present analytical figures suggest, leaving 5% still to be accounted for. It may be noted that Stein & Miller (1938) find that the sum of sulphur in methionine (0.08%), cystine (0.06%) and occurring as SO_4^{2-} (0.02%) agrees closely with the total sulphur of elastin (0.16%).

Comparison of the chromatograms suggests that elastin and reticular tissue from different sources may differ appreciably in composition. The residues from the exhaustive extraction of ox-hide, sheepskin and reticular tissue from fat deposits, which on the reported properties of collagen, elastin and reticular tissue, should consist mainly of elastin, do not give chromatograms similar to that of elastin from ligamentum nuchae, but, with the exception of the absence of hydroxyproline, give chromatograms more nearly resembling that of lymph-node reticular tissue. It appears, therefore, that the elastic fibres of skin are either appreciably different in composition from those of the ligamentum nuchae, or that they are less resistant to boiling water, and the residues obtained from skin consist of some other protein or proteins resembling reticular tissue in composition.

In view of the suggestions that have been made regarding the possible identity of reticulin and precollagen (Heringa & Weidinger, 1942) it is of interest

to note that with the exception of a lower proline and hydroxyproline content, the amino-acid distribution of the collagen and reticular tissue preparations are similar.

SUMMARY

1. Collagen has been prepared from ox-hide, elastin from ligamentum nuchae and reticular tissue from lymph nodes and fat deposits. Total nitrogen, and amide nitrogen have been determined and paper chromatograms of the hydrolysates examined.

2. Examination of the titration curve and chromatogram of elastin indicates that several amino-acids have still to be determined and that the reported values for arginine and alanine are low.

3. Tissues from different sources which stain similarly may vary appreciably in composition.

With the exception of a lower proline and hydroxyproline content, the amino-acid distribution of the reticular tissues was similar to that of collagen.

4. Examination of chromatograms of the residues obtained by the exhaustive extraction of skin suggests that the elastic fibres of skin either differ from those of ligamentum nuchae with respect to amino-acid composition or are less resistant to hot water.

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A Differential Method for the Detection of Small Differences in Mobility of Colloids in Electrophoresis

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The determination of differences between the mobilities of two colloids by the standard technique of measuring the boundary displacements in two separate experiments necessitates the rigorous prevention or control of movements of the boundaries due to causes other than the migration of the colloid in the electric field. The method described here has the advantage of eliminating possible complications of that kind. Its main feature is the simultaneous observation of the boundaries of two components *A* and *B*, in an electrophoresis run, in which a layer

of a solution of one component, *A* or *B*, is put on top of the mixture of *A* and *B*, the concentration of *A* or *B* being the same in both layers.

Two experiments are set up as shown in Fig. 1a, b. The boundaries are first brought into view (Fig. 1c) and, after the current has been started, are kept in position by compensation, i.e. by running buffer into the electrode vessel towards which the migration takes place. The distance between the two ascending boundaries is recorded at intervals. Experiments have also been made in which the supernatant liquid