THE UTILIZATION OF LYSINE IN THE BIOSYNTHESIS OF ELASTIN CROSSLINKS

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A polyfunctional amino acid which is probably the crosslink in elastin has recently been isolated from hydrolysates of purified bovine ligamentum nuchae by Partridge et al. (1963). Further studies by Thomas et al. (1963) have resolved two structural isomers which contain a pyridinium ring with four side chains located at the 1, 3, 4, 5 positions (desmosine) or at the 1, 2, 3, 5 positions (isodesmosine). Each side chain has a terminal carboxyl and an α-amino group indicating that all may be involved in peptide linkage in the protein. Thus, either isomer could serve as a link between as many as four different polypeptide chains. The empirical formula, $C_{24}H_{39}N_5O_8 \cdot H_2O$, and the structures suggested that either molecule could be formed from four lysine residues. One lysine would provide an ε-amino nitrogen in the formation of the pyridinium ring, while three other lysines would contribute the remainder of the ring structure and side chains with the loss of their ε-amino nitrogens during condensation. This report presents the results of two series of experiments designed to investigate this possibility.

Materials and Methods. Fertilized eggs and chickens of varying age were obtained from a local hatchery. From embryonic chicks, approximately 3 mm of ascending aorta containing the roots of the carotid arteries was chosen for study. From larger chickens, 0.5 - 1.0 cm of ascending aorta was excised from an area proximal to the point of exit of the carotid arteries. Embryonic aortas to be grown in tissue culture were excised with sterile instruments,

attached to a Millipore filter by means of a fibrin clot, and placed individually in a 16 x 150 mm test tube. For each experiment, 25-50 aortas were used. Four ml of a chemically defined tissue culture medium (TC 199) containing 100 units each of Panicillin and Streptomycin sulfate was added to each tube. Lysine-U-C^{14} was added to the medium at a level of 0.1 or 0.5 μc/ml. The tubes were stoppered and placed in a rotating drum in an incubator at 37° C for periods up to 12 days. The medium was changed every two days.

Elastin was isolated from the dry, fat-free samples by extraction with 0.1 N NaOH at 98° C for 1 hr according to the method of Lansing, et al. (1952). Portions of the residue remaining after alkali extraction were hydrolyzed in a nitrogen atmosphere in a sealed tube with approximately 1 ml of 6N HCl per mg of protein at 108° C for 72 hours. This period of hydrolysis was used routinely as desmosine, isodesmosine and valine were not completely released from the protein at shorter times. Appropriate correction factors were employed in the final calculations for threonine, serine, tyrosine and phenylalanine which were found to be partially destroyed during the course of hydrolysis.

Amino acid analyses of the elastin hydrolysates, equivalent to about 1 mg of protein, were performed on an automatic amino acid analyzer as described by Piez and Morris (1960). In this system, desmosine and isodesmosine chromatograph as two incompletely resolved peaks appearing between phenyalanine and hydroxylysine. (We are indebted to Dr. S. M. Partridge for samples of desmosine and isodesmosine which were used to standardize the analyzer.) As both isomers exhibit identical color yields (3.68 times leucine color yield) with the ninhydrin reagent, the total amount of the two isomers in any given hydrolysate could be easily calculated. Radioactivity determinations were made on the amino acids in the elastin hydrolysates by continuous scintillation counting of the effluent from the automatic amino acid analyzer as described by Piez (1962). Sufficient counts were obtained to give a standard counting error less than 6%.

Results. The amino acid composition of the elastin isolated from the

TABLE 1

The Amino Acid Composition of Elastin from Aortas of Chickens of Different Age

(Residues/1000 Total Residues)

	12 day Embryo	16 day Embryo	20 day Embryo	3 week Chick	4 week Chick	1 year Chicken
Hydroxyproline	24	25	22	23	22	23
Aspartic Acid	1.9	1.9	1.8	1.9	1.9	1.8
Threonine	4.2	3.4	3.6	3.1	3.5	4.6
Serine	5.4	4.1	3.2	5.1	5.7	4.1
Glutamic Acid	12	12	12	12	11	12
Proline	122	123	124	128	126	124
Glycine	352	352	351	352	352	352
Alanine	172	176	180	175	177	177
Half-cystine	0.5	0.5	0.6	0.4	0.3	0.6
Valine	177	177	177	176	176	174
Isoleucine	19	19	19	19	20	20
Leucine	62	59	56	58	57	58
Tyrosine	11	11	12	12	12	12
Phenylalanine	22	22	22	22	22	22
Quarter-Desmosine*	4.3	5.7	6.7	6.8	7.1	10.9
Lysine	5.7	4.6	3.9	3.6	3.5	1.6
Histidine	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Arginine	4.9	4.2	5.6	4.5	4.1	4.5
Amide-Nitrogen	(37.7)	(21.4)	(25.1)	(25.4)	(21.3)	(19.6)

* Desmosine plus isodesmosine.

aortas of chickens in six different age groups is shown in Table 1. The high content of nonpolar amino acids is characteristic of elastin prepared by alkali extraction of various tissues and has been noted previously in the case of elastin isolated from bovine ligamentum nuchae by Gotte et al. (1963). The amino acid compositions of the samples were identical except for lysine and desmosine (including isodesmosine). Desmosine plus isodesmosine are listed as quarter residues to indicate their probable equivalence to four lysine residues. The two isomers were present in approximately equal amounts. The age-dependent increase in the amount of crosslinking compound was accompanied by an equivalent decrease in lysine content, the total remaining constant at 10-11 quarter residues/1000 total residues. In the 12-day-old chick embryo approximately 40% of the total was desmosine and isodesmosine, while in the 1-year-old chicken 85% was present as the two isomers. The observed change is probably less than the actual change since it is likely that poorly crosslinked elastin (rich in lysine) would be removed by the alkalai extraction.

Table 2 shows the results of the experiments in which embryonic chick aortas were cultured in a medium containing lysine-U-C^{14}. In experiment 1, aortas from 16-day-old embryos were cultured for 4 days or 8 days in the presence of radioactive lysine. The results showed that there was synthesis of elastin throughout the period studied as indicated by the increase in lysine specific activity. The significant quantity of radioactivity found in desmosine and isodesmosine shows that lysine is a precursor of the cross-link and indicates that the crosslink was synthesized at approximately half the rate at which lysine was incorporated into the protein. Further results (experiment 2 in the table) revealed that if the aortas were cultured for only one day even in the presence of large amounts of radioactivity, no measurable amount of isotope was found in the crosslink. A series of pulse experiments were also performed, two of which are shown in the table. In experiment 3, aortas from 16-day-old embryos were cultured for 1 day in the presence of

TABLE 2

Incorporation of Lysine-U-C^{14} [a] into Lysine, Desmosine and Isodesmosine in Elastin of Chick Embryo Aorta in Tissue Culture

Exp.	Culture conditions [b]	Lysine, counts/μmole	Quarter-Desmosine, [c] counts/μmole
1	4 days with lysine-C^{14}	8,600	4,200
	8 days with lysine-C^{14}	14,200	6,000
2	1 day with lysine-C^{14}	89,600	< 100
3	1 day with lysine-C^{14}		
	7 days without	24,300	17,600
4	1 day with lysine-C^{14}		
	11 days without	73,700	78,100

[a] Present in the medium at 0.1 uc/ml in experiments 1 and 3 and at 0.5 μc/ml in experiments 2 and 4.

[b] Aortas were taken from 16-day-old embryos except in exp. 4 when 12-day-old embryos were used.

[c] Includes Isodesmosine.

lysine-U-C^{14} and allowed to grow in the same medium without radioactive lysine for the subsequent 7 days. Under these conditions, the specific activity of the crosslink was 65% of that found for the lysine. In experiment 4 aortas from 12-day-old embryos were allowed to grow for 1 day with radioactive lysine and 11 days without. In this case, the specific activity of the cross-link was slightly higher than that found in the lysine. Only trace amounts of radioactivity were found in other amino acids.

The amino acid composition of the elastin isolated from aortas after incubation was identical in all respects to that given in Table 1 for aortas of comparable age.

Discussion. The observation that the amino acid composition of elastin prepared from chicken aorta by hot alkali extraction does not change with increasing age except for a progressive decrease in the lysine content and an equivalent increase in the desmosine and isodesmosine content, suggests that lysine in peptide linkage in some type of a precursor is condensed to

form crosslinks. This interpretation is strongly supported by studies on the incorporation of lysine-U-C^{14} into elastin. Radioactivity is first found in the lysine and later in desmosine and isodesmosine. Pulse experiments indicate the continued accumulation of radioactivity in desmosine and isodesmosine after the removal of free lysine-U-C^{14}. These experiments do not rule out the participation of other compounds in addition to lysine in the biosynthesis of the crosslink but this seems unlikely in view of the structures of desmosine and isodesmosine.

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