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Biosynthetic Pathway of Desmosines in Elastin

By GILLIAN FRANCIS, RHYS JOHN and JOHN THOMAS
Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, U.K.

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1. Elastins purified by various methods from the ligamentum nuchae and the lungs of cattle of various ages were analysed for amino acid compositions and lysine-derived cross-links. 2. In fully mature elastins from adults the main cross-links were desmosine, isodesmosine and lysinonorleucine and the so-called aldol-condensation product. Trace amounts of merodesmosine were also found. 3. During the normal maturation of elastins the amounts of desmosine, isodesmosine and lysinonorleucine increased, whereas the aldol-condensation product and intact lysine residues decreased and merodesmosine remained the same. 4. Elastins from young animals contained significant amounts of dehydromerodesmosine whereas elastins from adults contained virtually nil. Evidence is presented which suggests that the biosynthetic pathway of desmosine and isodesmosine proceeds via the aldol-condensation product and dehydromerodesmosine.

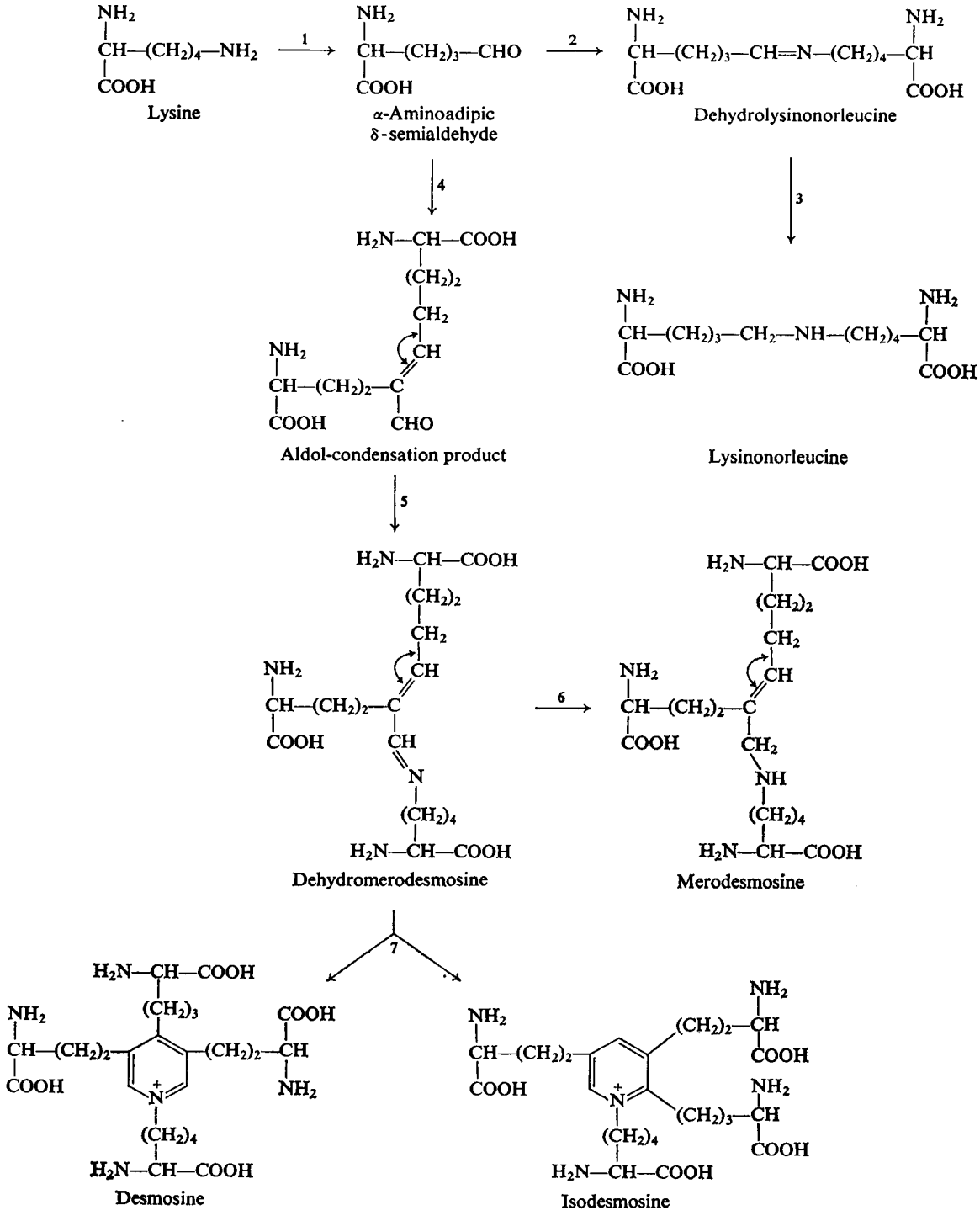
Elastin is a rubber-like insoluble protein present in mammalian connective tissues. There is evidence that the elastic fibres of these tissues are formed by aggregation of soluble protein subunits in the extracellular space (Sandberg *et al.*, 1969). The extreme insolubility of elastin is due to a considerable extent to the formation of covalent cross-links between groups on the side chains of lysine residues which lie in close proximity in adjacent polypeptide chains.

The first two cross-linking compounds to be isolated from elastin were desmosine and isodesmosine (Thomas *et al.*, 1963), and later other lysine-derived, but chemically simpler, cross-linking compounds were discovered. The structure of these compounds and the possible routes of their biosynthesis from lysine residues are presented in Scheme 1.

It is generally agreed that the key step in the biosynthesis of all these lysine-derived cross-links is the oxidative deamination of the ϵ-amino groups of selected lysine residues to yield residues of α-aminoadipic δ-semialdehyde (reaction 1, Scheme 1; Miller *et al.*, 1967). These aldehydic residues can then participate in Schiff-base reactions with ϵ-amino groups of other lysine residues or in aldol condensations with other similar aldehydic residues. Thus the cross-linking compound lysinonorleucine first isolated from acid hydrolysates of elastin by Franzblau *et al.* (1965) was shown to be a dicarboxylic diamino acid produced by the reduction *in vivo* of a Schiff-base precursor (dehydrolysinonorleucine) formed by the interaction of a residue of the semialdehyde with a lysine residue (Scheme 1, reactions 2 and 3). Similarly, the product derived from the aldol condensation of two semialdehyde residues, the so-called aldol-condensation product (see reaction 4, Scheme 1), was isolated as the corresponding alcohol derivative from alkaline hydrolysates of elastin that had been first treated with the reducing agent $NaBH_4$ (Lent *et al.*, 1969).

A complex compound derived from three lysine residues was detected in trace amounts in alkali-treated elastin by Starcher *et al.* (1967). This compound, merodesmosine, is probably formed from dehydromerodesmosine, an acid-labile compound resulting from the interaction of the aldol-condensation product of reaction 4 and the ϵ-amino group of a third lysine residue. This interaction (Schiff-base formation) can be catalysed in the laboratory by hot alkali and the subsequent reduction of the product with $NaBH_4$ yields acid-stable merodesmosine (see Scheme 1, reactions 5 and 6) which can then be isolated from acid-hydrolysed elastin. The possibility has therefore existed that the merodesmosine detected in elastin hydrolysates by Starcher *et al.* (1967) was an artifact arising from the experimental techniques employed. However, Paz *et al.* (1971) have subsequently reported the presence of both merodesmosine and dehydromerodesmosine in elastin isolated without exposure to alkali and reduction. It therefore seems probable that both compounds occur naturally in the elastin molecule.

The most complex of the elastin cross-linking compounds are desmosine and isodesmosine. Both are isomeric pyridinium compounds derived from the cyclization of the side-chain groups of four lysine residues. At the present time the biosynthetic route to these isomers is not clearly defined. A number of pathways are possible and the one suggested by Starcher *et al.* (1967), based on the interaction of



Scheme 1. *Structures of lysine-derived cross-links in elastin showing their possible synthesis from lysine molecules*
In elastin the α-amino and α-carboxyl groups form peptide bonds.

dehydromerodesmosine with a fourth lysine residue in the form of semialdehyde (reaction 7), is shown in Scheme 1. Unfortunately there is as yet little experimental evidence to support this pathway.

The present work was undertaken to throw light on the pathway of biosynthesis of the desmosines. Elastin in the mature animal is apparently turned over extremely slowly, if at all (Slack, 1954). If the type of biosynthetic pathway suggested in Scheme 1 is valid, it follows that as elastin molecules mature the number of lysine residues should diminish as more and more of them become involved in the formation of cross-links. It also follows from Scheme 1 that as the cross-links become more complex with maturity, so that larger amounts of the isomeric desmosines make their appearance, so also will the proportion of cross-links of lower complexity (i.e. based on two or three lysine residues) decrease. Determination of the number of lysine residues and the number and types of cross-linking compounds in elastin from animals of increasing maturity should therefore throw light on the precise biosynthetic route to the isomeric desmosines. A number of different sources of elastin have been employed in the study and four different isolation methods (of differing chemical severity) have been used to lessen the likelihood of artifacts resulting from isolation procedures.

Experimental

Purification of elastin

Elastins were isolated from the ligamentum nuchae and the visceral pleura of foetal, 1-week-old, 2–3-year-old and 10–12-year-old cattle. Elastins were also isolated from the aorta of cattle, chicks, humans and rats and from the visceral pleura and the parenchyma of the lungs of cattle and humans. The purification methods were based on the great insolubility of elastins and involved the removal of all other substances from the tissues. The procedures involved an initial extraction of the tissues with salt solution and organic solvents. The residues were then extracted by one of four procedures which involved either autoclaving or treatment with collagenase or formic acid or hot alkali (see John & Thomas, 1971). The insoluble materials remaining after the different treatments were taken as pure elastins.

Isolation of merodesmosine from acid hydrolysates of non-reduced and reduced elastins

The ligamentum nuchae from 1-week-old calves (total of 48) were cut into small pieces and homogenized in 1M-NaCl in a VirTis 45 homogenizer to give a creamy suspension. The residue remaining after centrifuging was extracted twice more with 1M-NaCl. The insoluble material was then washed repeatedly with water, defatted by suspending successively in chloroform–methanol (2:1, v/v), alcohol and acetone, and finally dried *in vacuo* to give a fine powder. Non-reduced elastin was extracted from a portion (200g) of this powder by treatment with hot 0.1M-NaOH (Lansing *et al.*, 1952), and a further portion (200g) was first reduced with KBH_4 before extraction with hot alkali. Reduction was performed by suspending the powder in 0.2M-Tris–HCl buffer, pH7.2 (2.5litres), adding KBH_4 (10g) with stirring and leaving overnight. Excess of KBH_4 was destroyed by acidifying with acetic acid and insoluble material was recovered by centrifuging and washed with water. The material was then extracted with hot alkali (Lansing *et al.*, 1952) to give reduced elastin.

Merodesmosine was extracted from non-reduced and reduced elastins (100g of each) by suspending in 5.7M-HCl (2.5litres) and refluxing in an atmosphere of N_2 for 24h. HCl was removed by repeated evaporation and the hydrolysate was dissolved in the minimum volume of water, cooled to 0°C and insoluble material removed by centrifuging. To the supernatant was added 0.2M-sodium citrate–HCl buffer, pH2.2 (6litres), and the solution was applied to a column (40cm×5cm) of Zeo-Karb 225 [8% cross-linked DVB (divinyl benzene; Permutit Co. Ltd., London W.4, U.K.); Na^+ form: 100–200 mesh]. The column was washed with 0.2M-sodium citrate–HCl buffer, pH4.25 (9.5litres) to elute the acidic, neutral and aromatic amino acids. Washing was then continued with 3.5litres of 0.35M-sodium citrate–HCl buffer, pH5.28, the first 2.5litres being discarded, and the remaining 1litre contained merodesmosine contaminated with desmosines, lysinonorleucine and lysine. This latter fraction was enriched in merodesmosine by lowering the pH to 2.2 with HCl, adding water (1litre) and passing the whole volume through a column (150cm×2.4cm) of Zeo-Karb 225 (8% cross-linked DVB; Na^+ form). The column was then washed with 0.35M-sodium citrate–HCl buffer, pH5.28 (1.5litres) and merodesmosine emerged in the next 750ml. This fraction was further enriched in merodesmosine by refractionation on the same column after the pH had been lowered to 2.2 with HCl. The column was developed with the pH5.28 buffer, fractions (20ml) were collected and merodesmosine was located by applying portions to an amino acid autoanalyser. The pooled merodesmosine fractions were desalted by passing through a column filled with sufficient Zeo-Karb 225 resin (8% cross-linked DVB; H^+ form) to remove all cations. After the column had been washed with water the merodesmosine was displaced with 0.2M-NaOH. From 100g amounts of non-reduced and reduced elastins 17mg and 72mg of merodesmosine were obtained respectively.

Amino acid analyses

Elastin samples (10mg in 5ml of constant-boiling HCl) were hydrolysed at 110°C for 72h in evacuated sealed glass tubes. Amino acid analyses were carried out on the Locarte Autoanalyser by the procedure of John & Thomas (1971).

Measurement of lysine-derived cross-links in elastin

Desmosine, isodesmosine, lysinonorleucine and merodesmosine. These were determined on the Locarte Autoanalyser by applying samples (4mg) of an acid hydrolysate of elastin to a 26cm column of sulphonated polystyrene resin. The region between the aromatic and basic amino acids was expanded by using an eluting buffer system of 0.2M-sodium citrate–HCl, pH 4.25 (80min), 0.35M-sodium citrate–HCl, pH 5.28 (65min) and 1.0M-sodium citrate–HCl, pH 6.65 (110min). The ninhydrin colour yields of desmosines was taken as four times, lysinonorleucine as twice and merodesmosines as three times that of leucine calculated on a molar basis.

Dehydromerodesmosine. As a routine this was measured by converting it into the acid-stable merodesmosine by reducing the elastin with KBH_4 before extraction and acid hydrolysis. The merodesmosine was then measured by using ninhydrin reagent on an amino acid autoanalyser as described above. The elastic tissues were extracted with salt, defatted with organic solvents, suspended (1g) in 0.5M-Tris–HCl buffer, pH 7.2 (100ml), and KBH_4 (50mg) was added. The suspension was stirred at room temperature for 2h, excess of KBH_4 destroyed by acidification with acetic acid to pH 3.0, the mixture centrifuged, and the elastin was washed with water to remove all salts and dried with organic solvents. Portions of reduced elastin (250mg) were purified by the above four procedures. Purified reduced elastins (10mg) were hydrolysed in 5.7M-HCl and amino acids and merodesmosine was measured on the autoanalyser. The dehydromerodesmosine concentration was the amount of merodesmosine recovered in excess of that present in non-reduced elastins.

Dehydrolysinonorleucine. This was determined in a manner analogous to that used for dehydromerodesmosine, by measuring the excess of lysinonorleucine in reduced elastin as compared with non-reduced elastin.

Aldol-condensation product. This was measured as the alcohol derivative in alkaline hydrolysates of elastin by the procedure of Lent *et al.* (1969). Reduced elastins (30mg), prepared by the four different extraction procedures described above, were hydrolysed in 2M-NaOH (2ml) at 105°C for 22h in stoppered polypropylene bottles. The hydrolysates were adjusted to pH2.2 with 2M-HCl, diluted to 20ml and 2ml samples were applied to a 26cm × 1.4cm resin column on the Locarte Autoanalyser. The eluting buffer system was 0.2M-sodium citrate–HCl, pH 3.25 (65 min), 0.2M-sodium citrate–HCl, pH3.8 (85 min), and 0.2M-sodium citrate–HCl, pH4.25, containing sufficient NaCl to give a 0.6M solution with respect to Na^+ (100min). The reduced aldol-condensation product gave a ninhydrin-positive peak emerging between leucine and tyrosine. The ninhydrin colour yield of the aldol-condensation product was taken as twice that of leucine calculated on a molar basis.

Reduction of elastin with KB^3H_4

Radioactive borohydride (from The Radiochemical Centre, Amersham, Bucks., U.K.) was prepared by diluting KB^3H_4 with non-radioactive KBH_4 to give a mixture containing 11 mCi/mol of KBH_4. Salt-extracted and defatted elastin (1g) was reduced with the borohydride mixture (50mg) as described previously and the reduced elastin was purified by the hot-alkali procedure. Portions of elastin were hydrolysed with 5.7M-HCl and others were hydrolysed with 2M-NaOH. The acid hydrolysates were concentrated to dryness on a rotary evaporator and dissolved in 0.2M-sodium citrate–HCl buffer, pH2.2. The alkaline hydrolysates were adjusted to give a final Na^+ concentration of 0.2M. The acid and alkaline hydrolysates (2mg) were each fractionated on a 26cm × 1.4cm resin column on the Locarte Autoanalyser by using the buffer system 0.2M-sodium citrate–HCl, pH3.25 (40min), 0.2M-sodium citrate–HCl, pH 4.25 (80 min), 0.35M-sodium citrate–HCl, pH5.28 (70 min) and 1.0M-sodium citrate–HCl, pH6.65 (100min). A fraction collector was attached to the bottom of the column and fractions were collected every 3min. Fractions were tested for radioactivity by adding portions (0.1ml) to 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. The components present in radioactive peaks were identified by combining similar fractions, adding an equal volume of water, adjusting the pH to 2.2 with strong HCl and separating on an autoanalyser, amino acids being located with ninhydrin reagent.

Results

Amino acid compositions of elastins purified by different methods

The elastin in ligamentum nuchae is readily purified compared with that of other mammalian tissues. Table 1 shows the similarity in the overall contents of amino acids in elastins extracted from

Table 1. *Amino acid compositions of elastins isolated by various methods from the ligamentum nuchae and visceral pleura of adult cattle*

The methods included the use of alkali, autoclaving, enzymes and formic acid. Results are expressed as amino acid residues/1000 residues.

	Ligamentum nuchae				Visceral pleura				
Isolation method ...	Collagenase	Autoclaved	Formic acid	Alkali	Collagenase	Collagenase+trypsin	Autoclaved	Formic acid	Alkali
Hyp	10.5	11.0	9.0	9.6	9.0	9.8	9.7	10.1	9.5
Asp	7.1	7.3	5.4	5.4	15.6	6.5	10.3	6.4	6.2
Thr	8.0	8.1	7.5	6.3	11.7	7.6	9.4	7.9	6.5
Ser	6.7	6.9	5.6	5.3	9.9	7.1	8.0	7.4	6.5
Glu	14.4	15.7	13.9	15.5	27.4	15.2	20.7	17.1	16.6
Pro	125.0	120.0	122.5	116.0	108.5	121.6	128.0	124.0	117.0
Gly	338.0	334.0	343.0	334.0	314.0	334.6	332.0	335.0	340.0
Ala	220.0	224.0	224.0	223.0	198.5	205.4	212.0	216.0	221.0
Val	140.0	142.5	144.0	145.8	131.5	141.5	139.0	141.0	145.0
Ile	26.0	25.4	24.5	27.6	27.0	28.1	25.3	26.0	25.9
Leu	58.4	60.8	57.7	64.0	70.0	65.8	62.0	61.0	62.4
Tyr	7.2	6.5	6.0	7.5	9.5	9.6	5.5	7.2	6.6
Phe	28.8	27.5	27.6	32.4	29.2	32.4	24.0	29.2	28.9
Lys	3.9	3.8	3.5	3.5	15.5	7.4	6.9	4.8	4.5
Arg	6.8	6.9	6.4	4.3	23.4	7.6	7.7	7.4	4.2
Total	1000.8	1000.4	1000.6	1000.2	1000.7	1000.2	1000.5	1000.5	1000.8

the ligaments of 2–3-year-old cattle by the four different methods of increasing severity described above. These analyses were also virtually identical with those for insoluble elastins isolated from foetal, 1-week-old and 12-year-old cattle by the same methods.

The elastin in the visceral pleura of cattle was more difficult to purify (John & Thomas, 1971). To obtain pleural elastins that were similar in composition to ligamental elastins it was necessary to use formic acid or alkali (Table 1). Autoclaving gave elastins that were very different. A tryptic digestion after collagenase treatment gave elastins that approximated to those of ligament.

Occurrence of merodesmosine and dehydromerodesmosine in elastin

Amino acid chromatograms of acid hydrolysates of ligament elastin contained the well-established cross-links isodesmosine, desmosine and lysinonorleucine. However, these chromatograms also contained an unknown ninhydrin-positive substance which travelled, on an expanded amino acid chromatogram, in a position between desmosine and lysinonorleucine (Fig. 1). This unknown substance was present in elastins isolated by different extraction procedures from a variety of tissues from

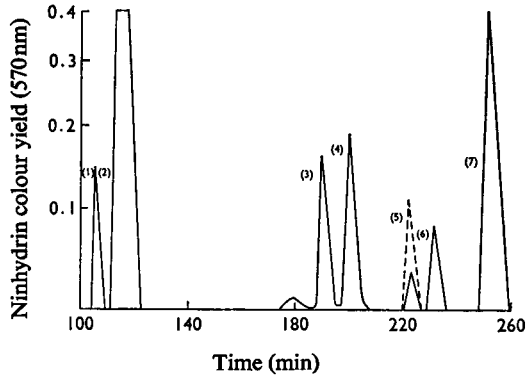


Fig. 1. *Position of merodesmosine on a chromatogram from an amino acid autoanalyser*

An acid hydrolysate of elastin (4mg sample) purified by formic acid extraction of calf ligamentum nuchae is shown. The dashed line shows the increase in merodesmosine concentration in elastin reduced before purification. The region between the aromatic and basic amino acids has been expanded. Peaks are (1) tyrosine, (2) phenylalanine, (3) isodesmosine, (4) desmosine, (5) merodesmosine, (6) lysinonorleucine and (7) lysine.

different animal species. These included the aortas, ligaments and lungs (visceral pleura and parenchyma) of cattle and humans, and the aortas of chicks and rats.

The possibility existed that this unknown substance was merodesmosine, the cross-link isolated by Starcher *et al.* (1967) from acid hydrolysates of bovine ligament elastin that had been treated with hot alkali before reduction with KBH_4. The unknown substance was isolated in a reasonable amount (17mg/100g of elastin) from acid hydrolysates of alkali-purified calf ligament elastin that had not been reduced. The behaviour of this unknown substance on different separation procedures was compared with that of merodesmosine isolated from acid hydrolysates of elastin that had been reduced before alkali extraction (yield 72mg/100g of elastin). The unknown substance and merodesmosine moved in the same positions on paper electrophoresis [0.1% aqueous $(NH_4)_2CO_3$, pH8.7 at 50V/cm for 1h in a Locarte High Voltage Electrophoresis Apparatus], on paper chromatography (butan-1-ol–pyridine–9M-ammonia, 1:1:1, by vol.) and on ion-exchange chromatography (amino acid autoanalyser). The chromatograms were developed with ninhydrin reagent. In each of these

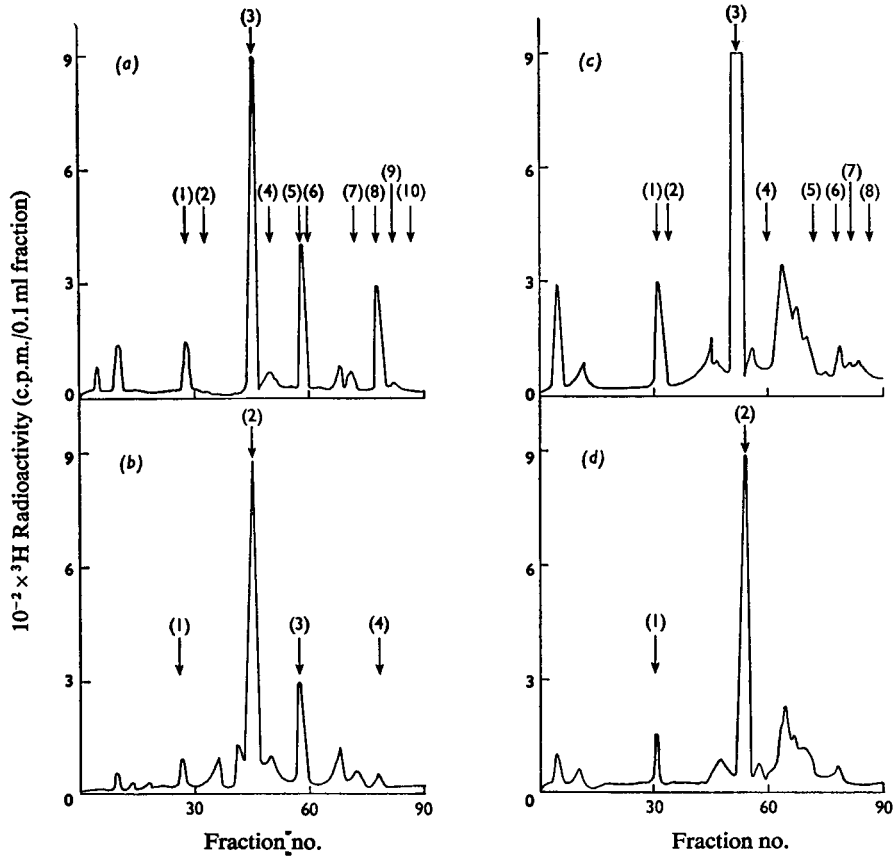


Fig. 2. *Distribution of ^3H in hydrolysates of reduced elastin from young and adult animals*

Elastins from the ligamentum nuchae of 1-week-old and 3-year-old cattle were reduced with KB^3H_4, purified with formic acid and samples (2mg) hydrolysed with acid and alkali and separated on a sulphonated polystyrene column. (*a*) Acid-hydrolysed elastin from young animals; peaks (1) ε-hydroxynorleucine, (2) glycine, (3) degraded aldol, (4) isoleucine, (5) ε-chloronorleucine, (6) tyrosine, (7) isodesmosine, (8) merodesmosine, (9) lysinonorleucine, (10) lysine. (*b*) Acid-hydrolysed elastin from adults; peaks (1) ε-hydroxynorleucine, (2) degraded aldol, (3) ε-chloronorleucine, (4) merodesmosine. (*c*) Alkali-hydrolysed elastin from young animals; peaks (1) ε-hydroxynorleucine, (2) glycine, (3) leucine+aldol, (4) tyrosine, (5) isodesmosine, (6) merodesmosine, (7) lysinonorleucine, (8) lysine. (*d*) Alkali-hydrolysed elastin from adults; peaks (1) ε-hydroxynorleucine, (2) leucine+aldol.

three different separation procedures the unknown substance and merodesmosine moved in identical positions and were free from other ninhydrin-positive materials. It was concluded that the unknown substance was merodesmosine. The higher yield of merodesmosine recovered from the reduced calf ligament elastin could be explained by the presence of the reducible precursor, dehydromerodesmosine, in calf elastin. It will be shown below that the amount of dehydromerodesmosine depended on the age of the animal, being high in the young and very low in the adult. The presence of dehydromerodesmosine and merodesmosine in elastins, purified without exposure to alkali, from different tissues, suggested that both compounds were normal constituents of elastin. The ninhydrin colour yield of merodesmosine was about three times that of leucine calculated on a molar basis. By using this as a measure of merodesmosine concentration the contents in the above tissues were estimated at about 0.25 residue/1000 amino acid residues in elastins isolated from the tissues listed previously.

Studies on acid and alkaline hydrolysates of elastin reduced with KB^3H_4

Calf and adult ligamentum nuchae were extracted with salt and organic solvents, reduced with KB^3H_4 and purified by hot-alkali extraction. Portions of the tritiated elastins were hydrolysed with either acid or alkali, fractionated by ion-exchange chromatography and fractions were collected and tested for both radioactivity and ninhydrin colour yield. When acid hydrolysates of elastins from calf were examined a prominent radioactive merodesmosine peak appeared (Fig. 2*a*). This peak was virtually absent in similar hydrolysates of elastins from adult (Fig. 2*b*). In hydrolysates of elastins of both ages other tritiated peaks were present, which could be identified by consideration of analogous experiments of Lent *et al.* (1969) and by reference to the patterns of radioactive peaks obtained when the elastins from calf and adult were hydrolysed with alkali (Figs. 2*c* and 2*d*). In acid hydrolysates the pre-glycine and pre-tyrosine peaks could be identified (by reference to the work of Lent *et al.*, 1969) as ε-hydroxynorleucine and ε-chloronorleucine respectively. In alkaline hydrolysates the chloro derivative was absent whereas the radioactive peak corresponding to ε-hydroxynorleucine increased. The large radioactive pre-isoleucine peak which appeared in acid hydrolysates (Figs. 2*a* and 2*b*) arose from the degradation of the reduced aldol-condensation product. This peak disappeared on alkaline hydrolysis and was replaced by that of the undegraded alcohol derivative of the aldol-condensation product, which occupied the position of leucine (Figs. 2*c* and 2*d*). This component could be separated from leucine and other amino acids by combining similar fractions and separating them on the autoanalyser by using the buffer system described in the appropriate Experimental section and developing with ninhydrin reagent. The tritiated ninhydrin-positive reduced aldol appeared only on chromatograms of alkaline hydrolysates of elastins that had been reduced before extraction.

Identical patterns of ^3H uptake were also seen in both acid and alkaline hydrolysates of tritiated elastin that had been purified by the autoclaving, enzymic and formic acid procedures.

The distribution of radioactivity in acid hydrolysates of tritiated elastin obtained in the present work differed in some respects from those obtained by Lent *et al.* (1969). Thus little uptake of ^3H by the desmosines was observed. This could be attributed to the mild reducing conditions used ($NaBH_4$/elastin ratio 1:20, w/w). Also in the present study little radioactivity was associated with lysinonorleucine, which under the fractionating conditions used was well separated from merodesmosine.

Proportions of lysine-derived cross-links in elastins extracted from the ligamentum nuchae of cattle of different ages

Elastins from cattle which included foetal, 1-week-old, 3-year-old and 12-year-old animals were purified by the usual methods. The concentrations of the various cross-links shown in Table 2 were determined on acid and alkaline hydrolysates. Irrespective of the purification procedure, the proportions of the individual cross-links were similar for a particular age. Since all these cross-links were derived from lysine residues any changes in their concentrations should be reflected by corresponding changes in the number of unmodified lysine residues. The most significant changes in concentrations during normal maturation of the animals occur in the desmosines, dehydromerodesmosine, aldol-condensation product and lysine residues.

For desmosine plus isodesmosine, from the mean values for the different extraction procedures, the concentration begins at 1.6 residues/1000 amino acid residues in elastin from foetuses, increases to 2.3 in the 1-week-old calf and reaches a maximum of 3.1 in the fully mature animal, a value that is maintained in the 12-year-old animal. In contrast the concentration of dehydromerodesmosine decreases with growth, being about 0.8 residue/1000 amino acid residues in the young and decreasing to trace amounts in the fully mature and old animals. For merodesmosine a low value of about 0.2 residue/1000 residues is present at all ages. Similarly, the content of lysinonorleucine (about 0.75 residues/1000 residues) shows little change with maturation. Dehydrolysinonorleucine is detectable in elastin from young animals (about 0.16 residue/1000 residues)

Table 2. *Concentrations of cross-links and lysine residues in elastins isolated by various methods from ligamentum nuchae of cattle of increasing age*

The animals were foetal, 1 week old, 3 years old and 12 years old. Purification methods involved alkali, autoclaving, collagenase and formic acid treatments. Concentrations are in residues/1000 amino acid residues. When cross-links are expressed as lysine equivalents desmosine and isodesmosine each equal four, dehydromerodesmosine and merodesmosine each equal three, and dehydrolysinonorleucine, lysinonorleucine and aldol-condensation product each equal two lysine residues.

	Foetal			1 week old				3 years old				12 years old		
Isolation method ...	Alkali	Autoclaved	Formic acid	Alkali	Autoclaved	Collagenase	Formic acid	Alkali	Autoclaved	Collagenase	Formic acid	Alkali	Autoclaved	Formic acid
Isodesmosine	0.73	0.82	0.75	1.06	1.09	1.06	0.89	1.26	1.31	1.29	1.28	1.16	1.18	1.19
Desmosine	0.79	1.01	0.81	1.37	1.30	1.29	1.15	1.74	1.73	1.82	1.75	1.70	1.75	1.76
Dehydromerodesmosine	0.76	0.62	0.71	0.91	0.79	0.85	0.81	0.04	0.03	0.04	0.10	0	0	0
Merodesmosine	0.18	0.17	0.18	0.22	0.26	0.22	0.23	0.22	0.22	0.19	0.19	0.21	0.23	0.24
Dehydrolysinonorleucine	0.22	0.13	0.15	0.10	0.07	0.15	0.25	0.04	0.03	0.02	0.02	0.08	0	0
Lysinonorleucine	0.78	0.66	0.50	1.14	0.83	0.69	0.61	0.87	0.85	0.84	0.85	0.86	0.82	0.80
Aldol	3.44	3.43	3.20	2.92	2.76	2.90	2.93	2.15	1.86	2.00	1.95	1.70	1.60	1.47
Lysine	9.10	10.80	9.00	6.10	7.00	8.50	5.90	3.50	3.80	3.90	3.50	2.30	2.60	3.00
Total lysine	26.88	28.93	25.61	27.53	27.03	28.59	24.76	22.40	22.19	22.75	22.13	19.65	19.85	20.06

but almost disappears in mature elastins from adults. Significant changes also occur in the concentration of the aldol-condensation product. Elastin from a foetus contains about 3.4 residues/1000 residues, decreases to 2.9 in the calf, to 2.0 in the adult and to 1.6 in the old animal. Changes also occur in content of lysine residues, there being 9.6 residues/1000 residues in elastin from a foetus, 6.9 in calf, 3.7 in the fully grown animal and 2.6 in the old animal. When all the lysine-derived cross-links are expressed as lysine equivalents and intact lysine residues are included in the total, the mean of the total lysine residues recovered from elastins extracted by the different methods decreased with age, being 27 residues for foetal, 28 for 1-week-old calf, 22 for the mature animal and 20 for the 12-year-old animal.

Effect of preincubation of ligament elastin in alkaline solution on the contents of lysine-derived cross-links

Treatment of salt-extracted and defatted elastins from 1-week-old and 3-year old cattle with alkali, before reduction and purification by various methods, produced an insoluble elastin in which the pattern of cross-links was different from that obtained for non-alkali-treated elastin. Studies at different pH values (10.6, 12.4 and 13.0) and times of incubation (1h and 2h) showed that elastic tissue kept at room temperature in 0.05M-NaOH (pH12.4) for 1h produced the most significant changes in the amounts of cross-links (see Table 3). Elastin from young animals that had been alkali-treated in this way contained slightly more desmosine, isodesmosine and lysinonorleucine than did the non-alkali-treated, but no increase was found in elastins from mature animals. However, significant changes were found in dehydromerodesmosines, aldol and lysine contents, especially in elastins from calf. Taking the average values of elastins purified by the various methods, preincubation in alkali produced a 2.5-fold increase in dehydromerodesmosine to 2.18 residues/1000 amino acid residues, decreased the aldol-condensation product by one-half to 1.54 and the lysyl residues from 6.3 to 5.3.

Preincubation with alkali also had an effect on the mature elastins, the dehydromerodesmosine increasing from an average of 0.03 to 0.51 residue whereas the aldol-condensation product decreased from 2.0 to 1.7 and lysine residues from 3.9 to 3.5.

Amounts of lysine-derived cross-links in elastins extracted from the visceral pleuras of cattle of different ages

Elastins were purified by the formic acid and alkali methods from the pleuras of cattle aged 1 week, 3 years and 12 years. The amounts of lysine cross-

Table 3. *Effect of incubation in alkaline solution before reduction and purification on the concentrations of cross-links and lysine residues in elastin*

The alkaline conditions were 0.05 M-NaOH for 1 h at room temperature. Elastins were purified from ligamentum nuchae of 1-week-old and 3-year-old cattle by alkali, autoclaving and formic acid treatments. Concentrations are expressed in residues/1000 amino acid residues and the total lysine equivalents as in Table 2. The control values from Table 2 are given in parentheses.

	1 week old			3 years old		
Isolation method ...	Alkali	Autoclaved	Formic acid	Alkali	Autoclaved	Formic acid
Isodesmosine and desmosine	2.52 (2.43)	2.64 (2.39)	2.34 (2.04)	2.89 (3.00)	2.92 (3.04)	2.84 (3.03)
Dehydromerodesmosine	2.24 (0.91)	2.06 (0.79)	2.15 (0.81)	0.52 (0.04)	0.45 (0.03)	0.56 (0.10)
Merodesmosine	0.22 (0.22)	0.26 (0.26)	0.23 (0.23)	0.22 (0.22)	0.22 (0.22)	0.19 (0.19)
Lysinonorleucine	1.15 (1.24)	0.91 (0.90)	0.86 (0.86)	0.74 (0.91)	0.95 (0.88)	0.87 (0.87)
Aldol	1.59 (2.92)	1.50 (2.76)	1.52 (2.93)	1.85 (2.15)	1.70 (1.86)	1.65 (1.95)
Lysine	5.50 (6.10)	5.90 (7.00)	4.60 (5.90)	3.60 (3.50)	3.60 (3.80)	3.40 (3.50)
Total lysine	28.44 (27.53)	28.24 (27.03)	25.86 (24.76)	22.56 (22.40)	22.59 (22.19)	22.05 (22.13)

Table 4. *Concentrations of cross-links and lysine residues in elastins isolated from visceral pleuras of cattle of different ages*

The animals were aged 1 week, 3 years and 12 years. Purifications involved alkali and formic acid methods. Concentrations are in residues/1000 amino acid residues and lysine equivalents are calculated as in Table 2.

	1 week old		3 years old		12 years old	
Isolation method ...	Alkali	Formic acid	Alkali	Formic acid	Alkali	Formic acid
Isodesmosine	0.93	0.94	1.00	1.20	1.13	1.19
Desmosine	1.37	1.39	2.00	1.90	1.89	1.88
Dehydromerodesmosine	0.79	0.78	0.10	0.07	0	0
Merodesmosine	0.23	0.22	0.25	0.21	0.23	0.18
Dehydrolysinonorleucine	0.21	0.18	0.01	0.03	0	0
Lysinonorleucine	1.08	0.82	1.10	0.90	0.93	0.73
Aldol	2.88	2.49	1.97	1.87	1.71	1.57
Lysine	6.90	6.20	4.50	4.80	4.40	4.80
Total lysine	27.50	25.50	23.71	23.64	22.45	22.22

links and intact lysine residues in the elastins of different ages is shown in Table 4. The changing patterns of cross-links and lysine residues is similar to those obtained for ligament elastins.

Discussion

Elastin is synthesized at a rapid rate by young growing animals but at a lower rate by adult animals. Apart from replacement of elastic tissues destroyed by disease processes and the normal processes of wear and tear, it is believed that once mature elastic fibres are laid down they are there for life. The synthesis of lysine-derived cross-links is a part of the natural process of maturation of these fibres. Hence the time to look for changes in the number and type of cross-links is in elastins isolated from young, growing animals. The amounts and types of cross-links can then be compared with those found in fully mature elastins from adult animals. In such studies caution must be observed in purifying the highly insoluble elastins, especially those present in young animals where the full complement of cross-links has not been attained. For example, covalent bonds may be broken by the chemical treatments employed in some purification procedures, causing solubilization of lightly-cross-linked elastin. For this reason several very different procedures have been employed in the present work.

The main source of elastin chosen for the study was that of the ligamentum nuchae of cattle. This tissue is readily available, highly enriched in elastin and readily purified by methods that do not involve acidic or alkaline media. The visceral pleura from the lungs of cattle is also a good source of elastin

but the protein was more difficult to purify from this source than from ligament. Only by using acidic and alkaline conditions could pleural elastins be obtained which approximated in chemical compositions to those of ligament. Taking amino acid compositions as a test for purity elastins extracted from both ligament and pleura by the different methods had overall compositions that were similar at all ages.

Elastins extracted from mature animals all contained the established cross-links lysinonorleucine and desmosine plus isodesmosine at concentrations of about 1 and 3 residues/1000 amino acid residues respectively. The detection of substantial amounts of the aldol-condensation product (about 2 residues/1000 amino acid residues in elastin from adults was obtained in the present study) confirmed earlier work (Lent *et al.*, 1969) which pointed to the product as an important cross-linking amino acid in mature elastin. However, the possibility still remained that hitherto unrecognized lysine-derived cross-links were present in elastin from adults. Chromatograms of acid hydrolysates of elastin from different tissues of different animal species all contained an unknown ninhydrin-positive peak. This compound has been identified as merodesmosine, thus confirming the conclusions of Paz *et al.* (1971) that merodesmosine and its reducible precursor, dehydromerodesmosine, occur naturally in the elastin molecule. However, the low concentrations of both these compounds (0.25 and 0.1 residue respectively in 1000 amino acid residues) in elastins from adults suggests that they are of minor importance only in the structure of mature elastins.

Elastins extracted from young animals when compared with those from adult animals show significant differences in the pattern of cross-linking compounds and lysine residues. Taking the contents of the desmosines as 100% in elastins from adults, those from the embryo were 50% and from the calf 75%. This increase in desmosines with maturity was balanced by a decrease in the content of residues of intact lysine and aldol-condensation product. Thus the elastin from adults contained only 60% of the amount of aldol found in embryonic material. This decrease with growth indicated that the aldol served two functions. About 60% of that present in the elastin from embryos remained unchanged, acting as an independent cross-link, whereas the remainder acted as an intermediate for the synthesis of the more complex cross-links of older elastins.

There are at least three possible pathways for the biosynthesis of desmosines from the aldol-condensation product. First, the aldol could undergo a Michael reaction with an α-amino-δ-adipic semialdehyde residue. Addition would occur across the aldimine bond to give a trilysyl dialdehyde residue which could then react via a Schiff base with the ε-amino group of an intact lysine residue. However, the presence of intermediates of this type should have been reflected in the appearance of new peaks on amino acid autoanalyser records, and the absence of such peaks suggests that this particular pathway is not operating. Secondly the aldol-condensation product could react with dehydrolysinonorleucine or thirdly (as shown in Scheme 1) via dehydromerodesmosine which would then react with a residue of α-amino-δ-adipic semialdehyde. If desmosines were synthesized via dehydrolysinonorleucine, appreciable amounts of this intermediate would be expected to occur in elastins from young animals. However, this compound is only present at concentrations of 0.17 residue/1000 amino acid residues, indicating that its sole function is to act as a precursor for the synthesis of lysinonorleucine. This is in contrast with dehydromerodesmosine, the content in elastins from young animals being about 0.8 residue/1000 amino acid residues. Since the total concentration of dehydromerodesmosine in elastin from embryos is nearer 1 residue, it would appear that 20% of this is stabilized by reduction of the aldimine bond to give merodesmosine. The remaining 80% must act as a precursor for the synthesis of desmosines. The amount of dehydromerodesmosine present in elastins from young animals could be increased by 2.5-fold by exposing the elastin to mild alkaline solution before extraction. This increase was balanced by a decrease of the same order in the contents of the aldol-condensation product and intact lysine residues. This indicated that the aldol and lysine residues were in the appropriate positions in the polypeptide chains in elastin which would allow for the synthesis of dehydromerodesmosine. This result, together with the recovery of reasonable amounts of dehydromerodesmosine in elastins from young animals and its virtual absence from elastins from adults, seems to be good evidence that the biosynthesis of desmosines proceeds by this route. To complete the synthesis of desmosines a residue of the semialdehyde would be required to give the tetrasubstituted pyridine ring.

Davis & Anwar (1970) by using model compounds showed that aliphatic aldimines reacted spontaneously with aliphatic aldehydes at room temperature to yield pyridinium compounds which were analogous to desmosine and isodesmosine. These authors suggested that desmosine synthesis could proceed via dehydromerodesmosine. However, an alternative pathway to isodesmosine via dehydrolysinonorleucine was postulated. In the present study the failure to detect significant amounts of dehydrolysinonorleucine in elastins isolated from growing animals gives little credence to the importance of this proposed pathway.

It should be pointed out that there is a large discrepancy between the contents of lysine residues (intact ones plus those involved in cross-linking)

in bovine insoluble elastins used in the present study and the contents in soluble elastins (tropoelastins) isolated by other workers. Sandberg *et al.* (1971) extracted a tropoelastin from the aortas of copper-deficient pigs with a lysine content of 45 residues/1000 amino acid residues and Sykes & Partridge (1972) extracted a tropoelastin from lathyritic chicks with a lysine content of 40 residues/1000 amino acid residues. Neither tropoelastin contained lysine-derived cross-links. In the present study the insoluble elastin isolated from the tissues of calves contained 27 lysine residues/1000 amino acid residues. The measurement of α-amino adipic semialdehyde in elastin was difficult because it moved on ion-exchange chromatography in a position near glycine. Lent *et al.* (1969) obtained 2–3 residues of α-amino adipic semialdehyde/1000 amino acid residues in bovine ligament elastin. Including this figure in the results obtained in the present work the total number of lysine residues recovered/1000 amino acid residues would then be 29–30. The discrepancy in lysine residues of 10–15/1000 amino acid residues between tropoelastins and insoluble elastins cannot be satisfactorily accounted for. One explanation could be that insoluble elastins contain other unidentified lysine-derived cross-links which were degraded during the extraction of elastin and the subsequent hydrolysis. Also it should be pointed out that in the present work elastins from adults contain 5 fewer lysine residues/1000 amino acid residues than elastins from young animals.

The normal process of maturation of elastic fibres should not be confused with that of senescence. A popular hypothesis of aging in animals suggests that the polypeptide chains in elastin become excessively cross-linked in older subjects. In the present study elastins from the ligamentum nuchae and visceral pleura of 10–12-year-old cattle were analysed. It was found that the contents of the natural lysine cross-links were no higher than those in 2–3-year-old animals; in fact the amount of aldol-condensation product tended to be lower. This decrease in lysine cross-links agreed with the detailed study made by John & Thomas (1972) on the effect of advancing age on the chemical compositions of elastins isolated from the aortas and lungs of human subjects.

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References

Davis, N. R. & Anwar, R. A. (1970) *J. Amer. Chem. Soc.* **92**, 3778–3782

Franzblau, C., Sinex, F. M., Faris, B. & Lampidis, R. (1965) *Biochem. Biophys. Res. Commun.* **21**, 575–581

John, R. & Thomas, J. (1971) *Int. J. Biochem.* **2**, 529–536

John, R. & Thomas, J. (1972) *Biochem. J.* **127**, 216–269

Lansing, A. I., Rosenthal, T. B., Alex, M. & Dempsey, E. W. (1952) *Anat. Rec.* **114**, 555–570

Lent, R. W., Smith, B., Salcedo, L. L., Faris, B. & Franzblau, C. (1969) *Biochemistry* **8**, 2837–2845

Miller, E. J., Pinnell, S. R., Martin, G. R. & Schiffmann, E. (1967) *Biochem. Biophys. Res. Commun.* **26**, 132–137

Paz, M. A., Henson, E., Blumenfield, O. O., Seifter, S. & Gallop, P. M. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1518–1523

Sandberg, L. B., Weissman, N. & Smith, D. W. (1969) *Biochemistry* **8**, 2940–2945

Sandberg, L. B., Zeikus, R. D. & Coltrain, I. M. (1971) *Biochim. Biophys. Acta* **236**, 542–545

Slack, H. G. B. (1954) *Nature (London)* **174**, 512–513

Starcher, B. C., Partridge, S. M. & Elsden, D. F. (1967) *Biochemistry* **6**, 2425–2432

Sykes, B. C. & Partridge, S. M. (1972) *Biochem. J.* **130**, 1171–1172

Thomas, J., Elsden, D. F. & Partridge, S. M. (1963) *Nature (London)* **200**, 651–652