Isolation and Chemical Characterization of Collagen in Bovine Pulmonary Tissues

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1. The contents of the fibrous proteins collagen and elastin in the pleural and parenchymal regions of bovine lungs were determined. The collagen content was approx. 70% (g/100g of salt-extracted defatted powder) in each tissue, and the elastin content was 28% in pleura and 13.5% in parenchyma. 2. Purification of the insoluble collagen from the pleura and parenchyma of bovine lungs by various methods was attempted. The collagen fractions isolated after incubation of the pulmonary tissues with the proteolytic enzymes collagenase ('collagenase-soluble' fraction) or pancreatic elastase ('elastase-insoluble' fraction) each contained approx. 87% of the total collagen initially present. 3. Both collagen fractions were chemically analysed for their amino acid and carbohydrate contents and were found to be similar to those of the intact interstitial collagens isolated from skin, bone and tendon. 4. The contents of the two aldimine cross-linking compounds, dehydrohydroxylysinoxorleucine and dehydrodihydroxylysinoxorleucine, were determined in the bovine pulmonary collagen fractions, and were found to decrease with increasing age of the animals, and were similar to the values found in intact collagens from bone and tendon.

Previous work from this laboratory on the fibrous proteins in lung has been concerned with elastin. John & Thomas (1971) reported that most of the elastin present in whole bovine lung was concentrated in the pleural and parenchymal regions. These pulmonary elastins had chemical compositions similar to the well-characterized elastins isolated from aorta and ligamentum nuchae of the same animals. John & Thomas (1972) also examined the effect of aging on the chemical compositions of elastins isolated from the aortas and pulmonary tissues of human subjects. During these various studies it became apparent that the pleura and parenchyma of lungs also contain substantial amounts of collagen and structural glycoproteins, and the present paper and that of Francis & Thomas (1975) describe the isolation and chemical characterization of these components from bovine lung.

Materials and Methods

Materials

Elastase (pancreatopeptidase, EC 3.4.21.11) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Collagenase EC 3.4.99.5; from *Clostridium histolyticum*, type III fraction 'A', pepsin (EC 3.4.23.1; 2×crystallized) and proteinase (EC 3.4.24.4; from *Streptomyces griseus*, repurified type VI, Pronase) were from Sigma (London) Chemical Co., London S.W.6, U.K. Trypsin (EC 3.4.21.4; 2×crystallized, salt-free) and α -chymotrypsin (EC 3.4.21.1)

were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. KB^3H_4 (3000mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Pure samples of hydroxylysinoxorleucine and dihydroxylysinoxorleucine were supplied by Dr. A. J. Bailey (Agricultural Research Council, Meat Research Institute, Langford, Bristol, U.K.). Pure samples of desmosine, isodesmosine, lysinoxorleucine and merodesmosine were isolated from elastin from bovine ligamentum nuchae (Thomas *et al.*, 1963; Francis *et al.*, 1973). Sephadex G-25 and G-10 were supplied by Pharmacia (G.B.) Ltd., London W.5, U.K. All other chemicals were of analytical grade and were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.

Initial treatment of bovine pulmonary tissues

The visceral pleura and parenchyma were removed from the lungs of 3-year-old cattle, scraped free of all adhering tissues and washed repeatedly with ice-cold water. The tissues were freeze-dried, cut into small pieces, mixed with solid CO_2 and powdered in a hammer-mill. The resulting fine powders were suspended in 1M-NaCl (1:500, w/v), stirred at 4°C for 24h, centrifuged at 15000g for 20min and the supernatant was discarded. Extraction with NaCl was repeated at least twice more and the residues were washed with water until salt-free. They were then defatted by dispersion in chloroform-methanol (2:1, v/v) for 24h at 4°C, filtered on a glass sinter with suction, washed successively with ethanol, acetone

and ether and finally dried *in vacuo*. The powders were chemically analysed for their nitrogen, hydroxyproline and carbohydrate contents and for their collagen, elastin and glycoprotein contents.

Attempted solubilization of collagen from bovine pulmonary tissues

The salt-insoluble and defatted powdered residues of pleura and parenchyma contained three groups of fibrous proteins, namely collagen, elastin and structural glycoproteins. Because of the insolubility of all these proteins the isolation of collagen in a pure form posed a considerable problem. Several different methods were tried to solubilize selectively either the collagen or the elastin and structural glycoproteins. These methods included treatments with concentrated solutions of urea and guanidine hydrochloride, mild alkali, mild acid and various proteolytic enzymes of different specificities.

Portions (0.5g) of the powdered residues were suspended separately in 50ml volumes of 8M-urea, 5M-guanidine hydrochloride, 0.5M-acetic acid and 0.1M-NaOH and stirred at 4° and 40°C for 48h. The suspensions were centrifuged at 15000g for 20min, the insoluble material was washed with water, and the washings were combined with the respective supernatants and passed through a glass sinter under suction. The soluble extracts were dialysed against water, concentrated on a rotary evaporator and freeze-dried. A further portion was suspended in 0.1M-NaOH and incubated in a boiling-water bath for 45min by the method of Lansing *et al.* (1952). The suspension was centrifuged and the residue washed with water at 98°C and dried with ethanol and acetone. The supernatant and washings were combined, the pH was adjusted to pH7 with HCl and the solution stored at 0°C.

For the enzyme-digestion experiments, 1g portions of the original powdered residues were incubated with enzyme (10mg) as suspensions in 100ml volumes at the appropriate pH. Pepsin was used in the presence of 0.05% acetic acid, pH3.5, trypsin, chymotrypsin and Pronase were in 0.1M-calcium acetate adjusted to pH8.0 with NH₃, collagenase was in 1mM-CaCl₂ adjusted to pH7.4 with NH₃ and pancreatic elastase was in 0.1M-(NH₄)₂CO₃, pH8.9. Mixtures were gently shaken at 40°C for 48h before centrifuging. Each residue was washed with 50ml of water and the washings and supernatants were combined and concentrated to about 20ml in a rotary evaporator at 40°C. In those cases in which Ca²⁺ ions were present these were removed by titration with oxalic acid. Precipitated calcium oxalate was separated by centrifugation and washed with small volumes of water. The supernatant and washings were combined and freeze-dried.

All the freeze-dried soluble extracts obtained by the various treatments described above were weighed and their hydroxyproline contents determined as an index of the presence of solubilized collagen.

'Collagenase-soluble' and 'elastase-insoluble' collagen fractions isolated from bovine pulmonary tissues

Analysis of the soluble fractions obtained by the various treatments described above established that the ones obtained by incubation with collagenase (designated 'collagenase-soluble') contained the highest content of hydroxyproline and hence the purest collagen (i.e. partially degraded collagen). In contrast, the soluble fractions obtained by treatment with elastase contained comparatively little hydroxyproline, but the insoluble residues (designated 'elastase-insoluble') remaining after such treatment contained hydroxyproline (and hence collagen) in amounts similar to those noted for the 'collagenase-soluble' fractions. The yield of the 'collagenase-soluble' fractions was improved by pretreating the original salt-extracted defatted residues with 5M-guanidine hydrochloride before incubation with collagenase. The 'collagenase-soluble' and 'elastase-insoluble' fractions were then selected for detailed chemical analyses.

In addition to 3-year-old animals the 'collagenase-soluble' and 'elastase-insoluble' fractions were also prepared from the pulmonary tissues of 1-week-old and 16-year-old animals.

Isolation of glycopeptides from the 'elastase-insoluble' collagen fraction from adult bovine pleura

The 'elastase-insoluble' pleural fraction (4g) was suspended in 150ml of 1mM-CaCl₂ adjusted to pH7.4 with NH₃ and was solubilized by digestion with collagenase (13mg) at 40°C. After 36h the pH was raised to 8.0 and digestion was continued by addition of Pronase (25mg) for 24h. The mixture was concentrated to a volume of 15ml on a rotary evaporator and separated on a Sephadex G-25 column (145cm×2.4cm) which was equilibrated and eluted with 0.1M-pyridine-acetate buffer, pH5.7. Fractions (10ml) were collected and portions (0.05ml) tested with ninhydrin and anthrone reagents by the method of Butler & Cunningham (1966). Two main peaks were seen; the first peak contained glycopeptides and the second peak contained only peptides. The glycopeptide-containing fractions were combined and freeze-dried. A portion (200mg) was hydrolysed with 2M-NaOH at 100°C for 24h under the conditions of Butler & Cunningham (1966). Under these circumstances the material was degraded to a mixture of glycopeptides and amino acids. The hydrolysate was desalted by passage through a small column

(10cm×0.9cm) of Dowex 50 (H⁺ form) and washing the column with water. The glycopeptides were eluted with 2M-NH₃. The eluate was concentrated on a rotary evaporator and the bulk of the free amino acids were removed by separation on a Sephadex G-10 column (75cm×1.5cm) which was equilibrated and eluted with 0.1 M-pyridine-acetate buffer, pH 5.7. Fractions (2.5ml) were collected and portions (0.05ml) tested with ninhydrin and anthrone reagents. Two main peaks were observed, the first containing glycopeptides and the second amino acids. The fractions containing glycopeptides were combined and freeze-dried. The glycopeptides were separated from residual ninhydrin-positive materials by preparative chromatography on washed Whatman no. 1 paper in the solvent system butan-1-ol-acetic acid-water (4:1:5, by vol.) for 96h. The glycopeptide material migrated as two slow-moving ninhydrin-positive spots (A and B) which were eluted from the paper with water and freeze-dried. When portions of each were applied to a Locarte amino acid auto-analyser glycopeptide A was eluted in a position close to a standard methionine sample and glycopeptide B was eluted in a position between standard samples of tyrosine and hydroxylysine. Hydrolysis of glycopeptide A in 0.2M-HCl at 100°C for 4h led to its partial conversion into glycopeptide B. Portions of glycopeptides A and B were each hydrolysed with 5.7M-HCl, after which hydroxylysine was identified and measured on the autoanalyser. Further portions were subjected to methanolic-HCl hydrolysis before analysis for galactose and glucose by g.l.c. Glycopeptide A contained hydroxylysine, galactose and glucose in the molar proportions 1:1.3:1.5. Glycopeptide B contained hydroxylysine and galactose in the molar ratio 1:1.3 and contained only trace amounts of glucose.

Preparation of intact collagens from other tissue sources

Insoluble collagens from the tendons of the tail of a 3-month-old rat and from the Achilles tendon of a 1-week-old calf were isolated as follows. The tendons were cut into small pieces, washed with ice-cold water, homogenized in 1M-NaCl (1:200, v/w) in a VirTis '45' homogenizer, centrifuged and the residues washed with water. After defatting with organic solvents as described under 'Initial treatment of bovine pulmonary tissues' the residues were dried *in vacuo*. This treatment yielded pure insoluble collagens from these tissue sources.

The leg bones were dissected from 24 1-day-old chicks and, after removal of the cartilaginous ends and the marrow, were scraped free of all other tissues. They were washed with cold water, homogenized in 1M-NaCl in a VirTis '45' homogenizer, washed free of salt and dried *in vacuo*. The powder was reduced with

KB³H₄ as described under 'Measurement of reducible cross-links in collagen' in order to stabilize collagen cross-links before the lengthy decalcification process. Calcium was removed by stirring the reduced powder (1g) with 0.5M-EDTA (100ml, adjusted to pH 7.4 with NaOH) at 4°C for 6 days, the EDTA being renewed each day. The insoluble material was recovered by centrifugation, washed with water and defatted with organic solvents as above. Insoluble collagen comprised 20% (w/w) of the bone. A section from the central shaft of the femur of a 3-year-old cow was treated in the same way after being powdered with solid CO₂ in a hammer-mill and the insoluble collagen recovered was 18.5% of the bone.

'Collagenase-soluble' and 'elastase-insoluble' fractions were prepared from the intact collagens of chick bones and of 1-week-old calf tendon by the methods described under 'Attempted solubilization of collagen from bovine pulmonary tissue'.

Analytical methods

Measurement of carbohydrate. Total carbohydrate was determined by the anthrone method of Yemm & Willis (1954). Galactose and glucose were determined by the g.l.c. method of Bhatti *et al.* (1970).

Measurement of hydroxyproline. The method of Stegemann & Stalder (1967) was used.

Measurement of nitrogen. The method was based on the use of Nessler reagent by Koch & McMeekin (1924).

Amino acid analyses. Samples (10mg in 5ml of constant-boiling HCl) were hydrolysed in evacuated sealed glass tubes. Collagen was hydrolysed for 24h and elastin for 48h. Amino acid analyses were performed on a Locarte autoanalyser by the procedure of John & Thomas (1971).

Determination of elastin in salt-extracted defatted powdered bovine pleura and parenchyma. The method of Lansing *et al.* (1952) was used. The powders were suspended in 0.1M-NaOH (1:50, v/w) in boiling-water bath for 45min and the insoluble material (elastin) was recovered by filtration through sintered glass, washed with water and after treatment with organic solvents dried *in vacuo*. The elastins were weighed and analysed.

Measurement of reducible cross-links in collagen. The cross-links measured were dehydrohydroxylysineonorleucine (Bailey & Peach, 1968) and dehydrodihydroxylysineonorleucine (Mechanic & Tanzer, 1970; Robins & Bailey, 1973). The structural formulae are shown in Scheme 1, the latter being shown in the enol and keto forms. These compounds are acid-labile because they contain aldimine bonds. However, reduction of the collagen converts them into the acid-stable hydroxylysineonorleucine and dihydroxylysineonorleucine respectively, which can then be isolated from acid hydrolysates of collagen on an auto-

Scheme 1. Structural formulae of the cross-links derived from lysine and hydroxylysine that have been shown to occur in collagen

analyser and measured with ninhydrin reagent. Because of the low content of these cross-links in collagen it was necessary to analyse 60mg amounts of collagen hydrolysates and to perform a preliminary fractionation on a resin column followed by re-fractionation of those fractions that contained the cross-links. To facilitate the localization of the cross-links during the first fractionation procedure they were radioactively labelled by using KB^3H_4 in the preliminary reduction step.

Collagen (1g) was suspended in 0.05M- Na_2CO_3 , pH 7.4 (100ml), and stirred with 30mg of KB^3H_4 (prepared by diluting radioactively labelled material with unlabelled KBH_4 to give a mixture containing 11mCi/mmol of KB^3H_4) at room temperature (19°C) for 1h. Excess of KBH_4 was destroyed by acidification with acetic acid to pH 3.0, the mixture centrifuged, the residue washed with water and dried with organic solvents. Portions of the reduced collagen (120mg) were hydrolysed in constant-boiling HCl (20ml) for 24h at 105°C. After removal of HCl on a rotary evaporator, the hydrolysate was dissolved in 0.2M-sodium citrate-HCl buffer, pH 2.2, and fractionated on a column (26cm $\times$ 0.9cm) of sulphonated polystyrene on a Locarte autoanalyser. The eluting buffer system was 0.2M-sodium citrate-HCl, pH 4.25 (for 90 min), 0.35M-sodium citrate-HCl, pH 5.28 (for 120 min) and 1.0M-sodium citrate-HCl, pH 6.65 (for 125 min). A fraction collector was attached to the bottom of the column and fractions (2.5ml; collected every 5 min) were tested for radioactivity by adding portions (0.05ml) to 10ml of scintillation liquid [0.6g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 7g of 2,5-diphenyloxazole in 750ml of toluene and 250ml of 2-methoxyethanol] and counting on a Packard Tri-Carb liquid-scintillation counter. ^3H -labelled dihydroxylysinonorleucine merged in fractions 34-37. These fractions were combined, diluted with an equal volume of water and the pH was adjusted to 2.2 with HCl. One-half of the volume was then re-fractionated on the same column but the autoanalyser and ninhydrin reagent were used to quantify the material. The eluting buffer system was 0.2M-sodium citrate-HCl, pH 4.25 (for 150 min), 0.35M-sodium citrate-HCl, pH 5.28 (for 105 min) and 1.0M-sodium citrate-HCl, pH 6.65 (for 40 min).

^3H -labelled hydroxylysinonorleucine emerged from the column during the preliminary fractionation in fractions 39-42. These were combined, diluted, acidified and one-half of the volume was re-fractionated and analysed as described above except that the eluting buffer system was that used in the preliminary column fractionation.

In the second fractionation procedure when ninhydrin was used as colour reagent both the dihydroxylysinonorleucine and hydroxylysinonorleucine moved as double peaks. Separate experiments

showed that authentic preparations of these compounds also moved as double peaks and gave ninhydrin colour yields of twice that of leucine when calculated on a molar basis. The concentrations of these cross-links in collagen were expressed as residues/tropocollagen molecule (300000g).

Detection and measurement of ϵ -(γ -glutamyl)lysine and ϵ -(γ -glutamyl)hydroxylysine cross-links in bovine pleural 'elastase-insoluble' collagen. The method of detection was that of Pisano *et al.* (1969), who examined these cross-links in fibrin. The method consisted of cyanoethylation of the collagen followed by acid hydrolysis, a procedure under which any lysine or hydroxylysine residue involved in this type of linkage would yield free lysine or hydroxylysine, whereas un-cross-linked lysine and hydroxylysine would yield the *N*-carboxyethyl derivatives, which would be eluted in different positions from lysine and hydroxylysine on an autoanalyser.

A portion (50mg) of pleural 'elastase-insoluble' collagen, which had been digested with collagenase and pronase (described above), was incubated with shaking in a mixture of 2ml of aq. 10% (v/v) triethylamine and 2ml of acrylonitrile in a stoppered tube at 37°C for 9 days. The contents were evaporated to dryness on a rotary evaporator and hydrolysed in

5.7M-HCl at 105°C for 24h in a sealed glass tube. After removal of HCl on a rotary evaporator the hydrolysate was dissolved in 0.2M-sodium citrate-HCl buffer, pH 2.2, and fractionated on a column (26cm $\times$ 0.9cm) of sulphonated polystyrene resin on a Locarte autoanalyser. The eluting buffer was 0.35M-sodium citrate-HCl, pH 5.28, and fractions were collected from the bottom of the column at 4min intervals. Those fractions eluted where standard lysine and hydroxylysine had previously been found to be eluted were combined separately, diluted with water and their pH was adjusted to 2.2 with HCl. They were then re-fractionated on the same column, being eluted with 0.35M-sodium citrate-HCl, pH 5.28, and the lysine and hydroxylysine were measured with ninhydrin reagent.

Results

Analysis of initial salt-extracted defatted powdered bovine pulmonary tissues

The analyses for pleural and parenchymal powders prepared from a 3-year-old animal are shown in Table 1. Elastin contains less than 2% (w/w) of hydroxyproline and it was calculated that only a

Table 1. *Analysis of initial salt-extracted defatted powdered bovine pleura and parenchyma*

The results are expressed in g/100g of tissue powder. Glycoprotein concentrations were those reported by Francis & Thomas (1975).

Tissue source	Nitrogen	Carbohydrate	Hydroxyproline	Collagen	Elastin	Glycoproteins
Pleura	16.6	0.94	9.76	68.0	28.0	4.0
Parenchyma	16.3	4.10	9.10	72.0	13.5	15.5

Table 2. *Attempted solubilization of collagen from salt-extracted defatted pleura and parenchyma of 3-year-old cattle*

Results for parenchyma are in parentheses. For further details see the text.

Treatment	Time of treatment (h)	Temperature (°C)	Material solubilized (g/100g)	Hydroxyproline solubilized (g/100g)
8M-Urea	48	4	1.0	0.5
	48	40	3.3 (4.2)	2.6 (0.7)
5M-Guanidine hydrochloride	48	4	1.5	1.0
	48	40	6.5 (16.0)	1.9 (4.2)
0.5M-Acetic acid	48	4	1.2	0.9
	48	40	4.7 (1.4)	3.4 (0.2)
0.1M-NaOH	48	4	1.0 (7.0)	0.7 (0.7)
	48	40	42.5 (39.5)	41.2 (31.5)
	0.75	98	71.9 (86.5)	95.0 (96.0)
	72	40	59.9 (61.0)	88.0 (87.0)
Collagenase	72	40	10.8 (29.1)	6.4 (6.2)
Chymotrypsin	72	40	9.9 (31.7)	7.5 (7.6)
Trypsin	72	40	46.4 (10.5)	39.8 (1.3)
Pepsin	72	40	48.0 (51.5)	28.4 (20.5)
Pronase	72	40	38.2 (39.0)	10.0 (12.3)
Pancreatic elastase	18	40		

negligible amount (3–5%) of the total hydroxyproline in the powders was due to elastin. Since collagen is the only other animal protein known to contain hydroxyproline (about 13.5%), the collagen present in the powders could be determined by measuring hydroxyproline. It was established that the contents of collagen in the pleural and parenchymal powders were 68 and 72% respectively.

Attempted solubilization of collagen from bovine pulmonary tissues

The results of attempts to solubilize the collagen from the salt-extracted defatted powdered pleura and parenchyma prepared from 3-year-old animals are presented in Table 2. Incubations at 4°C for 48 h in solutions of concentrated urea or guanidine hydrochloride, dilute acid or dilute alkali dissolved little hydroxyproline-containing material (hence collagen). Apart from dilute alkali, incubations at 40°C extracted only slightly more collagen and structural glycoproteins. Incubations in 0.1M-NaOH at 98°C for 45 min dissolved all the collagen and structural glycoproteins and left insoluble elastin.

The results of digestion with proteolytic enzymes (Table 2) can best be considered in the light of the observations of Thomas & Partridge (1960) that pure elastin is not solubilized by treatments at 40°C with chymotrypsin, trypsin, pepsin or collagenase but is solubilized by pancreatic elastase and pronase. Bacterial collagenase has been shown by Mandl *et al.* (1964) to dissolve collagen, and the other proteinases listed above have been shown (e.g. by Drake *et al.*, 1966) to have only limited solubilizing effects on collagen. Francis & Thomas (1975) have reported that all the above proteinases other than collagenase solubilized the structural glycoproteins present in pulmonary tissues.

Table 2 shows that digestion of the salt-extracted defatted pleura and parenchyma with each of the above proteinases had various solubilizing effects on the hydroxyproline-containing material (hence collagen). Collagenase solubilized about 87% of the total collagen present and did not affect elastin or the structural glycoproteins. Chymotrypsin and trypsin dissolved only small amounts of collagen but dissolved the structural glycoproteins. Since pepsin has no action on elastin the material solubilized by this enzyme from pleura was a mixture of collagen (40% of total) and structural glycoproteins. In the case of parenchyma, pepsin had little solubilizing effect on collagen but dissolved part of the structural glycoproteins. Pronase dissolved the elastin, the structural glycoproteins and between 20 and 28% of total collagen in both tissues. Since pancreatic elastase is known to dissolve elastin and structural glycoproteins, and since only about 11% of total collagen was

solubilized, most of the collagen remained in the insoluble residue after treatment with this enzyme.

In summary, no single treatment was capable of isolating the total collagen, either in a soluble degraded form or in an insoluble intact form. However, the fractions obtained which seemed to possess the highest concentrations of collagen (as judged from hydroxyproline measurements) were the 'collagenase-soluble' and the 'elastase-insoluble' fractions. These fractions contained about 87% of the collagen initially present. The collagen present in the 'elastase-insoluble' fraction was still only sparingly soluble when stirred in solutions of 5M-guanidine hydrochloride or in 0.5M-acetic acid at room temperature for 24 h.

Chemical compositions of the 'collagenase-soluble' and 'elastase-insoluble' collagen fractions isolated from bovine pulmonary tissues

Fractions prepared from salt-extracted defatted pleura and parenchyma of 3-year-old animals had the amino acid and carbohydrate compositions shown in Table 3. The main features of the composition are the high contents of glycine, proline, alanine and hydroxyproline, the presence of hydroxylysine and the low content of carbohydrate. These analyses are very similar to those reported by Eastoe (1967) for the fibrillar collagens isolated from bone, skin and tendon. The great bulk of the carbohydrate present in the pulmonary fractions was identified as galactose and glucose. Glycopeptides were isolated from the pleural 'elastase-insoluble' fraction which were similar to those isolated by Butler & Cunningham (1966) and Cunningham & Ford (1968) from skin collagen and by Spiro (1969) from tendon collagen. These glycopeptides contained the monosaccharide galactose and the disaccharide glycosylgalactose, each of which was linked by an *O*-glycosidic bond to the hydroxyl group of a hydroxylysine residue.

The compositions of the 'collagenase-soluble' and 'elastase-insoluble' collagen fractions indicated that these fractions were substantially free from elastin and structural glycoproteins. However, the presence of trace amounts of desmosine, isodesmosine, mero-desmosine and lysinonorleucine (known cross-linking compounds present in elastin) and galactosamine indicated slight contamination with elastin and structural glycoproteins.

Contents of 'reducible cross-links' in 'collagenase-soluble' and 'elastase-insoluble' collagen fractions isolated from bovine pulmonary tissues

It was pointed out under 'Analytical methods' that the reducible cross-links of dehydrohydroxy-lysinonorleucine and dehydrodihydroxylysinonorleucine were stabilized to acid hydrolysis by reduction

Table 3. *Compositions of 'collagenase-soluble' and 'elastase-insoluble' collagen fractions prepared from 3-year-old cattle*

Results are expressed as residues/1000 amino acid residues.

	'Collagenase-soluble'		'Elastase-insoluble'	
	Pleura	Parenchyma	Pleura	Parenchyma
Hyp	117.5	95.0	113.0	120.0
Asp	45.2	56.5	45.9	47.7
Thr	15.6	21.0	15.2	17.6
Ser	32.6	43.7	24.6	34.1
Glu	65.0	86.4	74.3	73.0
Pro	114.5	107.0	115.0	99.0
Gly	325.0	307.0	331.0	333.4
Ala	106.5	100.0	110.0	93.5
Val	27.4	17.1	24.4	22.4
Met	5.9	6.3	6.5	7.1
Ile	13.8	11.7	14.4	15.8
Leu	24.2	33.0	24.6	28.3
Tyr	5.3	9.3	4.5	6.4
Phe	12.7	16.1	13.6	15.8
Hyl	7.7	8.7	8.7	7.4
His	5.4	6.5	7.0	6.8
Lys	25.2	27.6	24.5	25.4
Arg	47.6	47.4	43.3	45.3
Total	1000.1	1000.3	1000.5	999.0
Carbohydrate (g/100g)	1.30	2.52	0.94	2.21

Table 4. *Contents of reducible cross-links in pulmonary collagen fractions and intact collagens isolated from various tissues of animals of different ages*

Pulmonary collagen fractions ('elastase-insoluble' and 'collagenase-soluble') were prepared from reduced salt-extracted defatted pleura and parenchyma of bovine lungs. Results for parenchyma are in parentheses. Intact insoluble collagens were isolated from bones and tendons. Concentrations of dihydroxylysinoxonleucine and hydroxylysinoxonleucine are expressed as residues/300 000g collagen.

Tissue source	'Elastase-insoluble'		'Collagenase-soluble'	
	Dihydroxylysinoxonleucine	Hydroxylysinoxonleucine	Dihydroxylysinoxonleucine	Hydroxylysinoxonleucine
Bovine pleural and parenchymal fractions prepared from animals aged:				
1 week	0.19 (0.38)	0.15 (0.29)	0.29 (0.36)	0.21 (0.24)
3 years	0.07 (0.05)	0.13 (0.10)	<0.05 (<0.05)	0.19 (0.16)
16 years	<0.05 (<0.05)	0.10 (0.09)	<0.05 (<0.05)	0.14 (0.15)
Intact insoluble collagens isolated from:				
1-day-old chick bones	1.85	0.30		
3-year-old cow bone	0.44	0.21		
1-week-old calf tendon	0.08	0.34		
3-month-old rat tendon	0.05	0.41		

of the salt-extracted defatted pleural and parenchymal powders with KB³H₄ before digestion with enzymes. Figs. 1(c) and 1(d) show the distribution of ³H after the preliminary separation on a resin column of acid hydrolysates of 'elastase-insoluble' and 'collagenase-soluble' pleural fractions prepared from a 3-year-old animal. Also included in Fig. 1 are the radioactivity

patterns given by comparable pleural fractions prepared from a 1-week-old animal (Figs. 1a and 1b) and from a 16-year-old animal (Figs. 1e and 1f). The main radioactive components at peaks 3 (dihydroxylysinoxonleucine) and 4 (hydroxylysinoxonleucine) seen in the traces of both fractions from the 1-week-old animal decreased with increasing age of

Fig. 1. Distribution of ${}^3\text{H}$ in acid hydrolysates of reduced collagen fractions from bovine pleura of animals of different ages. Salt-extracted defatted pleural powders from bovine lungs of 1-week-old, 3-year-old and 16-year-old animals were reduced with KB^3H_4 , the 'elastase-insoluble' and 'collagenase-soluble' collagen fractions prepared, hydrolysed with acid and separated on a sulphonated polystyrene column. (a) 'Elastase-insoluble' and (b) 'collagenase-soluble' fractions from 1-week-old animal. (c) 'Elastase-insoluble' and (d) 'collagenase-soluble' fractions from 3-year-old animal. (e) 'Elastase-insoluble' and (f) 'collagenase-soluble' fractions from 16-year-old animal. The positions of amino acids are (1) tyrosine, (2) phenylalanine, (3) dihydroxylysine, (4) hydroxylysine, (5) hydroxylysine, (6) lysine, (7) 'Fraction C' (histidinohydroxymerodesmosine), (8) lysine.

Fig. 2. Distribution of ${}^3\text{H}$ in acid hydrolysates of reduced intact collagens from bone and tendon

The insoluble collagens from decalcified defatted bones from chicks (1 day old) and cattle (3 years old), and from salt-extracted defatted tendons from calf (1 week old) and rat (3 months old) were reduced with KB^3H_4 , hydrolysed with acid and separated on a sulphonated polystyrene column. (a) Chick bone; (b) bovine bone; (c) rat tendon; (d) bovine tendon. Positions of amino acids are (1) tyrosine, (2) phenylalanine, (3) dihydroxylysinonorleucine, (4) hydroxylysinonorleucine, (5) hydroxylysine, (6) lysinonorleucine, (7) 'Fraction C' (histidinohydroxymersodesmosine), (8) lysine.

the animal, showing that the concentrations of these reducible cross-links decreased on aging. A similar decrease was observed in the collagen fractions isolated from the parenchyma of the same animals.

Measurements of the reducible cross-links by using ninhydrin (see Table 4) established that the contents in both collagen fractions prepared from pleura and parenchyma decreased with increasing age of the

animals. The total concentration of both cross-links in the fractions was about 0.5 residue/300000g in a 1-week-old animal, 0.2 residue/300000g in a 3-year-old animal and 0.1 residue/300000g in a 16-year-old animal. The concentration of dehydrohydroxylysino-norleucine was less affected by aging than that of dehydrodihydroxylysino-norleucine, the latter being hardly detectable in old animals. No detectable amounts of hydroxylysino-norleucine or dihydroxylysino-norleucine occurred at any age in pleural or parenchymal collagen fractions which had not been reduced with KBH_4 . Small amounts of lysino-norleucine were observed in the fractions, but the concentration did not exceed 0.05 residue/300000g in any fraction.

Contents of reducible cross-links in intact collagens isolated from bone and tendon

Fig. 2 shows the distribution of ^3H after separation on a resin column of acid hydrolysates of insoluble KB^3H_4 -reduced intact collagens isolated from different types of bone and tendon. All the collagens examined contained the radioactive components at peaks 3 and 4 and therefore contained both aldimine cross-links. However, there was a difference in the relative proportions of the components depending on the type of collagen. Thus component 3 (dihydroxylysino-norleucine) was the main component in bone collagens isolated from chicks (Fig. 2a) and cattle (Fig. 2b), and component 4 (hydroxylysino-norleucine) was the main component in tendon collagens isolated from rat (Fig. 2c) and calf (Fig. 2d). Component 7 seen in the radioactive trace of rat tail-tendon collagen was first observed by Bailey *et al.* (1970) and was called 'Fraction C', but was later identified as histidinohydroxymerodesmosine by Tanzer *et al.* (1973).

The concentrations of the reducible cross-links measured with ninhydrin in these collagens are included in Table 4. Apart from chick bone collagen (total of 2 residues/300000g) the other tissue collagens had total concentrations of about 0.5 residue/300000g. No lysino-norleucine or other elastin cross-linking compounds were observed in any collagen sample.

In order to test whether any reduction *in vivo* of the aldimine bonds of the reducible cross-links had occurred in these collagens, attempts were made to measure the concentrations of dihydroxylysino-norleucine and hydroxylysino-norleucine in acid hydrolysates of collagens that had not been reduced with KBH_4 . Neither compound could be detected.

The concentrations of dehydrohydroxylysino-norleucine and dehydrodihydroxylysino-norleucine (measured in reduced forms with ninhydrin) in the 'collagenase-soluble' and 'elastase-insoluble' collagen

fractions prepared from chick bone and 1-week-old calf tendon were very similar to the concentrations in the intact collagens of both tissues. The 'collagenase-soluble' and 'elastase-insoluble' fractions contained 80–85% of the total insoluble collagen initially present in each tissue. This recovery of collagen was similar to that obtained for the same fractions isolated from pulmonary tissues.

Occurrence of ϵ -(γ -glutamyl)lysine and ϵ -(γ -glutamyl)-hydroxylysine cross-links in the bovine pleural 'elastase-insoluble' collagen fraction

After cyanoethylation of 'elastase-insoluble' collagen, followed by acid hydrolysis, variable amounts of lysine and hydroxylysine were detected, but the amounts of each did not exceed 0.15 residue/300000g of collagen. In view of these small amounts and the possibility that not all the free ϵ -amino groups of lysine and hydroxylysine had reacted with acrylonitrile the presence and importance of ϵ -(γ -glutamyl)lysine and ϵ -(γ -glutamyl)hydroxylysine cross-links in collagen is doubtful.

Discussion

Methods were examined for isolating collagen in a pure form from the salt-extracted defatted visceral pleural and parenchymal regions of the lungs of 3-year-old cattle. The collagen content of both tissues was about 70% (w/w), the remaining material being identified as elastin and structural glycoproteins. The great insolubility of these three classes of proteins presented difficulties when trying to isolate pure collagen from pulmonary tissues. The methods used were based on the selective solubilization of either the collagen or of elastin and structural glycoproteins. Methods involving treatments with reagents which would not be expected to break covalent bonds were unsuccessful. Other methods involved digestion with proteinases of different specificities, and of the enzymes studied collagenase and pancreatic elastase produced the purest forms of collagen. Digestion of pulmonary tissues with collagenase solubilized about 87% (w/w) of the total collagen, whereas digestion with pancreatic elastase had a limited solubilizing effect on collagen and about 87% of the total collagen remained insoluble. The 'collagenase-soluble' and 'elastase-insoluble' materials were substantially free from elastin and structural glycoproteins, and chemical analyses showed them to be collagenous in nature. The amino acid and carbohydrate compositions of these collagen fractions were more similar to those of the interstitial collagens found in bone, skin and tendon (see Eastoe, 1967) than of the basement membrane collagens found in glomeruli (Kefalides,

1967). They possessed lower hydroxyproline, hydroxylysine and carbohydrate contents than the basement membrane collagens and the pleural 'elastase-insoluble' fraction was shown to contain both galactose and glucosylgalactose units each linked by an O-glycosidic bond to the hydroxyl group of a hydroxylysine residue, unlike the basement membrane collagens in which the carbohydrate occurs almost exclusively in the form of disaccharide units. Also, unlike basement membrane collagens, the pulmonary collagens contained no half-cystine.

The pulmonary collagen fractions were shown to contain the reducible cross-links dehydrohydroxylysinonorleucine and dehydrodihydroxylysinonorleucine. When expressed as residues of cross-link/300000g of collagen the values in young cattle were 0.5, in mature cattle 0.2 and in old cattle 0.1. The values for bone collagens in 1-day-old chicks was 2 residues and in adult cattle 0.7 residue/300000g of collagen. The comparable value for calf tendon and 3-month-old rat tendon was about 0.4. Robins *et al.* (1973) have suggested that the above reducible cross-links are the most recently synthesized cross-links in collagen and during maturation of animals are converted into more stable ones of unknown identity without an increase in the total. One possibility is that the aldimine bonds are reduced *in vivo*. This is known to occur during the maturation of elastin where the aldimine cross-links dehydrolysinonorleucine and dehydromerodesmosine are converted into lysinonorleucine and merodesmosine. However, in the present work there was no evidence that any *in vivo* reduction of aldimine cross-links occurred in collagen isolated from various mammalian tissue sources.

Tanzer & Mechanic (1970) have reported that reduced preparations of calf skin collagen contained lysinonorleucine. In the present study trace amounts of this compound together with traces of desmosine, isodesmosine and merodesmosine were detected in the pulmonary collagen fractions. It has been established that all these compounds are cross-linkers in elastin (Francis *et al.*, 1973) and since pulmonary tissues contain elastin it was concluded that their presence in pulmonary collagen fractions arose from contamination with elastin.

Another type of lysine-derived cross-link, ϵ -(γ -glutamyl)lysine, has been shown to occur in fibrin during blood clotting (Pisano *et al.*, 1969) and in hair medulla protein (Harding & Rogers, 1971). This cross-link and the analogous ϵ -(γ -glutamyl)hydroxylysine were detected in such small amounts in pleural collagen that they were regarded as artifacts without real significance in the cross-linked collagen structure.

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