EVIDFNCE FOR DITYROSINE IN ELASTIN[1]

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Previously unrecognized amino acids have in recent years been isolated from hydrolysates of bovine elastin. Their chemical constitutions are compatible with conclusions that two of them, desmosine and isodesmosine, result from the condensation of side chains of four lysine residues (Thomas et al., 1963) and the other, lysinonorleucine, from two lysine residues (Franzblau et al., 1965). Studies with radioisotopic lysine have shown the gradual conversion of this amino acid into the three apparent crosslinks (Miller et al., 1964; Partridge et al., 1964) and indicate that crosslinking between peptide chains occurs in elastin for some time following the elaboration of elastic tissue. In our studies of the composition of elastin in the developing human aorta, unknown ninhydrin-positive materials were eluted in the basic region of the amino acid chromatogram (LaBella and Vivian, 1967). Certain characteristics of one of these materials indicated aromaticity, suggesting its possible derivation from tyrosine residues of elastin. In this report data on the unknown are presented which show (a) by means of tyrosine-C^{14} that it is derived from tyrosine, and (b) that its chromatographic properties are identical to those of standard dityrosine.

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MATERIALS AND METHODS

Segments of the aortas of 12-day old chick embryos were fixed to a Millipore filter with a fibrin clot and placed individually into 18 x 150 mm test tubes. Three ml of Eagle's medium (Eagle, 1959) was added and the tubes were incubated at 37°C. After 2 days the medium was discarded, and 3 ml of fresh medium containing 1 μC of uniformly labelled tyrosine-C^{14} (297 μC/mM, ICN Corp.) was added. On day 5, 3 ml of fresh medium (without C^{14}) was added to the original 3 ml, and incubation was continued for 3 more days. On day 8, 25 cultures were removed and the aortas pooled; the medium in the remaining tubes was replaced by 3 ml of fresh medium without C^{14}. Two more pools of cultures were removed, one at 11 and one at 14 days. The aortas were rinsed several times with saline, dehydrated in acetone, and extracted with hot chloroform:methanol (3:1) for 3 hours. The defatted aortas were extracted twice with 0.1N NaOH at 100°C for 20 minutes, washed with water, washed with acetone, and air-dried. Approximately 1 mg of purified elastin was obtained from each pool and was hydrolyzed in 10 ml of 6N HCl for 24 hours at 110°C in a sealed vial under nitrogen. HCl was removed under vacuum, the residue dissolved in 0.3 ml of 0.1N HCl and placed on the ion-exchange column. Amino acid analyses were carried out on a Technicon instrument, using a 0.63 x 75 cm column and an 8 hour separation time, as previously described (LaBella et al., 1966). The effluent stream from the column was split so that one portion was reacted with ninhydrin and the other monitored for C^{14} and fluorescence at 405 mμ. Dityrosine was prepared by incubating a mixture of L-tyrosine, hydrogen peroxide, and horseradish peroxidase as described by Gross and Sizer (1959). The reaction mixture was fractionated on Dowex 50 with pyridine acetate buffer, tyrosine being eluted at pH 4.5 and dityrosine at pH 7. The purified dityrosine migrated appropriately on the paper chromatographic system of Gross and Sizer (1959).

RESULTS

Radioactivity was detected in only two regions in the chromatograms of elastin hydrolysates: the areas corresponding to tyrosine and to a ninhydrin-positive substance (U) eluted between lysine and histidine (Figure 1). Tyrosine decreased from 12 to 6.6 residues and U increased from trace amounts to 4.6 residues over the culture period (Table I). These findings

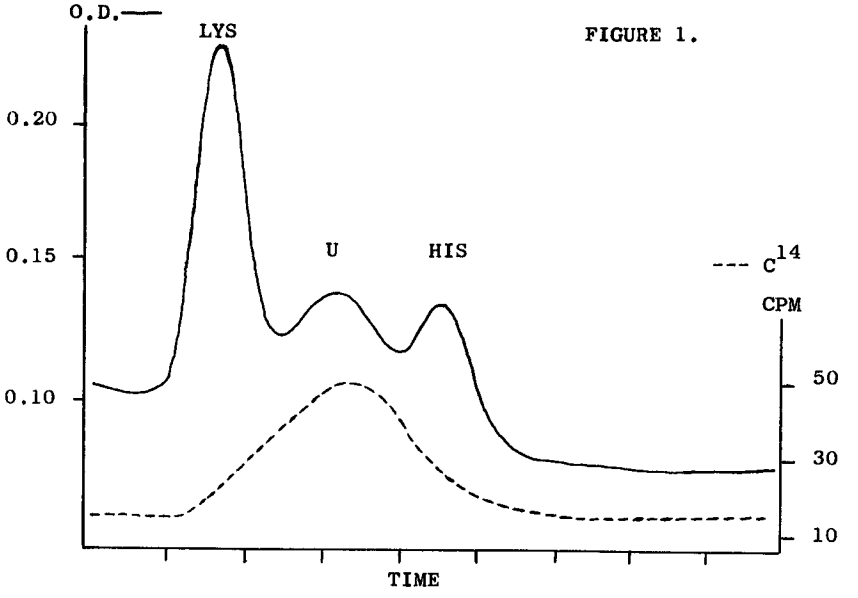

FIGURE 1.

Figure 1. Portion of amino acid chromatogram of elastin from 14-day culture of chick embryo aorta.

TABLE I

INCORPORATION OF TYROSINE-C^{14} INTO TYROSINE AND U OF ELASTIN FROM CULTURED CHICK EMBRYO AORTAS.

	moles/1000 total moles			cpm/mg elastin		
	8 day	11 day	14 day	8 day	11 day	14 day
Tyrosine	12	10	6.6	1270	2060	670
U	< 0.5	< 0.5	4.6	200	490	460

suggest a precursor-product relationship. In Table I, it is also seen that C^{14} in tyrosine and in U increased over the first two periods in which

tyrosine-C^{14} was present in the medium. Total C^{14} in elastin was lowest in the oldest culture, indicating loss of labelled components in the period between 11 and 14 days.

Dityrosine was subjected to chromatography on the same system used to fractionate elastin hydrolysates and emerged in the "U position". The elution of material in the "U position", as influenced by several alterations in the conditions of the ion-exchange column, was compared to the elution of dityrosine under the same conditions. The addition of methanol to all buffers shifted dityrosine to the left on the chromatogram, the degree of displacement dependent upon the methanol concentration, and U was displaced identically. Lowering the column temperature from 60°C to 40°C caused dityrosine to emerge as a very broad peak much later than histidine and just prior to arginine. Under these same conditions U was not evident in the region between lysine and histidine, but a small broad peak was detected in the chromatogram of the elastin hydrolysate corresponding exactly to the dityrosine position. Dityrosine and U were each retarded 65 minutes: the only other amino acids which were significantly retarded, about 50 minutes, were tyrosine and phenylalanine. The use of a system employing a 0.63 x 140 cm column and a 21 hour separation time caused dityrosine to emerge prior to lysine, in contrast to its post-lysine position in the 8 hour system. The positions of the other amino acids relative to one another were unaltered during separation on the long column. An unidentified peak, presumed to be U which was absent from region between lysine and histidine, corresponded exactly to the dityrosine position in the 21 hour system.

DISCUSSION

The compound U which is derived from tyrosine, as determined from the isotope incorporation studies, was identified as dityrosine on the basis of its similar fluorescence maximum and its chromatographic behavior on Dowex 50 relative to pure dityrosine. It is doubtful that any other sub-

stance would migrate identically under the several different conditions employed. Absolute proof of the structure of U will only be achieved, however by its isolation in pure form.

Dityrosine had not been reported in proteins, or naturally occurring in the free state as far as we know, until Andersen (1964, 1966) isolated it from resilin, an elastin-like protein in insect cuticle. Andersen showed that tyrosine-C^{14} was incorporated into dityrosine of resilin, into trityrosine present in lesser amounts, and into tetratyrosine present only in traces, and he suggested that the conversion is enzyme mediated on a "proresilin". Although Andersen proposes a crosslinking role for the polytyrosines, it remains to be shown that tyrosine residues on different chains are involved in their formation.

Our "pulse" labelling experiments show that there is a continuous formation of U from tyrosine residues in preformed elastin. A similar process has been shown for the desmosines (Partridge et al., 1964; Miller et al., 1964) and lysinonorleucine (Franzblau et al., 1965), crosslinks derived from lysine residues in elastin. Andersen (1966) proposed that some type of crosslinking occurs in elastin prior to lysine condensation, because relative insolubility is achieved in this protein before extensive desmosine and lysinonorleucine formation has occurred. We have shown (LaBella and Vivian, 1967) that in human elastin the concentration of U is highest in the earliest specimen examined, the 14-week old fetus. Lysine crosslinks are still at a low concentration at this stage. In this earlier work we found also that only traces of ninhydrin-positive material in the "U position" are present in elastin of the newborn. This significant finding indicates that dityrosine in elastin is of a transient nature and may be an intermediate in another process, perhaps the formation of a polyphenolic type of crosslink. The loss of total C^{14} (tyrosine plus dityrosine) in elastin between 11 and 14 days of culture may be accounted for by its presence in a polyphenolic substance which has been retained by the Dowex column after the conventional

amino acid separation was completed. A study of the distribution of tyrosine-C^{14} among the products of hydrolysis of elastin from older cultures or from whole animal experiments may indicate the ultimate fate of di-tyrosine in the protein.

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