IDENTIFICATION OF A NEW CROSSLINKING AMINO ACID IN ELASTIN

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Recently, Partridge et al. (1963) described the occurrence in elastin of two hitherto unrecognized amino acids for which evidence has now accrued to show their origin from lysine residues in a "pre-elastin" (Partridge et al., 1964; Miller et al., 1964). These substances, called desmosine and isodesmosine, are in fact quaternary pyridinium compounds with four side-chain substituents that function in crosslinking of elastin chains (Thomas et al., 1964). An examination of peptide fractions of elastin selected for enrichment with respect to the desmosines revealed the presence of an additional amino acid, called X_4, also previously undescribed (Franzblau et al., 1965). The present communication presents data to show that X_4 can be represented by the formula, N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine.

EXPERIMENTAL PROCEDURES AND RESULTS

X_4 was isolated from acid hydrolysates of elastin of bovine ligamentum nuchae as described by Franzblau et al., (1965). In brief this method consists of fractionation on Dowex 50 x 8 by means of elution with solutions of HCl followed by rechromatography of the crude X_4 fraction on a sulfonated polystyrene-divinylbenzene resin.

The electrophoretic mobility of X_4 was studied using Whatman #1 paper and a Spinco Model R electrophoresis cell at 300 volts. Buffers used were: acetic acid - formic acid

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(pH 1.9), pyridine-acetic acid (pH 3.5 and pH 6.4), barbital (pH 8.9), and sodium carbonate (pH 10.9). X_4 migrated as a single band in all systems and, from the data obtained, one could estimate its isoelectric point to be between pH 8.6 and pH 10.9.

Reactions with fluorodinitrobenzene (FDNB) of X_4 and of the copper complex of X_4 were studied as follows. First, X_4 was dinitrophenylated directly with FDNB by the method of Sanger (1945). The copper complex of X_4 was prepared by treatment with copper carbonate, dinitrophenylated, and the copper stripped from the DNP derivative by treatment with acid and then H_2S. An aliquot of this derivative was then treated once more with FDNB, and the new DNP derivative isolated. A spectrum of the DNP derivative of the copper complex of X_4 revealed absorption at 385 mμ characteristic of DNP derivatives of N-substituted amino acids such as sarcosine, pipecolic acid and proline. In contrast, the derivative of X_4 formed by dinitrophenylation in two stages had a major absorption peak at 355 mμ with a broad shoulder at 385 mμ; in fact its absorption spectrum was almost identical with that of the fully dinitrophenylated X_4 formed directly omitting the step in which a copper complex was formed. The spectrum of the fully dinitrophenylated X_4 could be reproduced by a model system consisting of two equivalents of DNP-alanine and one of DNP-sarcosine.

In a Technicon amino acid analyzer employing a column similar to that described by Spackman et al. (1958), the DNP derivative of X_4 formed from the copper complex (and then stripped of copper) eluted as a skewed peak which after ninhydrin reaction showed relatively high absorption at 440 mμ. This in part was due to the intrinsic absorption by the dinitrophenyl group. This behavior is reminiscent of that of ε-DNP-lysine. From the molar extinction coefficient of the DNP group and the number of leucine equivalents yielded in the ninhydrin reaction, one could infer that this derivative contained two primary α-amino groups and one DNP-substituted secondary amino group. Taken with the spectral data of the fully dinitrophenylated derivative, one could conclude that X_4 per se contained primary α-amino groups and secondary amino groups in a ratio of 2:1.

Nuclear magnetic resonance studies were performed with X_4 in both deuterium oxide

and in trifluoroacetic acid. The spectra in trifluoroacetic acid unequivocally showed the absence in X_4 of C-CH_3 groupings and confirmed the absence of an aromatic nucleus (Franzblau et al., 1965). In a positive way, the NMR spectra revealed groups characteristic of amino acids and presence of protons on carbon atoms adjacent to an electron-withdrawing group such as would occur in CH_2-N. Thus NMR data supported the results obtained by dinitrophenylation indicating the presence of both primary α-amino groups and an imino group. The NMR spectrum was compared with that of lysine and found almost identical except for one significant difference. Where lysine shows a peak at 3.1τ due to the ϵ-NH_3^+ group, X_4 shows a corresponding peak at 2.8 τ.

Manometric determinations of the carbon and nitrogen contents of X_4 were performed by the methods of Van Slyke (1929) and Van Slyke et al. (1941). Because the hygroscopic nature of X_4 precluded accurate weighing of samples, analyses were performed using aliquots of a solution of X_4 containing 0.78 mg of N in 25 ml of water. Table I shows the results of triplicate analyses as ratios from which one can infer a structure for X_4. That the analyses are in fact consistent with the postulated structure, N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine is shown by comparison with the theoretical ratios also shown in the table.

Formol titration of X_4 showed two equivalents of a group with an apparent pK at pH 8.0 and one with a pK at 10.2. The titration was performed using X_4 in an amount providing 59 micro-equivalents of N, and complete titration consumed 60 micro-equivalents of NaOH. Thus, these groups satisfactorily accounted for the nitrogen content of X_4.

At this juncture, all of the analytical data obtained for X_4 were consistent with the structure of N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine. Accordingly, the chemical synthesis of this compound was undertaken.

Synthesis of N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine was accomplished by the scheme shown in Figure 1.

Comparison of X_4 with N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine showed the two compounds to be identical with respect to chromatographic behavior, electrophoretic mobility,

TABLE I. MANOMETRIC ANALYSES OF X_4

Ratio	Found	Theoretical for N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine
Total Nitrogen* / Total Amino Nitrogen**	1.61	1.50
Total Amino Nitrogen** / Total α-Amino Nitrogen***	1.02	1.00
Total Carbon / Total Nitrogen*	3.95	4.00
Total Carbon / Total α-Amino Nitrogen***	6.13	6.00

* Kjeldahl method (μM/ml)
** Nitrous acid-N_2 method (μM/ml)
*** Ninhydrin-CO_2 method (μM/ml)

dinitrophenylation, spectral behavior of DNP derivatives and spectral behavior in the infrared. In contrast, X_4 could be distinguished from N^{ϵ}-(4-amino 4-carboxybutanyl)-lysine or N^{δ}-(4-amino 4-carboxybutanyl)-ornithine which were also synthesized. N^{ϵ}-(2-amino 2-carboxyethyl)-lysine (lysinoalanine) was prepared from ribonuclease as described by Bohak (1964), and it too was readily distinguished from X_4. A further difference between X_4 and lysinoalanine consistent with their respective proposed structures, is that the former does not react with sodium periodate whereas, as shown by Bohak, lysinoalanine by this treatment yields lysine, ammonia, formaldehyde and glyoxal.

The biosynthesis of X_4 was studied in experiments modeled after those of Partridge et al. (1964) and Miller et al. (1964) in which it was shown that the desmosines derive from lysine residues in a "pre-elastin". Table II shows the results of an experiment in which 10 day old Mount Hope chick embryos were incubated and, at various periods, a group was sacrificed. Aortas were removed, elastin prepared by the method of Miller et al. (1964), and the protein analyzed for contents of lysine, isodesmosine, desmosine and X_4. As shown by the other investigators, there occurred an increase in desmosine and isodesmosine contents with increase

FIGURE 1

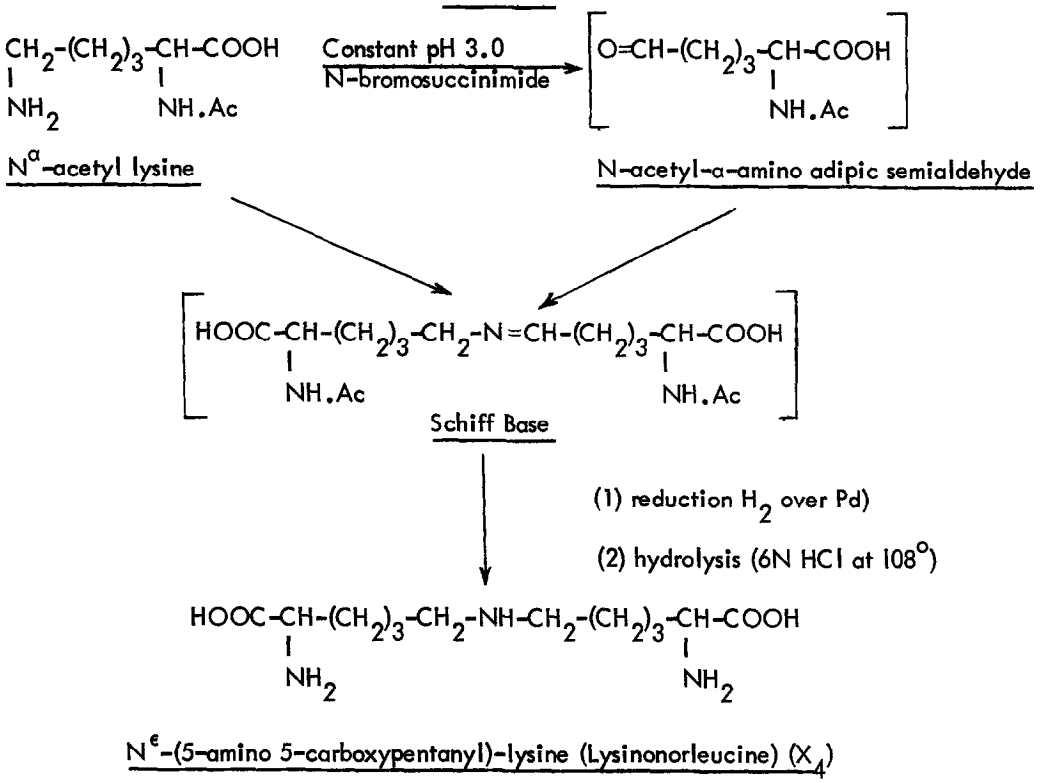

TABLE II. DETERMINATION OF LYSINE, DESMOSINES AND X_4 IN ELASTIN OF AORTAS OF CHICK EMBRYOS AND OF CHICKS

	AGE OF EMBRYOS OR OF CHICKS				
	10 Day	12 Day	15 Day	17 Day	6 Mo.
Lysine	10.3	8.2	6.8	4.6	2.4
Isodesmosine	2.1 (0.52)	2.5 (0.62)	3.0 (0.74)	3.4 (0.82)	4.8 (1.2)
Desmosine	2.2 (0.55)	1.9 (0.50)	2.4 (0.60)	2.9 (0.72)	4.8 (1.2)
X_4	0.53 (0.27)	0.63 (0.32)	0.72 (0.36)	0.84 (0.42)	1.0 (0.50)

* The numbers in parentheses indicate residues per 1000 total amino acid residues, of desmosine, isodesmosine or X_4, whereas the other numbers indicate leucine equivalents in the ninhydrin determination.

in age of embryo, and concurrently a decrease of lysine. Significantly, the data revealed that the contents of X_4 in elastin also increased with age of embryo, at a rate quite similar to that for the desmosines.

DISCUSSION AND SUMMARY

The properties of a newly described amino acid, X_4, found in hydrolysates of purified elastins have been studied in analytical detail and compared with those of synthetically prepared model compounds. The data indicate that X_4 has analytical features consistent with the formula, N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine and indeed is indistinguishable from a sample of this compound that was synthesized. Studies on the appearance, as a function of time, of this amino acid in the aortic elastin of chick embryos, show that it follows the already established pattern of the desmosines, i.e., it increases in content with age of embryo as the content of lysine residues decreases. Partridge, in a personal communication, has reported independent studies with radio-lysine that show lysine to be a precursor of X_4. Thus, the evidence is cogent that N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine, in the manner of the desmosines, is a crosslinking amino acid in elastin.

Chemically, N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine is related to N^{ϵ}-(2-amino 2-carboxyethyl)-lysine prepared by Bohak (1964) by chemical modification and degradation of ribonuclease. This substance was given the trivial name, lysinoalanine. Using this system of designation, one could then refer to X_4 as lysinonorleucine. Chemically, this compound is synthesized by oxidation of an ϵ-amino group of lysine to an aldehyde, coupling of the aldehyde with the ϵ-amino group of a second lysine molecule through a Schiff base, and reduction of the Schiff base. Experiments are planned to determine whether biochemically analagous reactions occur in formation of lysinonorleucine in elastin.

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REFERENCES

Bohak, Z., J. Biol. Chem., 239, 2878 (1964).
Franzblau, C., Sinex, F.M. and Faris, B., Nature, 205, 802 (1965).
Miller, E.J., Martin, G.R. and Piez, K.A., Biochem. Biophys. Res. Commun., 17, 248 (1964).
Partridge, S.M., Elsden, D.F. and Thomas, J., Nature, 197, 1297 (1963).
Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Tesler, A. and Ho, P.-L., Biochem. J., 93, 30C (1964).
Sanger, F., Biochem. J., 39, 507 (1945).
Spackman, D.H., Stein, W.H. and Moore, S., Anal. Chem., 30, 1190 (1958).
Thomas, J., Elsden, D.F. and Partridge, S.M., Nature, 200, 651 (1963).
Van Slyke, D.D., J. Biol. Chem., 83, 425 (1929).
Van Slyke, D.D., Dillon, R.T., MacFadyen, D.A. and Hamilton, P., J. Biol. Chem., 141, 627 (1941).