INVESTIGATION OF THE NATURE OF THE INTERMEDIATES INVOLVED IN DESMOSINE BIOSYNTHESIS

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Desmosine and isodesmosine have been suggested as cross-linking compounds between the polypeptide chains of elastin (Thomas et al., 1963; Partridge et al., 1964). Studies on the incorporation of radioactivity into the desmosines from lysine-U-C^{14} (Miller et al., 1964, 1965; Partridge et al., 1966) and age-dependent changes in the amount of the desmosines and lysine in chick aorta elastin (Miller et al., 1964; Franzblau et al., 1965) have shown that peptide-bound lysine is utilized in desmosine synthesis. Further studies have shown that in states of copper deficiency (Miller et al., 1965) and lathyrism (O'Dell et al., 1966), desmosine synthesis is inhibited and that the lysine content of elastin from the aortas of copper-deficient and lathyritic animals is markedly increased.

It has been proposed (Partridge et al., 1964) that the synthesis of desmosine and isodesmosine from lysine proceeds through an aldehyde intermediate formed by deamination and oxidation at C-6 in lysyl residues of elastin. A recent study (Miller and Fullmer, 1966) in support of this proposal has demonstrated that the low lysine content of elastin isolated from aortas of control chicks is associated with increased aldehyde reactivity when compared to elastin from aortas of lathyritic chicks in which the lysine content is considerably higher. In the present communication, we give further evidence for the existence of intermediates in the biosynthesis of the desmosines, and present evidence that one of these is the δ-semialdehyde of α-amino adipic acid.

MATERIALS AND METHODS

16-day-old chick embryo aortas were incubated for varying intervals in a chemically defined medium as previously described (Miller et al., 1965). Tissue culture tubes containing aortas and media were flushed once daily with 95% oxygen, 5% carbon dioxide. Lysine-U-C^{14} and β-aminopropionitrile fumarate (BAPN) were added to the media used for incubation as required.

One-day-old chicks were raised for 3 weeks on a commercial chick ration, and lathyrism was induced in the experimental animals as previously described (Miller and Fullmer, 1966).

Elastin was isolated as the insoluble aortic residue either by extracting the tissues with boiling 0.1 N NaOH for 1 hour (Miller et al., 1964) or by exhaustive extraction of the homogenized tissues in a number of solvents as previously described (Miller and Fullmer, 1966). Collagenase treatment, however, was omitted in these studies.

Performic acid oxidation of aortas from tissue culture and of elastin isolated from the older chicks by solvent extraction was carried out according to the procedure of Moore (1963) as applied to insoluble proteins.

Amino acid analyses of the 72-hour hydrolysates of elastin samples were performed on an automatic amino acid analyzer (Piez and Morris, 1960) as modified for accelerated analyses (Miller and Piez, 1966), and radioactivity in the column effluent was monitored continuously (Piez, 1962).

The identity of the amino acid which traveled in the position corresponding to α-amino adipic acid in the amino acid chromatograms was verified in two separate chromatographic systems. For this purpose, the appropriate fraction obtained during amino acid analyses was desalted on a Dowex 2-X10 column according to the method of Brenner et al. (1965). An aliquot of the desalted material was chromatographed on a thin-layer system (Eastman Chromogram, Type K301R2, Silica Gel) for 4 hours using n-propanol and ammonia (70:30 v/v) as the developing liquid. A further aliquot was applied to Whatman 3MM paper, and electrophoresis was carried out for 3 hours at 84 volts per cm using a buffer composed of pyri-

dine, glacial acetic acid, and water (200:8:1800 v/v/v) at pH 6.4. In both chromatographic systems, the spots were located by spraying with ninhydrin. Radioactivity associated with the material chromatographed in the thin-layer system was determined by removing serial fractions of the silica gel and counting the samples in a liquid scintillation counter. The developed electrophoretic paper was scanned for radioactivity in a gas flow strip counter.

RESULTS AND DISCUSSION

Table I shows the results of a typical experiment in tissue culture, in which BAPN was added to the medium at different stages of incubation. In these experiments, elastin was isolated from the aortas by hot alkali extraction. Data for groups 1 and 2 indicate that desmosine synthesis continued after the removal of isotope, while lysine specific activity decreased during the subsequent 5 days. The presence of BAPN during the entire incubation period suppresses all radioactivity incorporation into the desmosines (group 3). If, however, the addition of BAPN was delayed until after the initial 24-hour labeli period (group 4), near normal isotope incorporation into the desmosines without a decrease in lysine specific activity was observed. Desmosine synthesis appear to proceed by utilizing intermediates formed during the first day of incubation.

TABLE I

The Influence of β-aminopropionitrile Fumarate on the Synthesis of the Desmosines After Exposure of Embryonic Chick Aortas to Lysine-U-C^{14} for 24 hours

Group	Experimental Conditions	Specific Activity (cpm/μmole) Lysine	Quarter-desmosine and isodesmosine
1	Control - Day 1	42,400	8,400
2	Control - Day 6	23,500	17,500
3	BAPN (50 μg/ml), Days 1-6 (inclusive)	22,200	0
4	BAPN (50 μg/ml), Days 2-6; no BAPN on Day 1	44,600	15,000

Information concerning the nature and number of the intermediates formed during desmosine synthesis was sought by examining the radioactivity pattern obtained during amino acid analyses of elastin isolated from aortas which had

been incubated with lysine-U-C^{14} for 48 hours with or without BAPN (10 µg/ml). As shown in the top portion of figure 1, ten radioactive peaks are routinely found in the elastin chromatograms from control aortas, whereas similar chromatograms from aortas incubated in the presence of BAPN show absence of radioactivity in all peaks except lysine, desmosine and lysinoalanine. The latter amino acid is most likely formed via cysteine and lysine combination during hot alkali extraction (Bohak, 1964) and therefore represents an artifact in these samples. N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine (lysinonorleucine) (Franzblau et al., 1965) is eluted in the region between lysinoalanine and ammonia and significant amounts of radioactivity are not incorporated into this amino acid during short incubation times employed in these studies. Similar radioactivity patterns were observed in hydrolysates of whole aortas as well as the aortic residues after extraction with aqueous solvents, suggesting that the radioactive peaks did not arise during alkali treatment.

The presence of radioactivity in the glycine and leucine regions of amino acid chromatograms of lysine-labeled elastin has previously been observed (Cleary et al., 1966). Since the addition of BAPN to the tissue culture medium inhibits the appearance of radioactivity in these and other regions, it seems likely that the radioactive peaks represent components derived from the alteration of lysine during desmosine biosynthesis.

The middle portion of Figure 1 depicts the radioactivity pattern observed in the hydrolysates of elastin from aortas which had been oxidized with performic acid prior to alkali extraction. A significant proportion of the total radioactivity is associated with the α-amino adipic acid peak. This amino acid is the expected oxidation product of the δ-semialdehyde of α-amino adipic acid which has been proposed as the first intermediate derived from lysine in the cross-linking of both elastin (Partridge et al., 1964) and collagen (Bornstein et al., 1966).

In the thin-layer system, the ninhydrin-reactive material derived from the α-amino adipic acid region of the amino acid chromatogram exhibited an R_f value

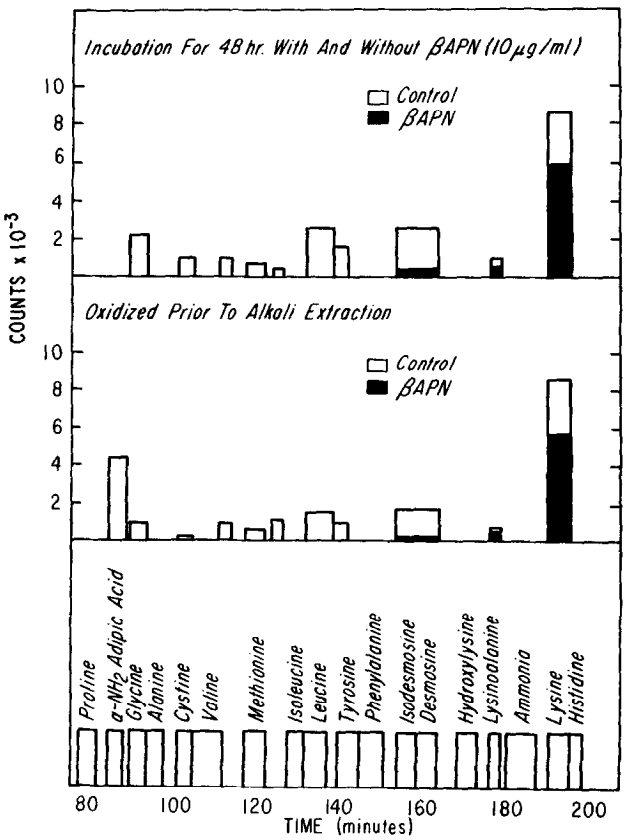

Fig. 1. Schematic representation of a portion of a typical amino acid chromatogram, indicating the positions of the amino acids and observed radioactivity patterns. Bars on the lower portion of the figure represent only position and width of the amino acid peaks.

of 0.43 which corresponded to the value observed for authentic α-amino adipic acid. During electrophoresis, the material migrated 9 cm toward the anode, as did authentic α-amino adipic acid. In both systems, the α-amino adipic acid spot contained radioactivity while the areas above and below it were free of counts.

Elastin from aortas incubated with BAPN contained no radioactive α-amino adipic acid, suggesting that the lathyrogen prevents the oxidative deamination of lysyl residues. Furthermore, oxidation of the aortas always resulted in a significant decrease in radioactivity associated with other regions of the chromatogram, sugggesting that some of these radioactive peaks represent break-

down products of the δ-semialdehyde of α-amino adipic acid arising during hydrolysis.

Further evidence that the α-amino adipic acid present in our amino acid chromatograms represents a stable derivative of an intermediate formed from lysine, was obtained in studies on elastin isolated from 3-week-old control and lathyritic chicks. The results are presented in Table II and indicate that an inverse relationship exists between the lysine and α-amino adipic acid contents, similar to that previously demonstrated between lysine content and aldehyde reactivity (Miller and Fullmer, 1966).

Table II

Partial Amino Acid Composition of Performic Acid Oxidized Chick Aorta Elastin

	Residues/1000 Total Residues	
	3-week control	3-week lathyritic
Lysine	9.2	14.0
α-Amino Adipic Acid	3.9	1.8
Quarter-Desmosine plus Isodesmosine	5.8	5.4

REFERENCES

Bohak, Z., J. Biol. Chem., 239, 2878 (1964).
Bornstein, P., Kang, A.H., and Piez, K.A., Proc. Natl. Acad. Sci. U.S., 55, 417 (1966).
Brenner, M., Niederwieser, A., and Pataki, G., in Thin-layer Chromatography, edited by E. Stahl, Academic Press, Inc., New York, p. 397 (1965).
Cleary, E.G., Sandburg, L.B., and Jackson, D.S., Biochem. Biophysic. Research Commun., 23, 139 (1966).
Franzblau, C., Sinex, F.M., Faris, B., and Lampidis, R., Biochem. Biophysic. Research Commun., 21, 575 (1965).
Miller, E.J., Martin, G.R., and Piez, K.A., Biochem. Biophysic. Research Commun. 17, 248 (1964).
Miller, E.J., Martin, G.R., Mecca, C.E., and Piez, K.A., J. Biol. Chem., 240, 3623 (1965).
Miller, E.J., and Fullmer, H.M., J. Exptl. Med., 123, 1097 (1966).
Miller, E.J., and Piez, K.A., Anal. Biochem., 16, 320 (1966).
Moore, S., J. Biol. Chem., 238, 235 (1963).
O'Dell, B.L., Elsden, D.F., Thomas, J., Partridge, S.M., Smith, R.H., and Palmer, R., Nature, 209, 401 (1966).
Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Tesler, A., and Ho, P., Biochem. J., 93, 30c (1964).
Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Tesler, A., and Ho, P., Nature, 209, 399 (1966).
Piez, K.A., and Morris, L., Anal. Biochem., 1, 187 (1960).
Piez, K.A., Anal. Biochem., 4, 444 (1962).
Thomas, J., Elsden, D.F., and Partridge, S.M., Nature, 200, 651 (1963).