possessed the characteristic note. In a similar fashion, 2-butoxy-3-propylpyrazine (**19**) exhibits only a low medicinal character.

Based on three compounds, it appears that the oxygen of the methoxy group can be replaced by sulfur with a quantitative but not qualitative change in character. Thus, it can be seen that while the intensity of 2-methylthio-3-isobutylpyrazine (**21**) may be lower than the oxygen analog (**6**), the bell pepper note remains similar. Analogously, 2-methylthio-6-isobutylpyrazine (**22**) possesses this note, but with lower intensity than the 2,3 isomer (**21**).

Infrared and mass spectral data of previously unpublished compounds have been deposited in the microfilm edition of this volume of the journal.

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Quantitative Isolation and Partial Characterization of Elastin in Bovine Muscle Tissue

H. Russell Cross, Gary C. Smith,* and Zerle L. Carpenter

Three tissues (muscle, aorta, and *ligamentum nuchae*) from one young animal and one old animal were utilized as sources of connective tissue to compare staining properties and amino acid composition to ascertain the purity of elastin isolated from bovine muscle. Elastin preparations from the *triceps brachii* and *biceps femoris* muscles were essentially identical in amino acid composition to elastin from *ligamentum nuchae* in both young and old animals. The ease with which elastin can be purified varies considerably with the source, but by the use of extraction procedures of increasing severity, the end product from tissues of different kinds and from animals of different ages approaches constancy of composition. It can be concluded that elastin isolated as the residue remaining after extraction with 0.1 *N* NaOH at 98° for 45 min yields a material that is relatively homogenous in composition and varies little with tissue source or animal age.

Although considerable research has been reported concerning the role of connective tissue in determining the tenderness of meat, the majority of the related literature has dealt with collagen (Cross *et al.*, 1972; Goll *et al.*, 1963; McClain, 1969; McClain *et al.*, 1965; Wilson *et al.*, 1954). Collagen has been extensively investigated, both with regard to its physical and chemical properties and to determine its relationship to tenderness. Elastin has been less extensively researched, perhaps because it forms a lesser proportion of connective tissue than collagen and because of the inherent difficulty in studying a protein which is characteristically insoluble during heating (Partridge *et al.*, 1955).

The historical assumption that elastin is present in muscle tissue in only small amounts has been challenged by Partridge (1966), who found considerable amounts of elastin in the muscles of the beef rump. The necessity to consider the singular effects of elastin, rather than total connective tissue, on muscle tenderness suggests that a quantitative method for determining small quantities of elastin in muscle tissue needs to be developed. The methods presently available for the isolation of elastin fibers are based on the insolubility of elastin and its resistance to hydrolytic reagents. The ease with which elastin can be purified varies considerably with the connective tissue source. Most of the purification methods reported in the literature have been conducted on an elastin-rich tissue such as aorta and *ligamentum nuchae* (Gotte *et al.*, 1963; Lansing *et al.*, 1952; Partridge, 1962). Bendall (1967) isolated elastin from muscle tissue using numerous extractions with NaOH and various fat solvents. Contrary to results reported by Partridge (1966), Bendall found significant amounts of elastin only in the *semitendinosus* muscle of the bovine. Little additional work has been reported on the isolation of elastin in muscle tissue; thus, procedures for isolating elastin without undue hydrolytic damage appear to be needed. More specifically, the objective of this study was to develop a quantitative assay for elastin derived from muscle tissue and to document age-associated changes in bovine elastin.

Department of Animal Science, Texas A&M University, College Station, Texas 77843.

EXPERIMENTAL PROCEDURE

Four tissues (*ligamentum nuchae*, aorta, *triceps brachii*, and *biceps femoris*) were utilized from a young Hereford female (388 days of age) and an old Hereford female (3660 days of age) as sources of bovine elastin.

Purification of Elastin. Purified elastic-fiber prepara-

tions were obtained from two bovine tissues, *ligamentum nuchae* and aorta. Both tissues were dissected free from adhering tissues, frozen in liquid nitrogen, powdered, and extracted with excess 1% NaCl in a low-speed mechanical shaker. Agitation was continued until the extract gave a negative test for protein with trichloracetic acid solution. Three 1-hr extractions with 1% NaCl were normally required to obtain a negative test for protein. The samples were then suspended in distilled water and disintegrated in a high-speed blender. The samples were washed several times with distilled water and finally recovered in a low-speed centrifuge (1500 × *g*). The washed preparations were partially defatted with acetone by shaking for 2 hr in a low-speed mechanical shaker and dried overnight in air at room temperature.

From the dried, partially defatted tissue, samples of purified elastin were prepared by each of the following procedures.

Autoclaved Elastin. The dried tissue was suspended in distilled water, autoclaved at 1 atm pressure for 45 min, refluxed with acetone for 1 hr, dried in warm air overnight, and sequentially powdered and autoclaved until the effluent gave a negative reaction for protein. Four sequential periods of powdering and autoclaving were normally required to achieve purification. Finally, the preparation was refluxed in diethyl ether for 1 hr and dried in a vacuum desiccator.

Autoclaved Elastin Extracted with 0.5 N Sodium Hydroxide at 25°. Extraction of the dried, partially defatted tissue was continued by using a mechanical shaker with an excess of 0.5 *N* NaOH at 25° for three or four periods of 1 hr each or until the extract gave a negative test for protein.

Elastin Extracted with 0.1 N Sodium Hydroxide at 98°. The dried, partially defatted tissue was digested with 0.1 *N* NaOH at 98° for 45 min, according to the procedure of Lansing *et al.* (1952).

Elastin Extracted from Muscle Tissue. The Lansing *et al.* (1952) method of isolating elastin is based on the assumed insolubility of elastin in 0.1 *N* NaOH at 98° as contrasted with the solubility and rapid extraction of collagen and reticulin constituents of the tissue following removal of sarcoplasmic and myofibrillar proteins. The weight of the dry residue remaining after thorough extraction represents the weight of elastin in the tissue.

The actual procedure for elastin extraction from muscle consisted of the following. Duplicate samples of 2–3 g of tissue were weighed in 50-ml round-bottomed polypropylene centrifuge tubes. The muscle tissue was extracted with 1.1 *M* potassium iodide plus 0.1 *M* potassium phosphate buffer, pH 7.4 (10 ml/g of wet tissue), for 3 hr as outlined by Helander (1957). The tubes were centrifuged for 10 min at 1400 × *g*; extraction was repeated for an additional 3 hr; the tubes were centrifuged again; and a final extraction of 2 hr duration completed the procedure. The tissue was washed twice with distilled water and the residue was recovered with low-speed centrifugation (1400 × *g*). Exhaustive extraction of the muscle tissue was performed to ensure complete removal of myofibrillar and sarcoplasmic proteins. Removal of the latter proteins was assumed to be complete when a white residue was achieved which evidenced a complete lack of visible redness in the final residue.

An excess of 2:1 chloroform–methanol was added to extract the lipid from the stromal fraction. The samples were agitated in a low-speed mechanical shaker at 2° for 2 hr. Following fat extraction, the samples were washed three times with distilled water to remove the solvent. It was essential that no trace of the chloroform–methanol remained prior to alkaline hydrolysis to prevent foaming of the solution upon subsequent heating. The samples were hydrolyzed in 0.1 *N* NaOH (10 ml/g of wet tissue) at 98° for 50 min. The residue was washed with distilled water, recovered by centrifugation, and the tubes were immersed in hot water to remove any surface contaminants from the tube. The tubes were dried for 24 hr at 102°, cooled, and weighed. Elastin content was reported as mg/g on a whole tissue basis (WTB) or on a moisture and fat-free tissue basis (MFFB).

Rate of Alkaline Hydrolysis. Samples of *ligamentum nuchae,* aorta, and stromal protein from the muscle were suspended in a measured volume (100 ml/g of dry weight) of 0.1 *N* NaOH and the tubes were placed in a 98° water bath. Triplicate tubes were removed at 5- or 10-min intervals, centrifuged, dried for 24 hr at 102°, and weighed to determine the weight loss as a percent of the original dry weight.

Changes in Staining Character with Partial Hydrolysis. Changes in the staining character of elastin after mild treatment with NaOH solution were investigated. Samples of autoclaved *ligamentum nuchae* preparation were further extracted with 0.1 *N* NaOH at 98° for 30 or 45 min or with 0.5 *N* NaOH at 25° for 4, 8, 24, or 48 hr. Samples of the muscle elastin were further extracted with 0.1 *N* NaOH at 98° for 50, 60, or 80 min. Following extraction, the samples were washed and dried in a vacuum desiccator.

Five dye solutions were investigated. The dyes used were: aniline blue (C.L. 42755), methyl green (C.L. 42590), fast green (C.L. 42053), orcein (C.L. 14689), and bromphenol blue (C.L. 240980). The dyes were prepared in the following manner: aniline blue (10 mg/l. in 1% acetic acid), methyl green (100 mg/l. in Na_2CO_3–HCl buffer, pH 8.0), fast green (10 mg/l. in 1% acetic acid), orcein (50 mg/l. in 70% ethanol containing 0.1 *N* HCl), and bromphenol blue (6.3 mg/l. in Na_2CO_3–HCl buffer, pH 8.8). The elastin samples (10 mg) were first equilibrated with the solvent used for dissolving the dye by shaking with an excess of solvent for 1 hr at 20°. The solvent was then poured off, replaced by 1 ml of dye solution, and allowed to stain for 24 hr with intermittent shaking. The fibers were allowed to settle to the bottom of the flask and the dye solutions were decanted into 0.5-ml light path cuvettes. The absorbance of the dye which had not been taken up by the elastic fibers was determined at the wavelength of maximum absorption for the specific dye solution used.

Elastin Staining. In order to ascertain the purity of the purified elastic preparation, the orcein procedure of Romeis (1948) and the Orcinol-New Fuchsin procedure as described by Fullmer and Lillie (1956) were used on the elastin extracted from muscle by hot alkali. Both methods stain elastin red and do not stain collagen or reticulin. To detect the presence of collagen or reticulin, the procedure of Humason and Lushbaugh (1960) was used. In this procedure, elastin stains red, reticulin stains black, and collagen stains blue.

Amino Acid Analysis. Tissue samples from *ligamentum nuchae,* aorta, *biceps femoris,* and *triceps brachii* were analyzed to determine amino acid composition on a modified Beckman 120 amino acid analyzer. Samples were prepared as follows. Dried samples (approximately 1.5 mg) were weighed accurately into the hydrolysis tube and 1.0 ml of reagent grade 6 *N* HCl was added. (Note: If excess acid is added, it is difficult to obtain sufficient vacuum due to the "bubbling" of the acid during evacuation.) Oxygen-free nitrogen was bubbled through the sample for 3–5 min. The samples were evacuated for 2 min with an efficient water aspirator. If the samples had a tendency to boil, they were chilled to −10° in a freezer before evacuating. The hydrolysis tubes were sealed under vacuum and placed in an oven at 110 ± 2° for 72 hr. Following hydrolysis, the tubes were removed and cooled to room temperature. If there was any visible sediment, the sample was filtered through Whatman No. 3 filter paper into a 250-ml round-bottomed flask and evaporated to

Table I. Amino Acid Composition of Elastin Preparations from Four Sources in Hereford Females 388 and 3660 Days of Age

	g of amino acid/100 g of protein							
	Ligamentum nuchae			Aorta				
Amino acid	Autoclaved at neutral pH	Autoclaved and extracted with 0.5 *N* NaOH at 25°	Autoclaved and extracted with 0.1 *N* NaOH at 98°	Autoclaved at neutral pH	Autoclaved and extracted with 0.5 *N* NaOH at 25°	Autoclaved and extracted with 0.1 *N* NaOH at 98°	*Triceps brachii*, extracted with 0.1 *N* NaOH at 98°	*Biceps femoris*, extracted with 0.1 *N* NaOH at 98°
				388-Day-old Hereford				
Lysine	0.39	0.45	0.46	2.47	1.15	0.57	0.51	0.48
Histidine	0.01			0.68	0.14			
Arginine	0.92	0.67	0.64	2.82	1.36	0.67	0.61	0.63
Aspartic acid	0.87	0.79	0.71	3.30	1.86	0.76	0.67	0.65
Threonine	0.97	0.88	0.72	2.21	1.46	0.74	0.69	0.74
Serine	0.82	0.71	0.71	1.91	1.34	0.68	0.73	0.78
Glutamic acid	2.28	2.18	2.01	5.90	3.73	2.06	2.13	2.06
Proline	12.84	13.03	13.02	10.41	11.60	13.94	13.56	12.97
Glycine	21.99	22.62	22.57	16.73	20.33	24.74	23.06	22.94
Alanine	17.71	18.38	18.29	14.11	16.38	18.55	17.97	17.87
Valine	24.53	24.51	24.48	20.36	22.23	24.62	24.21	23.97
Methionine	0.29	0.30	0.07	1.30	0.68	0.09	0.04	0.08
Isoleucine	2.90	2.98	3.15	3.75	3.31	3.19	3.19	3.34
Leucine	7.40	7.69	8.12	7.77	7.52	8.16	7.94	8.19
Tyrosine	1.26	1.07	1.11	2.40	1.58	1.22	1.37	1.43
Phenylalanine	4.81	4.43	4.42	4.85	4.53	4.52	4.53	4.39
Hydroxyproline[a]	1.72	1.60	1.58	1.71	1.60	1.55	1.51	1.49
				3660-Day-old Hereford				
Lysine	0.66	0.21	0.22	0.65	1.05	0.53	0.23	0.29
Histidine	0.01			1.11	0.50			0.03
Arginine	0.72	0.61	0.59	0.43	2.20	0.72	0.56	0.64
Aspartic acid	0.91	0.76	0.64	2.32	2.96	0.84	0.71	0.74
Threonine	0.97	0.88	0.71	1.72	2.03	0.83	0.70	0.69
Serine	0.83	0.70	0.69	1.50	1.65	0.66	0.82	0.89
Glutamic acid	2.27	2.14	1.98	4.28	5.34	2.39	1.89	2.24
Proline	12.77	13.00	13.01	11.40	10.78	13.89	13.09	12.78
Glycine	22.13	22.53	22.51	19.17	17.83	23.14	24.01	23.72
Alanine	17.40	18.29	18.19	15.84	14.76	18.90	17.96	17.73
Valine	24.34	24.48	24.43	22.45	20.79	23.78	23.02	23.84
Methionine	0.26	0.28	0.05	0.79	1.12	0.27	0.12	0.09
Isoleucine	2.88	2.98	3.17	3.50	3.66	3.35	3.12	3.47
Leucine	7.36	7.68	8.10	7.71	7.82	8.34	8.16	7.95
Tyrosine	1.26	1.07	1.12	1.91	2.14	1.40	1.42	1.65
Phenylalanine	4.82	4.41	4.40	4.92	4.62	4.89	4.64	4.48
Hydroxyproline[a]	1.75	1.62	1.59	1.83	1.61	1.57	1.52	1.48

[a] Determined separately.

dryness in a rotary evaporator. The dried film was redissolved with an exactly measured volume of pH 2.2 citrate buffer to bring the sample to the concentration of approximately 0.05 μmol of each amino acid. The integrator on the analyzer had a measuring range of about 0.005 to 0.3 μmol, and accuracy was not suitable beyond these limits.

The amino acid concentrations were determined from a computer program which was designed to calculate data from an automatic amino acid analyzer equipped with a digital integrator. The amino acids in this study were reported in the condensed form (as found in the peptide chain, without the water added during hydrolysis) rather than the free form and values were reported in grams of amino acid/100 g of protein. Hydroxyproline was determined separately using the procedure outlined above for the amino acid analyzer because it was difficult to quantitate hydroxyproline due to the interference of aspartic acid.

Hydroxyproline. A second determination of hydroxyproline content was obtained for *ligamentum nuchae*, aorta, and muscle tissues using the procedure (Method II) outlined by Woessner (1961).

Percent Recovery of Elastin. Elastin previously isolated from muscle tissue was subjected to identical isolation procedures as detailed previously to establish quantitative accuracy in determining elastin content from muscles. Realizing that it was impossible to duplicate the biological conditions under which the elastin was originally isolated from muscle, such data should provide an indication of the relative accuracy of the procedure.

RESULTS AND DISCUSSION

Evidence for proof of purity is one of the necessary stages in the development of a procedure for the quantitation of a specific protein found in heterogeneous substances. The more obvious methods for verification of the purity of a protein *via* solubility properties cannot be used because elastin is insoluble in its native state. Various staining techniques have proven useful in identifying preparations of elastin, but the staining characteristics of elastin can be altered when it is exposed to prolonged acid or alkaline treatment (Partridge, 1962). Partridge *et al.* (1955) used alkaline and acid extractions to yield a product of constant weight and constant amino acid composition which gave the staining reactions typical for elastin fibers. The

latter researchers concluded that this evidence was sufficient to provide proof that the isolate fraction was a chemically homogeneous protein.

In the present study, the amino acid composition of elastin isolated from muscle tissue was compared with that of elastin isolated from *ligamentum nuchae* and aorta tissues. Techniques for purification of progressive severity were applied to the *ligamentum nuchae* and aorta and the amino acid composition of the subsequent products was examined after each procedure to determine if a protein of constant composition resulted from each procedural step. Since severe extraction procedures could alter the composition and chemical properties of elastin, the purified protein preparations were examined for changes brought about by partial hydrolysis. Such changes should be reflected by changes in the dye binding capacity.

The results of amino acid analyses of material prepared from three types (four sources) of tissue from a young animal (388 days of age at slaughter) are presented in Table I. Elastin was prepared by autoclaving at a neutral pH, by extraction with 0.5 *N* NaOH at 25°, or by extraction with 0.1 *N* NaOH at 98°. Amino acid analyses for the *ligamentum nuchae* revealed that the protein fraction prepared by either type of alkali extraction was generally similar in amino acid composition to that produced by autoclaving alone. Elastin preparations from the *triceps brachii* and *biceps femoris* muscles were essentially the same in amino acid composition as that of *ligamentum nuchae* treated in the same manner. The composition of muscle elastin is closest in composition to that from *ligamentum nuchae* produced by extraction in 0.1 *N* NaOH at 98° for 45 min. The minor differences in amino acid composition observed between *ligamentum nuchae* elastin prepared by extraction with 0.1 *N* NaOH at 25° and elastin prepared by extraction with 0.1 *N* NaOH at 98° provide presumptive evidence that both preparations are relatively pure.

The elastin preparation extracted from aorta by use of alkali at 98° (Table I) was similar in composition to elastin from *ligamentum nuchae* and muscle tissues, but the elastin preparation produced by autoclaving at neutral pH had a higher content of lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, methionine, and tyrosine, and a lower content of proline, glycine, alanine, and valine than either of the other tissues. This suggests that the fraction yielded by autoclaving the aorta at neutral pH contained elastic fibers and a contaminating protein which had an amino acid composition which was very different from that of elastin. Analyses of the protein from alkaline hydrolysates revealed that the contaminating substance was removed by treatment with hot alkali. The attempt to extract autoclaved aorta elastin with cold dilute alkali (0.5 *N* NaOH) resulted in considerable purification, but clearly did not remove all of the contaminants. The fraction produced by autoclaving and extraction with 0.1 *N* NaOH at 98° was similar in amino acid composition to that determined for samples of ligament and muscle tissue.

The amino acid analyses from three tissues derived from a mature animal (3660 days of age at slaughter) and prepared in the same manner as that previously described and reported for a young bovine are also reported in Table I. With the exception of percent lysine, the amino acid composition of "mature" *ligamentum nuchae* is essentially identical to that from the same tissue in the young bovine. Miller *et al.* (1964), working with aorta from chickens of varying ages (ranging from a 12-day embryo to a chicken which was 1 year of age), demonstrated that the lysine content of elastin decreased with advancing age. Partridge *et al.* (1966) studied the biosynthesis of desmosine and isodesmosine and reported that four molecules of lysine were incorporated into each of these unique amino acids. These authors also demonstrated that the cyclization process was slow, which provides an explanation for

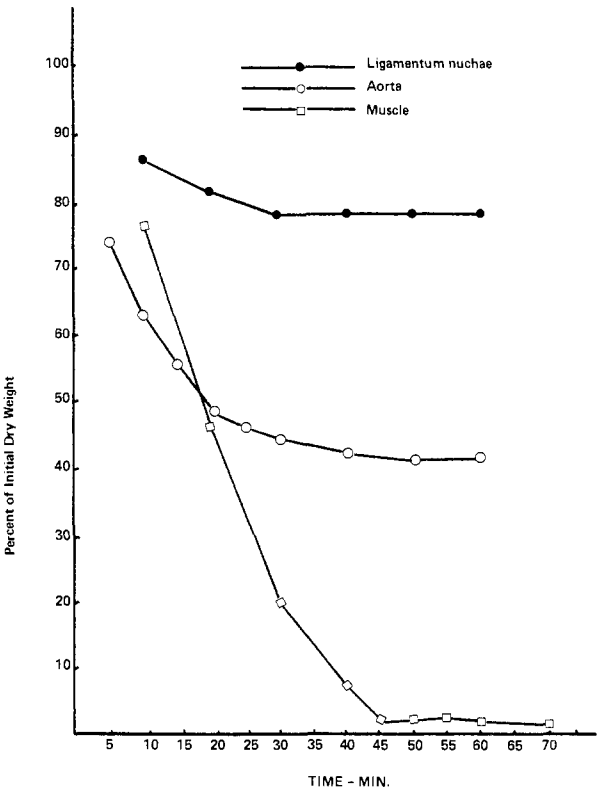

Figure 1. Time course for rate of removal of material other than elastin from the *ligamentum nuchae*, aorta, and muscle stroma. Samples were extracted in 0.1 *N* NaOH at 98°.

the observed variation in the lysine concentration of elastin from animals of different ages. In the present study, desmosine and isodesmosine concentrations were not determined, since pure samples of these amino acids, necessary for use in standardizing the amino acid analyzer, could not be obtained. Elastin from the aorta of the older animal did not decrease in lysine content to the extent observed for the ligament or muscle from the older animal, thus indicating either a lesser degree of crosslinking or a lesser degree of purification for the aorta sample. As was observed for the elastin from the young bovine, the elastin from mature muscle is very similar to that prepared from the hot (98° alkali-treated) *ligamentum nuchae* and aorta. The hydroxyproline values reported in Table I are very similar to those reported by Gotte *et al.* (1963), but lower than the 1.9% value reported by Neuman and Logan (1950) for assays of the *ligamentum nuchae* and aorta of cattle. The latter researchers used a colorimetric procedure which may not have the same degree of sensitivity as that of an amino acid analyzer. The values in Table I were somewhat higher than those obtained from the same tissue using the Woessner (1961) procedure (1.5 *vs.* 1.3%). The average hydroxyproline value for elastin from muscle tissue was 1.50%.

The results of the elastin staining studies, using the procedures of Romeis (1948), Fullmer and Lillie (1956), and Humason and Lushbaugh (1960) and elastin extracted from muscle by use of hot alkali, were negative for collagen and reticulin and positive for elastin. In view of the amino acid composition data reported previously for muscle isolates, it would not seem likely that contaminates detectable by staining techniques were present.

Differences in the rate of alkaline hydrolysis and removal of substances other than elastin for three tissue sources are presented in Figure 1. The *ligamentum nuchae* and aorta were defatted with acetone, powdered, and dried in a vacuum desiccator, while the muscle stroma fraction was defatted for 2 hr in 2:1 chloroform–methanol and dried in a vacuum desiccator. *Ligamentum nuchae*, which

Table II. Absorption of Dyes from Solution by Elastin Fiber Preparations from Bovine *Ligamentum nuchae* and *Biceps femoris*

Dye solution	Wavelength of dye, mμ	Absorbance[b] of dye	Absorbance of the supernatant[a]								
			Ligamentum nuchae						*Biceps femoris*, autoclaving with 0.1 N NaOH at 98°		
			Control, autoclaved at neutral pH	Autoclaving with 0.1 N NaOH at 98°		Autoclaving with at 0.5 N NaOH at 25°					
				30 min	45 min	4 hr	8 hr	24 hr	48 hr	50 min	60 min	80 min
Fast green	625	1.60	0.34	0.32	0.28	0.24	0.22	0.18	0.15	0.30	0.27	0.22
Methyl green	630	0.36	0.15	0.15	0.13	0.09	0.08	0.05	0.01	0.14	0.13	0.10
Aniline blue	600	0.31	0.08	0.08	0.09	0.10	0.15	0.20	0.40	0.10	0.10	0.13
Orcein	500	0.90	0.59	0.62	0.62	0.65	0.66	0.68	0.69	0.62	0.64	0.65
Bromphenol blue	590	1.78	0.54	0.60	0.60	0.64	0.69	0.71	0.78	0.61	0.63	0.70

[a] Decreased absorbance of the supernatant is indicative of increased absorption by elastin. [b] Measured at the wavelength of maximum absorption for the specific dye used.

Table III. Recovery of Muscle Elastin[a]

Elastin added, mg[b]	Elastin recovered, mg	% recovery
100	95.1	95.1
50	48.2	96.3
25	24.3	97.1
10	9.6	96.0
5	4.8	96.5

[a] The added elastin was subjected to the procedure outlined for the isolation of elastin tissue from muscle. [b] The added elastin originated from muscle tissue previously subjected to the identical isolation procedure.

contained approximately 78% elastin, reached constancy of weight within 30 min of hydrolysis in 0.1 *N* NaOH at 98°. Aorta (42% elastin) required 50 min, while muscle stroma (2.5% elastin) required only 45 min to achieve constant weights. Since the amino acid composition of the three samples is very similar, differences in the rate of alkali hydrolysis could be ascribed either to differences in the mean diameter of the elastin fibers or to the presence of contaminating proteins. Since Partridge *et al.* (1963) reported that there are differences in the degree to which elastin samples are cross-linked, the latter property could provide a third explanation for differences in the rate of hydrolysis. Data of the present study indicating high lysine content in aorta from the older animal may indicate less crosslinking of elastin in aorta from old animals.

Because of the cross-linked structure of elastin, it would be expected that any partial hydrolysis that occurs through the use of alkaline or acidic reagents would give rise to rupture points in the chemical framework owing to hydrolysis of susceptible peptide bonds in the backbone chains. Each rupture point should result in the appearance of a new α-carboxyl and a new α-amino group attached to the free ends of the broken chains. A second type of hydrolysis which could be encountered involves the liberation of ammonia from asparagine and glutamine residues. These reactions would give rise to a greater number of charged groups and would be expected to result in a change in the staining characteristics of the fibers.

The uptake of dyes for samples of *ligamentum nuchae* elastin treated for various times with 0.1 and 0.5 *N* NaOH at 98° is presented in Table II. Two groups of dyes which vary in specificity were identified. The first two, fast green and methyl green, are excellent dyes for staining collagen under the conditions used but are relatively ineffective for staining unmodified elastin. However, as the liberation of free α-carboxyl and α-amino groups within the backbone of elastin progresses, the density of staining increases and more of the dye is removed from the solution. The second group of dyes, including aniline blue, orcein, and bromphenol blue, effectively stains the untreated elastin, but mild hydrolytic damage brought about by the action of dilute sodium hydroxide results in a progressively lower uptake of the chromagenic substances.

The ability of the collagen dyes (fast green and methyl green) to effectively stain *ligamentum nuchae* elastin and muscle elastin gradually increased with prolonged treatment (decrease in optical density) and two of the elastin stains (aniline blue and bromphenol blue) began to lose (increase in optical density) their ability to effectively stain elastin with prolonged treatment periods. The findings presented in Table II indicate that prolonged treatment with dilute sodium hydroxide will result in hydrolysis of the elastin preparation, but hydrolysis is minor up to and beyond the point (50 min) required for removal of material other than elastin from the muscle stroma fraction.

Recovery percentages for elastin which was subjected to identical isolation procedures, as detailed for quantitation of elastin from muscle, are presented in Table III. Realizing that it is impossible to duplicate the biological conditions under which the elastin was originally isolated from muscle, these data provide an indication of the relative accuracy of the procedure. Percent recovery values were all relatively high. The minor losses which were noted can be attributed either to experimental error or to loss due to continued hydrolysis in hot alkali. The procedure identified in the present study for the isolation and quantitation of elastin from muscle tissue is supported by presumptive evidence of purity, and empirical evidence suggests that percents of recovery exceeding 95% are possible in isolating elastin from bovine muscle.

Several conclusions can be drawn from the preceding data concerning the isolation and purification of elastin from different tissues. The ease with which elastin can be purified varies considerably with the source, but by the use of extraction procedures of increasing severity, the end product from tissues of different kinds and from animals of different ages approaches constancy of composition. Samples of elastin purified by prolonged contact with dilute sodium hydroxide displayed signs of hydrolytic damage which were revealed by changes in absorption capacity for various dyes. It can be concluded that the procedure outlined for isolating elastin from muscle tissue yields a material that is relatively constant in amino acid composition and varies little with tissue source or age.

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Oxidation of Sodium [U-^{14}C]Palmitate into Carbonyl Compounds by *Penicillium roqueforti* Spores

Clemence K. Dartey and John E. Kinsella*

Spore suspensions of *P. roqueforti* metabolized sodium palmitate into carbon dioxide and a variety of carbonyl compounds, including a homologous series of methyl ketones. Both D-glucose and L-proline suppressed the catabolism of palmitate to CO_2 but stimulated the rate of formation of carbonyl compounds, including methyl ketones. Spore concentrations of 6.3×10^8 spores/ml produced the optimum yield of (32.4%) carbonyl compounds from the incubation of 5 m*M* of palmitate in the presence of 20 m*M* of D-glucose under optimum conditions of pH and temperature; *i.e.*, pH 6.5 (0.1 *M* phosphate buffer) and 30°. Analyses of carbonyl compounds formed from [U-^{14}C]palmitate revealed that a homologous series of labeled methyl ketones, C3 to C15 inclusive, was produced. Pentadecanone contained the highest radioactivity, followed by tridecanone and undecanone, with the lower methyl ketones containing varying amounts of radioactivity. Appreciable amounts of carbonyl compounds other than methyl ketones (*i.e.*, labeled, saturated, and unsaturated aldehydes) were also produced from [^{14}C]palmitic acid.

Growing cultures of *Penicillium roqueforti* oxidize fatty acids into their corresponding methyl ketones with one less carbon atom (Franke and Heinen, 1958; Hammer and Bryant, 1937; Lawrence, 1966; Stärkle, 1924; Stokoe, 1928). Gehrig and Knight (1958) reported that only the spores of *P. roqueforti* oxidize fatty acids; however, Rolinson (1954), Vinze and Ghosh (1962), and Lawrence and Hawke (1968) demonstrated the oxidation of fatty acids into methyl ketones by the mycelium of *P. roqueforti*.

The addition of simple sugars (including D-glucose) and amino acids (including L-proline) stimulates oxygen uptake and formation of 2-heptanone from octanoic acid by the spores of *P. roqueforti* (Lawrence, 1965a). The formation of methyl ketones increased when metabolic carbon dioxide was retained in the incubation medium, but nonaeration of the incubation system decreased fatty acid oxidation (Lawrence, 1966). Enrichment of air with carbon dioxide increased the growth of *P. roqueforti*; however, these increases showed variation with both temperature and strain of the organism (Golding, 1940).

In contrast to mammalian, plant, and bacterial systems, a fatty acid oxidation system has not been isolated from fungi because of difficulty in disrupting the mycelia and obtaining cell-free active enzymes. However, a number of investigators showed that *P. roqueforti* oxidizes fatty acids *via* β-oxidation (Gehrig and Knight, 1963; Hammer and Bryant, 1937; Katz and Chaikoff, 1955; Lawrence, 1966; Lawrence and Hawke, 1968; Stärkle, 1924). These investigators agreed that both the β-oxidation reaction and ketone formation proceed simultaneously. The β-oxoacyl-CoA formed by β-oxidation reaction is deacylated and subsequently decarboxylated to a methyl ketone. The acetyl-CoA formed by complete β-oxidation is further oxidized *via* the tricarboxylic acid cycle to CO_2 and H_2O.

Fatty acids are actually toxic to *P. roqueforti* and the degree of toxicity depends upon chain length, concentration of acids, and the pH of the incubation medium (Franke *et al.*, 1962; Lawrence, 1966; Lawrence and Hawke, 1968). Pressman and Lardy (1956) reported that the saturated fatty acids uncouple phosphorylation and myristic acid exhibited the greatest effect, which decreased progressively from myristic acid as the fatty acid carbon chain was lengthened or shortened. Thus, it has been suggested that conversion of fatty acids to methyl ketones may be a detoxifying mechanism.

P. roqueforti is important in the manufacture of Blue and Roquefort type cheeses. Methyl ketones are the principal compounds responsible for the unique flavor of Blue cheese (Anderson, 1966; Day, 1965; Dartey and Kinsella, 1971; Hawke, 1966; Patton, 1950). Metabolism of milk fat by *P. roqueforti* and the production of methyl ketones is important in the development of Blue cheese flavor. Although palmitic acid is the major fatty acid of milk fat, little is known of its metabolism by *P. roqueforti* during cheese ripening. Palmitic acid may serve as a precursor for some of the methyl ketones found in the cheese. While the major methyl ketones are presumably derived directly from the corresponding fatty acids by oxidation, the concentrations of C7 and C9 methyl ketones exceed the molar ratios of the corresponding C8 and C10 fatty acids in milk fat, indicating their possible derivation from longer chain fatty acids.

In this study the factors affecting the metabolism of palmitic acid by the spores of *P. roqueforti* were deter-

Department of Food Science, Cornell University, Ithaca, New York 14850.