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Determination of the Myofibrillar and Connective Tissue Proteins in the Bovine Diaphragm[1]

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The contents of myosin, actin, collagen, and elastin of the dome and costal regions of the adult bovine diaphragm and the separated intracellular and extracellular fractions have been determined by use of analytical chromatographic methods developed to quantitate the unique amino acids that occur in these proteins. This proposed direct chemical approach is based on the determination of the myofibrillar myosin and actin and collagen contents of skeletal muscles from the amounts of N^{τ}-methylhistidine and 5-hydroxylysine found in their acid hydrolysates, respectively. Elastin can also be determined from the amounts of desmosine or isodesmosine found. The costal diaphragm contained 11.0% actin and 23.0% myosin, corresponding to 21.1% and 44.0% of the myofibrillar proteins (52.3% of the total protein), 43.0% other SDS-soluble intracellular proteins, and 4.73% SDS-insoluble extracellular muscle proteins, corresponding to 2.62% collagen, 0.13% elastin, and 1.8% transcellular matrix proteins.

The quantitative determination of the myofibrillar and connective tissue proteins in vertebrate skeletal muscle tissues has always been a difficult analytical problem. However, in order to understand the molecular and cellular mechanisms involved during muscle contraction and cell development, under both normal and myopathic conditions, it is necessary to quantitatively establish the levels of these muscle proteins in either skeletal muscle tissues, meats, or tissue cell culture specimens. One major method presently in use for determining the myofibrillar myosin and actin contents of muscle tissues involves the quantitative extraction, solubilization, and separation of the contractile proteins by one- or two-dimensional electrophoresis on polyacrylamide gels (Hanson and Huxley, 1957; Szent-Gyorgi et al., 1955; Potter, 1974; Yates and Greaser, 1983; Murakami and Uchida, 1985), followed by densitometry. The results obtained by this method of quantitating contractile proteins in muscles showed considerable differences in their myosin and actin contents. Yates and Greaser (1983) have discussed the problems associated with this method of quantitative contractile proteins and pointed out that most of the variation was attributed to densitometric protein measurements of stained gels because more than one protein frequently comigrates in a single band. The quantitation of protein concentration in the stained protein bands has also been carried out by eluting the separated myosin subunits and actin bands and determining their concentrations either from the extracted dye (Murakami and Uchida, 1985) or by amino acid analysis corrected for comigrating protein bands (Yates and Greaser, 1983). These measurements have shown that the molar ratio of myosin to actin in skeletal muscles is 1.0:6.0 (Potter, 1974; Murakami and Uchida, 1985). Other methods used for determining the myosin to actin mass ratios have shown similar variations (Huxley and Hanson, 1957), primarily due to differences in the myofibrillar volume and protein content of the myofibrils among muscle types, as determined by quantitative electron microscopy (Eisenberg and Kuda, 1976; Page and Surayk-Droske, 1979).

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The quantitation of N^{τ}-methylhistidine [His(τ-Me)], a unique basic amino acid found in both myosin and actin (Elzinga et al., 1973; Vandekerckhove and Weber, 1978, 1979; Elzinga and Collins, 1977; Huszar, 1984; Maita et al., 1987), has been proposed as an index for determining these two principal myofibrillar proteins in tissues (Perry, 1970; Hibbert and Lawrie, 1972) and their turnover rates in skeletal muscle (Young and Munro, 1978; Harris and Milne, 1981, 1987). This quantitation is based on three major findings: that actin and its isoforms contain 1 mol of His(τ-Me) at position 73 in their amino acid sequence (Elzinga et al., 1973; Vandekerckhove and Weber, 1978; 1979; Vandekerckhove et al., 1986); that myosin prepared from fast-twitch white muscle fibers contains 1 mol of His(τ-Me) at position 755 in each of its two heavy chains (Maita et al., 1987); and that His(τ-Me) is absent from all other proteins (Huszar, 1984; Zarkadas et al., 1988). Various measurements of the His(τ-Me) content of vertebrate skeletal muscle made by a variety of chromatographic methods (Fitch et al., 1986; Hancock and Harding, 1984; Ashworth, 1987) have yielded variable amounts of His(τ-Me) among the muscle tissues studied (Asatoor and Armstrong, 1967; Haverberg et al., 1975). Although some of this variation was attributed to the presence of variable amounts of fast, slow, or mixed fast/slow classes of myosin heavy-chain (MHC) isoforms in different muscle types (Huszar, 1984), most of this variation was due to incomplete separation of such small amounts of His(τ-Me) from other compounds present in such complex tissue hydrolysates by the multicolumn systems employed (Hancock and Harding, 1984). Another complication has been the presence in skeletal muscles of variable amounts of balenine (Carnegie et al., 1983; Harris and Milne, 1987), a histidine dipeptide, β-alanyl-L-N^{τ}-methylhistidine, which must be extracted from muscle tissues prior to acid hydrolysis.

The extracellular connective tissue proteins of skeletal muscle, i.e., collagen, elastin, muscle fiber ghosts proteins, proteoglycans, etc. (Hay, 1981; Miller and Gay, 1982; Foster, 1982; Paz et al., 1982; Loewy et al., 1983; Carrino and Caplan, 1983, 1986; Cheah, 1985), which are involved in maintaining proper alignment of the muscle fibers, have also been implicated in a variety of morphogenetic and developmental processes (Wiens et al., 1984; Parthasarathy and Tanzer, 1987) and can influence either the synthesis of non-actin myofibrillar proteins or the assembly of cardiac contractile proteins into myofibrils (Wiens et al., 1984). Thus, the anatomical arrangement of the connective tissue in each level of muscle organization, i.e., epimysium, perimysium, and endomysium, obviously influences the function of the muscle (Borg and Caulfield, 1980; Kovanen et al., 1984; Sanes, 1986; Anmailley and Timpl, 1986). Low levels of 5-hydroxylysine [Lys(5-OH)] and structural collagen defects have for example been reported recently as prime factors in avian muscular dystrophy (De Michele et al., 1985).

The purpose of the present study was to quantitatively establish the levels and variation of all methylated basic amino acids, Lys(5-OH), desmosine (Des), isodesmosine (iDes), and related compounds in the adult bovine diaphragm and the two major muscle protein and connective tissue fractions, prepared and quantitative by the methods of McCollester (1962), McCollester and Semente (1964), and Laurent et al. (1981). The bovine diaphragm was selected for this work since this skeletal muscle has a similar function in all mammals, and although the presence of all three types of fibers has been indicated, this muscle has a majority of slow-twitch red fibers (type I) and a capacity for combined aerobic and anaerobic metabolism (Gauthier et al., 1978; Davies and Gunn, 1972; Gauthier and Lowey, 1979; Carraro et al., 1983, 1985). The aim was to determine whether the levels of these unique basic amino acids in the diaphragm could be used for the determination of its myofibrillar and connective tissue proteins. These calculations are based on the total protein content of the diaphragm and the isolated intracellular muscle protein and extracellular matrix fractions determined by their detailed amino acid compositions.

MATERIALS AND METHODS

Materials. Types DC-4A (Lot No. 750) and DC-5A (Lot No. 746) cation-exchange spherical resins, sized to 9.0 ± 0.5 and 6.0 ± 0.5 μm, respectively, were purchased from Dionex Chemical Co., Sunnyvale, CA. The unusual amino acid standards were obtained as follows: N^6-lysinoalanine [N^6-(DL-2-amino-2-carboxyethyl)-L-lysine] from Miles Analytical Laboratories, Inc., Elkart, IN; the diastereoisomer mixture of 5-hydroxy-DL-lysine, N^6-methyl-L-lysine, N^6,N^6-dimethyl-L- and N^6,N^6,N^6-trimethyl-L-lysine bis(*p*-hydroxyazobenzenesulfonate) hydrate, N^{τ}-methyl-L-histidine, N^{τ}-methyl-L-histidine hydrate, N^{ω}-methyl-L-, N^{ω},N^{ω}-dimethyl-L-, and N^{ω},N^{ω}-dimethyl-L-arginine bis(*p*-hydroxyazobenzenesulfonate), D-glucosamine monohydrochloride, D-galactosamine monohydrochlorite, and 4-hydroxyproline from Calbiochem-Behring Corp., La Jolla, CA; DL-ornithine (5-aminonorvaline) from Schwarz/Mann, Orangeburg, NY; norleucine and L-2-amino-3-guanidinopropionic acid from Pierce Chemical Co., Rockford, IL; 3-nitro-L-tyrosine from Aldrich Chemical Co., Milwaukee, WI. The standard amino acid calibration mixture was purchased from Beckman Instruments, Inc., Palo Alto, CA. Bovine ligamentum nuchae elastin was purchased from Sigma, and Des and iDes were prepared as described previously (Zarkadas, 1979). All reagents and buffers were made with high-purity laboratory water (Zarkadas et al., 1987b) with use of activated carbon beds, mixed ion exchangers, glass distillation, and further deionization steps. All other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Experimental Procedures. *Sampling and Preparation of Muscle Tissue.* Samples of bovine diaphragm were excised as 5-cm-thick muscle sections from the left side of six commercial carcasses weighing approximately 270 kg each and were randomly selected from mature (8-year-old) Holstein–Friesian cows (Canada Grade C1) obtained from Abattoir Soulange, Les Cedres, Quebec. The samples (200 g each) were cleaned of adhering fat, cut into small cubes, ground, frozen (−173 °C), and lyophilized. They were then pulverized in an electric driven end runner coffee mill (Moulinex Canada Ltd., Weston, Ontario) to pass through a 40-mesh screen and stored at −20 °C until needed. Fresh muscle specimens of the costal diaphragm, however, were used for the preparation of intracellular and extracellular muscle protein fractions.

Extraction Procedure for the Dome Diaphragm. The samples used for the amino acid analysis of the diaphragm before and after extraction were excised primarily from the dome-shaped portion of this skeletal muscle, which is largely tendinous because of its proximity to the pleura. To effectively remove all traces of soluble histidine dipeptides, including balenine, a β-alanyl-L-N^{τ}-methylhistidine, known to be present in certain muscle tissues (Carnegie et al., 1983; Harris and Milne, 1981, 1987), samples (10 g) of the pulverized diaphragm were extracted with a mixture of chloroform, methanol, and water essentially as described by Bligh and Dyer (1959). The only

minor modification introduced was the use of a VirTis Model 45 homogenizer (VirTis, Gardiner, NY). Since the moisture of the lyophilized samples was low, these were adjusted to a final moisture of 80 ± 1%. The volumes of chloroform–methanol–water before and after dilution were kept at the specified levels of 1/2/0.8 and 2/2/1.8, respectively. The dilipidated muscle tissue in the methanolic layer was recovered by filtration, and the extraction procedure was repeated two more times. The muscle tissue residue was dried overnight at room temperature, ground and sieved to pass through a 40-mesh screen, and stored at −20 °C until needed for analysis.

Preparation of Intracellular SDS-Soluble (F1) and Extracellular SDS-Insoluble Protein (F2) Fractions from Costal Diaphragm. The selected specimens for this experiment were excised fresh from the periphery and the two crura (roots) of the costal region of the bovine diaphragm attached ventral to the lumbar vertebrae and consist primarily of striated skeletal muscle. The procedure employed for the extraction and preparation of a sodium dodecyl sulfate (SDS) soluble intracellular muscle protein fraction (F1) and an SDS-insoluble extracellular matrix protein fraction (F2) combines the original method of McCollester (1962) and McCollester and Semente (1964) and the procedures described by Laurent et al. (1981) as follows: Approximately 200 g of bovine skeletal diaphragm samples, excised fresh from mature Holstein–Freisian cows as before, were cut into small cubes and ground in an electric end runner mill. Approximately 10 g of ground and well-mixed samples was homogenized in 100 mL of 0.50 M $CaCl_2$ solution in a VirTis homogenizer (Model 45) for 10 s (full speed) at 4 °C. The homogenate was centrifuged at 4000*g* (SS-34 Sorvall rotor; 6000 rpm) for 20 min at 4 °C, and the supernatant was decanted through eight layers of cheesecloth to trap fat particles and retained at 4 °C so that muscle proteins could be precipitated with 5% trichloroacetic acid (TCA). The pellet was resuspended in 70 mL of phosphate-buffered saline (PBS: 0.15 M NaCl/0.02 M sodium phosphate buffer, pH 7.4) at 37 °C for 30 min; an equal volume of distilled water was added and rehomogenized for 3 min. The homogenate was again centrifuged at 4000*g* for 20 min, and the supernatants were combined.

The pellet was resuspended in 70 mL of 2% sodium dodecyl sulfate and rehomogenized for 3 min, and the supernatants were combined. The wash procedure with SDS was repeated a further four times as recommended for lung tissues by Laurent et al. (1981). The remaining pellet was then extracted a further three times with 70 mL of phosphate-buffered saline (PBS; pH 7.4) to remove the bulk of the SDS. The supernatants were combined, and the intracellular SDS-soluble skeletal muscle protein fraction (F1) was recovered by precipitation with 5% trichloroacetic acid and centrifugated as before. The precipitated muscle proteins (F1) were desalted by exhaustive dialysis at 4 °C against distilled water, 40% methanol (v/v), and again distilled water, freeze-dried, weighed, and stored at −75 °C. The pellet containing the SDS-insoluble connective tissue proteins (F2) was then rehomogenized in acetone and centrifuged at 4000*g* for 5 min at 4 °C and the supernatant discarded. This step was repeated and the pellet was dialyzed as before, dried under vacuum, weighed, powdered in the end runner mill to pass a 2-mm screen, and stored at −75 °C.

Procedures for Amino Acid Analyses. Amino acid analyses were carried out on either a conventional (Beckman Model 120C) or a fully automated amino acid analyzer (equivalent to Beckman Spinco Model 121MB). The automated instrument was equipped with a Varian Vista 402 chromatographic data reduction system (Varian Instruments Group, Walnut Creek, CA) to increase the sensitivity of the analysis and to enable quantitation of amino acids at the picomole level as described previously (Zarkadas et al., 1987b).

Complete amino acid analyses were carried out on each of the bovine diaphragm samples, before and after extraction, and the two major fractions, F1 and F2, prepared from three different animals. Bovine diaphragm muscle tissue samples (0.1 g) were hydrolyzed in Pyrex test tubes (18 × 150 mm) under vacuum (below 10 μmHg) with 10 mL of triple-glass-distilled constant-boiling HCl (6.0 M) at 110 °C in duplicate for 24, 48, 72, and 96 h, respectively, with the usual precautions described by Moore and Stein (1963) and Hunt (1985). The small amounts of insoluble materials formed during acid hydrolysis were removed by filtration (0.22-μm Millipore microfilters; Millipore Corp., Bedford, MA) and were washed with the same acid (6.0 M HCl). Foaming of hydrolysates was suppressed during evacuation by the addition of 5–10 μL of octanoic acid. The clear filtrate and washings were combined, evaporated to dryness in a Rotary Evapomix (Buchler Instruments, Fort Lee, NJ) at 45 °C, and brought to volume with 0.2 M sodium citrate buffer, pH 2.2. Norleucine and 3-nitrotyrosine, selected as the internal standards, were included in this step or prior to hydrolysis (Zarkadas et al., 1987b).

All samples were analyzed by the standard procedure described previously (Zarkadas et al., 1987b). The data reported for serine and threonine represent the average of values extrapolated to zero time of hydrolysis. Addition of phenol (20–15 μL) to the hydrolysates usually prevented chlorination of tyrosine. The values for valine, isoleucine, leucine, and phenylalanine are averages of data from 48, 72, and 96 h of hydrolysis. All others are reported as the average values from 24, 48, 72, and 96 h of hydrolysis. 4-Hydroxyproline was determined separately from a concentrated 24-h hydrolysate (equivalent to 0.1 mg of protein/analysis) using a single column (21 × 0.6 cm) packed with Dionex type DC-6A resin (Zarkadas et al., 1986). Recoveries of 4-hydroxyproline were calculated relative to alanine (Berg, 1982).

Methionine and cysteine were determined in separate samples (0.1 g) as their oxidation products by the performic acid procedure of Moore (1963). Tryptophan in the diaphragm muscle samples (0.1 g) was determined separately after alkaline hydrolysis (Hugli and Moore, 1972) by an improved chromatographic procedure using 3-nitrotyrosine [Tyr(NO_2)] as an internal standard (Zarkadas et al., 1986).

Determinations of the methylated basic amino acids, the diastereoisomers of Lys(5-OH), and related compounds were carried out with concentrated 96-h hydrolysates (equivalent to 100–250 μg of protein/analysis) by the single-microcolumn (50 × 0.28 cm) system using Dionex DC-4A (Zarkadas et al., 1987b) so that peaks adequate for these components could be obtained. The unusual amino acid calibration standards employed for peak identification and standardization of the instrument were prepared essentially as described previously, with Tyr(NO_2) as the internal standard. Recoveries of these unique basic amino acids were calculated on the protein content of each 96-h hydrolysate determined by the procedure described by Horstmann (1979).

Statistical Analysis. Data processing and linear regression analysis of the results were carried out by a Fortran computer program developed for this purpose. Analysis of variance conducted on the amino acid data for

a completely randomized block design (factorial) was carried out by the general linear model procedure (SAS, 1981), using the computing centre (VAX) of Datacrown, Inc., IBM, Toronto, Ontario.

Theory of the Method. *Calculation of the Protein Mass in the Diaphragm.* The protein mass of each acid hydrolysate was determined by the procedure described by Horstmann (1979). This method is based upon knowledge of the amino acid composition of the diaphragm and yields accurate estimates of the amount of protein present. According to this method, a mean residue weight (WE, g/mol) is calculated for the amino acids constituting the proteins in the diaphragm as

$$\mathrm{WE} = \sum_{i=1}^{20}(a_i b_i) \tag{1}$$

where a_i is the mole fraction of an amino acid i found in the analyzed aliquot and b_i is the molecular weight of amino acid residue i as described by Horstmann (1979). A conversion factor (F), which is the apparent average residue molecular weight increased in proportion to the missing tryptophan and cyst(e)ine values and is characteristic for each protein or protein mixture, can be calculated from

$$F = \mathrm{WE}/[1 - (a_{\mathrm{Trp}} + a_{\mathrm{Cys}})] \tag{2}$$

A conversion factor F' (g/mol) was also calculated according to eq 2 but for determining protein mass in the absence of tryptophan, cyst(e)ine, proline, and Pro(4-OH). These factors (F and F') are constants characteristic of each protein or protein mixture.

The amount of protein (P, μg) in each hydrolysate can then be calculated

$$P = F\sum_{i=1}^{18}x_i \tag{3}$$

where x_i values are the nanomoles of each amino acid i found in the analyzed aliquot.

Determination of a Specific Protein in the Diaphragm. The amount of a specific protein j present in the diaphragm and the separated intracellular and extracellular protein fractions can be calculated from the quantitative determination of a given unique amino acid i known to occur exclusively in that specific protein (j), according to

$$P_j = C_i\frac{M_r(P_j)}{n_i M_r(i)} \tag{4}$$

where P_j is the concentration of a specific protein j expressed in grams per kilogram of total protein, $M_r(P_j)$ is the molecular mass of protein j in daltons, c_i is the mean concentration in grams per kilogram of total protein of a unique protein-bound amino acid i found in the analyzed acid hydrolysate of the diaphragm or its protein fractions, n_i is the integer number of amino acid residues i per mole of protein j, and $M_r(i)$ is the anhydrous molecular weight of the unique amino acid i.

An analogous method to calculate the amount of a specific protein in skeletal muscle is

$$P_j = c_i\frac{[1000]}{n_i'}\frac{\mathrm{WE}(P_j)}{M_r(i)} \tag{5}$$

where $\mathrm{WE}(P_j)$ is the weight equivalent of a specific muscle protein j determined from eq 1 as described by Horstmann (1979) and n_i' is the number of amino acid residues, i, per 1000 amino acid residues. The moles of a specific protein per kilogram of total protein (X_j) can thus be calculated by dividing P_j in eq 4 and 5 by $M_r(P_j)$ to give eq 6, which also shows the relation between n_i and n_i'.

$$X_j = \frac{C_i}{M_r(i)}\frac{1}{n_i} = \frac{C_i}{M_r(i)}\frac{1000\mathrm{WE}(P_j)}{n_i' M_r(P_j)} \tag{6}$$

Determination of the Amount of a Mixture of Two Proteins. The amount of two proteins ($P_1 + P_2$) containing the same unique amino acid in both proteins can also be determined from the total amount (C_T) of that amino acid (eq 7). By substituting C_1 and C_2 by the amounts of each

$$C_T = C_1 + C_2 \tag{7}$$

protein from eq 4–6, the following relationships can be derived:

$$\sum_{j=1}^{2}[P_1 + P_2] = \frac{(X_1/X_2)M_r(P_1) + M_r(P_2)}{n_2 + (X_1/X_2)n_1}\frac{C_T}{M_r(i)} \tag{8}$$

$$\sum_{j=1}^{2}[P_1 + P_2] = \frac{(P_1/P_2) + 1}{n_2'/\mathrm{WE}(P_2) + (P_1/P_2)n_1'/\mathrm{WE}(P_1)}\frac{1000C_T}{M_r(i)} \tag{9}$$

The amount of the two proteins is dependent on the relative composition of the two proteins in the sample (X_1/X_2 or P_1/P_2), the number of residues of the unique amino acid (n_i or n_i') in the respective proteins, and the amount of the unique amino acid (C_T). Equations 8 and 9 are analogous to eq 4 and 5 for one protein with a unique amino acid. As $X_1/X_2 \to 0$ or $P_1/P_2 \to 0$, eq 8 and 9 will simplify to eq 4 and 5, respectively.

(a) Determination of Connective Tissue Proteins. In this study, an attempt was made to relate the amounts of the unusual protein-bound amino acids, which occur exclusively in vertebrate connective tissue proteins, i.e., collagen, elastin, etc., to the contents of these extracellular matrix proteins in the diaphragm. In this chemical approach the distribution of elastic fibers (elastin) in this mammalian skeletal muscle tissue could be calculated from the amounts of Des found in the acid hydrolysates of this tissue. Amorphous elastin (Foster, 1982), which was used as standard for comparison, contains 3 residues of Des per 1000 total amino acids, and has a mean residue weight of WE = 85.06 (Table V). The anhydrous $M_r(i)$ of Des is 454.54. The total collagen and collagen-like proteins (Porter and Reid, 1978; Anglister et al., 1976) of the diaphragm (per kilogram of protein) could also be determined from the amounts of the aLys(5-OH) diastereoisomer present in their acid hydrolysates. Since types I and III collagens accounted respectively for 61 and 35% of the recovered collagen in the muscle connective tissue in epimysium, perimysium, and endomysium, while type IV collagen accounted for the remaining 5% (Light and Champion, 1984; Light et al., 1985; Light, 1985), a mean for the diastereoisomers of Lys(5-OH) content of $n_i' = 10.0$ residues per 1000 total amino acid residues in muscle collagen could be computed from the relative distribution of collagen types and their respective Lys(5-OH) contents presented in Table I. The average residue weight (WE) for collagen is 91.1, and each of the diastereoisomers of Lys(5-OH) has an anhydrous M_r of 145.18. The following analytical conventions, derived from eq 5, can therefore be used for calculating

$$\text{amt of collagen } (P_C) = \text{amt of Lys(5-OH)} \times 63.3 \tag{5a}$$

$$\text{amt of elastin } (P_E) = \text{amt of Des} \times 62.4 \tag{5b}$$

The amount of total connective tissue in the diaphragm (in grams per kilogram of total protein) could then be calculated from the sum of collagen (P_C) and elastin (P_E) found in this tissue. An alternative method for calculating the amount of collagen and elastin involves substituting

Table I. Average Values for 5-Hydroxylysine and 4-Hydroxyproline Computed from the Known Distribution of Types I, III, and IV Collagens in Skeletal Muscle[a]

collagen type std	chain assoc	av no. res/1000 total res[b]		collagen type distribn,[a] % total muscle collagen	av no. res contrib by collagen type/1000 res	
		Pro(4-OH)	Lys(5-OH)		Pro(4-OH)	Lys(5-OH)
I	$[\alpha1(I)]_2\alpha2(I)$	98	10	61.5	60.3	6.2
III	$[\alpha1(III)]_3$	118	5	33.5	39.5	1.7
IV	$[\alpha1(IV)]_2\alpha2(IV)$	120	42	5.0	6.0	2.1
$\sum_{i=1}^{3} n_i'$					105.8	10.0

[a] Data taken from Light and Champion (1984) and Light et al. (1985). [b] Average values computed from the data of Miller and Gay (1982), Laurent et al. (1981), and Light (1985). (See also Table V.)

into eq 9 the total amount of Pro(4-OH) found in both proteins (Berg, 1982; Etherington and Sims, 1981; Rucker, 1982), the known Pro(4OH) contents of collagen (n_i' = 105.8; see Table I) and elastin (n_i' = 22; see Table V), the relative distribution (P_1/P_2) of these proteins per unit of tissue calculated from the Lys(5-OH) and Des contents (eq 5a and 5b), and the anhydrous molecular weight ($M_r(i)$ = 113.12) of Pro(4-OH).

(*b*) *Determination of the Myofibrillar Proteins Myosin and Actin.* The quantitation of protein-bound His(τ-Me), a unique basic amino acid known to occur exclusively in myosin and actin (Elzinga et al., 1973; Elzinga and Collins, 1977; Maita et al., 1987), can be used as an index for determining these two principal myofibrillar proteins in the diaphragm, provided considerable care is taken to extract all soluble histidine dipeptides, especially balenine (Carnegie et al., 1983; Harris and Milne, 1987), prior to acid hydrolysis of this tissue.

Sequence studies (Elzinga et al., 1973; Maita et al., 1987) have shown that 1 mol of actin (A) contains 1 mol of His(τ-Me) and that 1 mol of myosin (M) contains 2 mol of His(τ-Me). Thus, the total amount of His(τ-Me) in the diaphragm determined by the present method (Zarkadas et al., 1987b) represents the sum of the distribution of His(τ-Me) in the myosin and actin present in the diaphragm according to

$$C_T = C_A + C_M \tag{7}$$

where C_T (in grams per kilogram of the total protein) is the total protein-bound His(τ-Me) in myosin and actin, C_A is the amount (g) of His(τ-Me) in actin, and C_M is the amount (g) of His(τ-Me) in myosin in 1 kg of total protein.

The relative amounts of actin (M_r(A) = 41 782 (Elzinga et al., 1973)) and myosin (M_r(M) = 521 000 (Yates and Greaser, 1983)) per unit of tissue, expressed as moles per kilogram of total protein (see eq 6), are related to the distribution of His(τ-Me) in myosin and actin of the diaphragm as

$$\frac{\text{mol actin}}{\text{kg protein}}(A) = C_A\frac{1}{M_r(i)n_A} \tag{6a}$$

$$\frac{\text{mol myosin}}{\text{kg protein}}(M) = C_M\frac{1}{M_r(i)n_M} \tag{6b}$$

where n is the number of residues per mole of protein and $M_r(i)$ = 151.2 represents the anhydrous molecular weight of His(τ-Me).

Since n_A = 1 and n_M = 2, the amount of His(τ-Me) in actin and myosin can be related as

$$C_A = \frac{A}{M}\frac{1}{2}C_M \tag{6c}$$

Substituting C_A or C_M respectively in eq 7, the following relationships can be derived:

$$C_M = [2M/(A + 2M)]C_T \tag{6d}$$

$$C_A = [A/(A + 2M)]C_T \tag{6e}$$

Therefore, the amounts of myosin (P_M) and actin (P_A) (in grams per kilogram of total protein) can be calculated by substituting eq 6d and 6e, respectively, in eq 4 as follows:

$$P_M = C_M[M_r(M)/2M_r(i)] = \left[\frac{M}{A + 2M}\frac{M_r(M)}{M_r(i)}\right]C_T \tag{6f}$$

$$P_A = C_A[M_r(A)/M_r(i)] = \left[\frac{A}{A + 2M}\frac{M_r(A)}{M_r(i)}\right]C_T \tag{6g}$$

Combining eq 6f and 6g, the total amount of actin and myosin in the diaphragm can thus be calculated from the amount (C_T) of His(τ-Me) and the molar ratio of actin and myosin per kilogram of total protein by the following equation, which is analogous to eq 8

$$\sum_{j=1}^{2}[P_A + P_M] = \left[\frac{A/M}{A/M + 2}\frac{41\,782}{151.2} + \frac{1}{A/M + 2}\frac{521\,000}{151.2}\right]C_T \tag{8a}$$

$$\sum_{j=1}^{2}[P_A + P_M] = \left[\frac{276A}{A + 2M} + \frac{3446M}{A + 2M}\right]C_T \tag{8a'}$$

Substituting the molar ratio of actin to myosin reported by Murakami and Uchida (1985) (A:M = 6) in eq 8a, the total amount of actin and myosin in the diaphragm can be calculated as

$$\sum_{j=1}^{2}[P_A + P_M] = [207 + 431]C_T \tag{8b}$$

$$\sum_{j=1}^{2}[P_A + P_M] = 638C_T \tag{8b'}$$

Since the sum of myosin and actin in the myofibril accounts for 65% of the total myofibrillar protein by weight (Yates and Greaser, 1983), the totel myofibrillar protein in grams per kilogram of total protein in the diaphragm can also be calculated as

$$\text{amt myofibrillar protein} = \sum_{j=1}^{2}\frac{[P_A + P_M]}{0.65}C_T = 981C_T \tag{8c}$$

RESULTS AND DISCUSSION

The present studies further investigate the possible use of four unique basic amino acids as markers for determining the specific myofibrillar and connective tissue proteins in vertebrate skeletal muscle. The adult bovine diaphragm chosen as an example of a typical skeletal muscle for these studies is a dome-shaped sheet of skeletal muscle tissue, separating the thoracic and abdominal cavities. The muscle fibers are large and project radially from its central dome near the sternum to the periphery and the two crura of the diaphragm, thus giving at least two specific tissue sites (Gottschall, 1981): the costal region

Table II. Amino Acid Composition (Grams of Amino Acid per Kilogram of Total Proteins) of the Tendinous Dome of the Bovine Diaphragm before and after Solvent Extraction with a Methanol–Chloroform–Water Mixture[a]

	dome-shaped region of the diaphragm					
	control		extracted with methanol–chloroform–water		signif levels between treatments[a]	
amino acid	mean ± SEM[a]	CV[a]	mean ± SEM[a]	CV[a]	CV	F
aspartic acid	75.68 ± 0.48	12.66	85.20 ± 1.55	3.51	5.34	2.42[ns]
threonine	38.79 ± 0.78	4.73	44.52 ± 0.45	1.94	3.76	46.46[ns]
serine	33.62 ± 2.00	1.40	39.63 ± 1.98	9.58	10.18	1186.02*
glutamic acid	131.83 ± 2.22	3.96	135.21 ± 2.93	4.16	2.96	1.41[ns]
proline	67.98 ± 1.88	6.51	56.11 ± 2.26	7.74	3.31	515.21*
glycine	82.10 ± 3.97	11.35	67.97 ± 2.53	7.17	5.39	1.02[ns]
alanine	69.29 ± 1.97	6.67	65.56 ± 0.39	1.14	2.56	0.33[ns]
cysteine	5.57 ± 0.16	6.99	10.38 ± 0.10	1.94	4.43	818.87*
valine	59.01 ± 1.51	5.99	59.62 ± 0.53	1.70	3.91	7.08[ns]
methionine	22.54 ± 0.49	5.19	28.02 ± 0.25	1.73	1.91	19.61[ns]
isoleucine	44.72 ± 0.99	5.23	45.33 ± 0.34	1.45	5.06	0.06[ns]
leucine	82.42 ± 0.99	2.90	82.17 ± 0.66	1.55	2.85	0.98[ns]
tyrosine	33.35 ± 0.85	0.85	38.24 ± 0.45	2.25	2.07	37.85[ns]
phenylalanine	40.93 ± 1.37	7.87	42.34 ± 0.17	0.75	7.04	0.55[ns]
histidine	31.18 ± 1.53	11.55	25.61 ± 0.34	2.56	9.01	139.85*
lysine	78.03 ± 1.86	5.61	78.99 ± 1.67	4.07	5.01	0.04[ns]
arginine	67.89 ± 0.91	3.14	65.43 ± 0.34	0.98	3.15	10.83[ns]
tryptophan	14.77 ± 0.91	14.45	9.45 ± 0.15	3.04	4.54	5.56[ns]
4-hydroxyproline	15.75 ± 5.00	60.81	15.70 ± 1.35	16.52	5.34	0.001[ns]
5-hydroxylysine	1.234 ± 0.146	22.65	1.343 ± 0.11	28.54	4.97	1.40[ns]
isodesmosine	0.548 ± 0.018	6.29	0.761 ± 0.051	12.86	8.21	11.92[ns]
desmosine	0.638 ± 0.035	10.41	1.011 ± 0.014	2.55	4.51	58.12[ns]
ornithine	0.315 ± 0.018	10.91	0.441 ± 0.08	38.89	9.00	0.54[ns]
N^6-methyllysine	0.335 ± 0.006	34.86	0.077 ± 0.01	25.58	5.20	2.75[ns]
N^6,N^6,N^6-trimethyllysine	0.173 ± 0.018	20.86	0.487 ± 0.022	8.54	11.08	58.93[ns]
N^π-methylhistidine	0.893 ± 0.060	12.92	0.0			
N^τ-methylhistidine	0.409 ± 0.018	8.49	0.380 ± 0.004	2.15	4.30	1.15[ns]
ammonia	22.43 ± 1.92	20.10	7.21 ± 2.45	65.04	27.05	7.64[ns]
total AA	1000 ± 30.17		1000 ± 20.84			
total AA N	186.51		169.96			
total protein, g/kg dry wt	814.01 ± 19.72		970.62 ± 9.80			
WE,[b] g/mol	105.233		106.703			
F,[b] g/mol	106.731		108.455			
F',[c] g/mol	117.234		117.597			

[a] Mean values and standard errors of measurements (SEM) for 3 replicates and 48 determinations. Significance: F, values from analysis of variance between treatments; *, $P < 0.05$; ns, not significant; CV, coefficient of variation. [b] The WE and F constrants were calculated according to Horstmann (1979). [c] The conversion factor F' was also calculated according to eq 2 but for determining protein mass in the absence of tryptophan, cyst(e)ine, proline, and 4-hydroxyproline.

and a largely tendinous central dome, which can be compared in each animal species for their myofibrillar and connective tissue contents. Although the presence of all three muscle cell types with fast, mixed fast/slow, and slow properties have been indicated (Gottschall, 1981; Green et al., 1984), the bovine diaphragm has a majority (70%) of slow-twitch red fibers (type I) compared to smaller animals that contain between 60 and 70% mixed (types IIA and IIB) fast-twitch white fibers (Davies and Gunn, 1981; Gauthier et al., 1978; Green et al., 1984). Recent biochemical studies have also shown the presence of fast, slow, or both fast and slow classes of the MHC isoforms or other contractile proteins (Buckingham, 1985; Carraro and Catoni, 1983; Carraro et al., 1985; Gauthier et al., 1982; Gauthier, 1986; Lowey, 1986a, 1986b; Mahdavi et al., 1986). In addition, available evidence indicated that differences in the function of slow and fast muscles could be related to the amount and anatomical arrangement of connective tissue in the perimysium (Borg and Caulfiend, 1980; Kovanen et al., 1984).

To validate the use of His(τ-Me) as an index for determining the myofibrillar myosin and actin contents of skeletal muscles, accurate and detailed determination of the His(τ-Me) content of the bovine diaphragm was carried out at the picomole range by the single-column methodology described previously (Zarkadas et al., 1987b) as follows: first on the largely tendinous central dome region of the diaphragm before and after extraction (Bligh and Dyer, 1959) and second on the intracellular and extracellular muscle protein fractions separated from the costal diaphragm (McCollester, 1962; Laurent et al., 1981). A preliminary note illustrating the analytical capability of the present methodology and its application to complex tissue hydrolysates, i.e., bovine diaphragm, has been presented previously (Zarkadas et al., 1987b). One complication, however, often encountered in the analysis of His(τ-Me) in muscle tissue hydrolysates is the presence of variable amounts of soluble histidine dipeptides, including carnosine, anserine, and balenine, which on acid hydrolysis yield β-alanine, histidine, His(π-Me), and His(τ-Me) (Carnegie et al., 1983). Although the physiological function of anserine and balenine in skeletal muscle tissues has not been established, and only β-alanine and carnosine are now considered as putative neurotransmitters (Griffith, 1986), these dipeptides must be extracted from muscle tissues prior to acid hydrolysis. Thus, Table II summarizes the results obtained on the analysis of acid hydrolysates of the dome region of the diaphragm before and after extraction (Bligh and Dyer, 1959) by the present methodology (Zarkadas et al., 1987b).

The overall amino acid composition of the dome portion of the diaphragm before and after extraction and levels of statistical significance obtained from analysis of variance as presented in Table II represent the average values of three replicates and triplicate determinations obtained from duplicate 24-, 48-, 72-, and 96-h hydrolysates. The

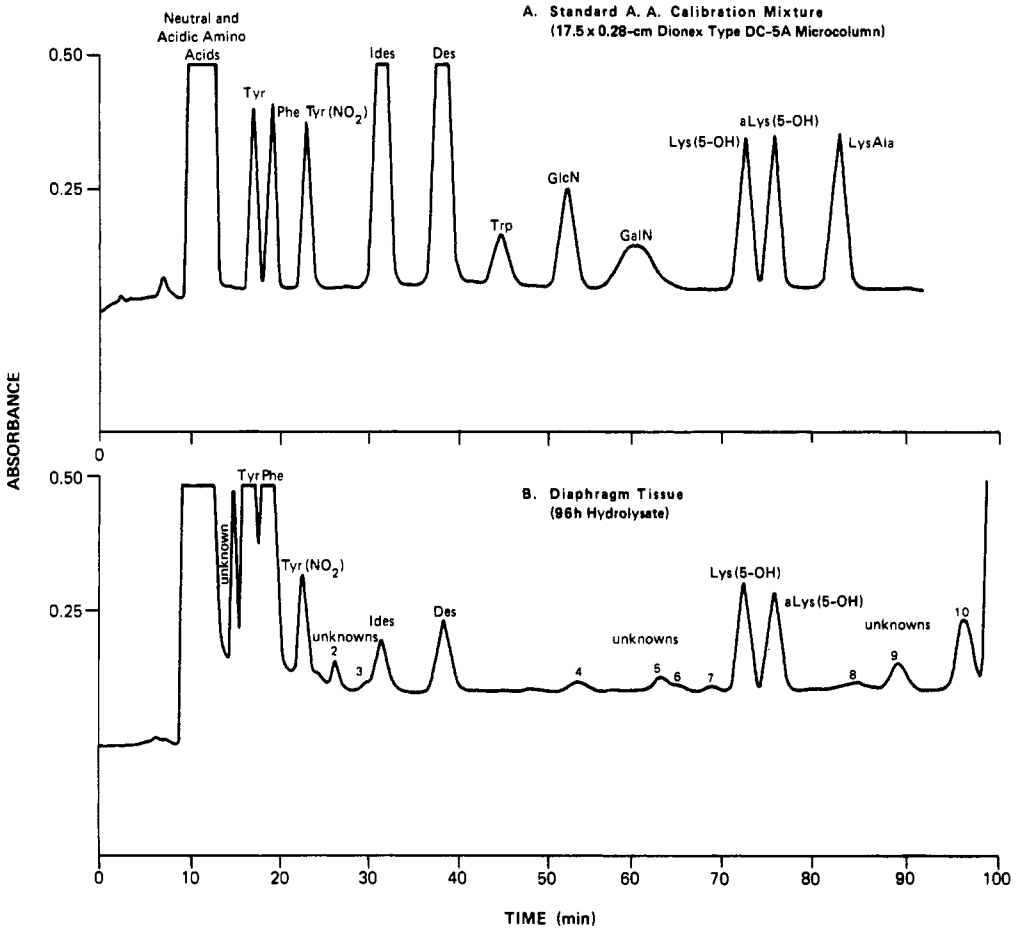

Figure 1. Chromatographic separation of 11 unique basic amino acids (0.5 nmol each) on an analytical 17.5 × 0.28 cm microcolumn of Dionex DC-5A resin: (A) separation of a synthetic amino acid calibration mixture; (B) analysis of a 96-h acid hydrolysate of the dome region of the diaphragm. The curves show absorbance at 570 nm. Key: Tyr(NO_2), 3-nitrotyrosine; iDes, isodesmosine; Des, desmosine; GlcN, glucosamine; GalN, galactosamine; Lys(5-OH), 5-hydroxylysine; aLys(5-OH), *allo*-5-hydroxylysine; LysAla, N^6-lysinoalanine.

values in Table II show deviations of less than 100 ± 2.5% from the average values obtained between three animals within the same treatment. The least variability in tissue amino acid content was found when the results were expressed as grams of amino acid per kilogram of anhydrous and fat- and ash-free tissue protein, since the influence of both fat and moisture (Zarkadas et al., 1987a) is eliminated. The protein concentration of individual hydrolysate samples was determined by the procedure described by Horstmann (1979). The average weight equivalent (WE, g/mol) and conversion factors F and F' (g/mol) obtained are listed in Table II and can be used in all subsequent quantitations of this tissue following standard procedures (Horstmann, 1979). The apparent differences noted in the levels of aspartic acid, glutamic acid, Pro(4-OH), Lys(5-OH), and those amino acids undergoing destruction on acid hydrolysis were not statistically significant. There was, however, significant variation in the content of serine, proline, and cyst(e)ine, and especially histidine between treatments. The result of Table II shows that practically all of the His(π-Me), 18.05% of the total histidine, and 7.22% of His(τ-Me) found in the diaphragm have been effectively extracted at ambient temperatures by this procedure (Bligh and Dyer, 1959), and no doubt most of these as soluble dipeptides. These results are in accord with those reported by Carnegie et al. (1983) for the porcine diaphragm. Quantitative amino acid analysis of the soluble extracts indicated that the total free amino acids and soluble peptides, expressed as protein content, was very small (0.75%).

The values obtained for protein-bound His(τ-Me) in the extracted specimens of the bovine diaphragm show high reproducibility, low coefficient of variation, and within the precision of the chromatographic procedure (100 ± 2.5%), recoveries were found to be quantitative (Table II). The presence of considerable amounts of iDes and Des in the acid hydrolysates of the dome portion of the diaphragm is highly significant. An accurate determination of the levels of iDes, Des, and the diastereoisomers of Lys(5-OH) in acid hydrolysates (96 h) of the dome region of the diaphragm was also carried out by a rapid analytical procedure using a 17 × 0.28 cm microcolumn of Dionex DC-5A resin (Zarkadas et al., 1986). Typical chromatographic separations obtained for iDes and Des on this column are illustrated in Figure 1. As may be seen in Figure 1, the total Lys(5-OH) contents of these samples were from the values obtained for one of its diastereoisomers, aLys(5-OH), after epimerization (96 h). Although previous work from this laboratory showed that the determination of Lys(5-OH) can be made from the sum of the values obtained from its diastereoisomers, after epimerization in 6 M HCl at 110 °C for 96 h (Zarkadas, 1975), in the present study it was found that another unknown ninhydrin-positive compound(s) present in the diaphragm appeared to coelute with Lys(5-OH), thus interfering with its quantitation (Figure 1B). Because of this, the aLys-(5-OH) diastereoisomer is now being routinely used for quantitating this unique to collagen basic amino acid after epimerization. Figure 1B also shows the complete separation of iDes and Des and the separation of a number of

Table III. Elution Times and Contents of Unknown Ninhydrin-Positive Compounds Separated from the Bovine Diaphragm (See Figure 2)

unknown[a] compd	elution time, min	bovine diaphragm, nmol/μg protein mean ± SEM[b]	CV[c]	F[c]
Dome Region:[d] Extracted with Methanol–Chloroform–Water				
2	78	4.00 ± 0.27	13.53	11.76[ns]
3	122	0.46 ± 0.01	2.31	
17	365	4.43 ± 0.95	42.73	1.43[ns]
Costal Region				
Fraction F1: SDS-Solubilized Intracellular Muscle Proteins				
2	77	1.06 ± 0.23	17.88	15.40[ns]
7	137	0.46 ± 0.06	22.62	2.19[ns]
8	149	3.60 ± 0.10	2.51	12.19[ns]
10	211	21.34 ± 0.98	5.36	6.87[ns]
11	238	0.82 ± 0.09	12.24	7.22[ns]
12	255	0.74 ± 0.07	2.30	213.53**
13	261	4.91 ± 0.24	11.02	0.27[ns]
17	365	0.89 ± 0.18	5.49	13.95[ns]
Fraction 2: SDS-Insoluble Extracellular Matrix Proteins				
2	77	1.98 ± 0.21	3.36	119.05**
7	137	3.31 ± 0.02	1.34	0.21[ns]

[a] Analyses of diaphragm hydrolysate samples (100 μL equivalent to 100–400 μg of protein) were carried out as described previously (Zarkadas et al., 1987b). [b] Mean values and standard errors of measurements (SEM) for 3 replicates and 12 determinations. The relative concentrations of the unknown peaks were calculated by assuming a relative response factor (RRF) equal to that of the internal standard (3-nitrotyrosine, RRF = 1,000). [c] Significance: F, values from analysis of variance; **, $P < 0.01$; ns, not significant; CV, coefficient of variation. [d] Values for the control samples have been reported previously (Zarkadas et al., 1987b).

as yet unidentified ninhydrin-positive peaks, designated 2–10. Although the identity of these compounds has not been established, further detailed studies to ascertain the nature of these and the rather large number of other unknown compounds (Table III) are required. This chromatographic procedure has an advantage over other methods in that complete separation of the elastin cross-links and the diastereoisomers of Lys(5-OH) is possible in a single analysis in less than 1.25 h (Zarkadas et al., 1986). Thus, the data presented in Table II indicate that the dome portion of the bovine diaphragm is a highly tendinous muscle tissue containing high levels of collagen and elastin but lower amounts of myofibrillar proteins.

One major limitation of the muscle tissue extraction procedures is that often high incidences of unemptied muscle cell segments are encountered during homogenization. Although the mechanisms responsible for the emptying phenomenon of the muscle cells are not known, it appears that the high incidence of these unemptied cell segments is related to irreversible contraction of muscle cells during homogenization at ambient temperatures (McCollester, 1962; McCollester and Semente, 1964). Because the muscle cell membrane is extremely thin, conventional methods of cellular disruption and extraction are unable to produce a satisfactory dissolution and separation of the intracellular structures from the membranes of contracted muscle tissues. McCollester (1962) has shown that if the homogenization is carried out in 50 mM $CaCl_2$ close to 0 °C followed by a wash with neutral phosphate-buffered saline (PBS; pH 7.4) and incubation at 37 °C for 30 min, the subsequent addition of water to the cell segments produces an abrupt and dramatic dissolution of the remaining intracellular proteins, leaving behind the membranes as empty, thin-wall tubes.

In order to show whether or not soluble amino acids and histidine dipeptides have contributed to the protein-bound His(τ-Me) of the diaphragm reported in Table IV, the intracellular and extracellular muscle protein fractions were separated and prepared as free as possible from all soluble amino acids and peptides, so that these fractions could form convenient standards for comparison. The procedure employed for their separation from the costal diaphragm combines the original method of McCollester (1962) and the SDS washing procedure described by Laurent et al. (1981). Both fractions were subjected to amino acid analysis by the present procedures (Zarkadas et al., 1987b). The results presented in Table IV show that, even after exhaustive dialysis (Laurent et al., 1981), the separated SDS-soluble muscle protein fraction (F1) contained 54.78% protein compared to the extracellular SDS-insoluble connective tissue residue (F2) that contained only 31.25% protein. The quantity of the intracellular muscle protein fraction F1 found in the bovine costal diaphragm is on the average 95.27% of the total protein while the average quantity of SDS-insoluble extracellular matrix represents 4.73% of the total muscle protein. Since the protein concentration in each fraction was determined from their amino acid compositions (Horstmann, 1979), also reported in Table IV, it is unlikely to cause large errors in the relative protein values reported for these fractions. Deviations from these averages may reflect variations in the thickness of the tendinous septa surrounding the myofibrils. The average weight equivalents and conversion factors obtained from both fractions F1 and F2 are given in Table IV, so that they can be used in all subsequent quantitations of protein in these fractions following standard procedures (Horstmann, 1979).

As shown in Figure 2A, the analysis of the intracellular muscle protein fraction (F1) isolated from the costal region of the bovine diaphragm (100 μL equivalent to 220 μg protein analysis) by the present method revealed the complete separation, at pH 4.501 and 71 °C, of His(τ-Me) from an unknown ninhydrin-positive peak, designated 17. In addition, a number of as yet unidentified minor and major ninhydrin-positive peaks, designated 2, 7, 8, and 10 were apparent (Figure 2A), and the results calculated relative to the internal standard [Tyr(NO_2)] are presented in Table III. Two of these major unknown compounds (8 and 10) found in relatively large amounts in most skeletal muscle tissues examined and in highly purified actins from avian, porcine, and bovine skeletal muscles were investigated. Similarly, the unknown compound (17) has also been found in variable amounts in other proteins and tissues. As may be seen in Figure 2B the analysis (100 μL equivalent to 65 μg of fraction F2) of the SDS-insoluble extracellular matrix muscle protein fraction (F2) isolated from the costal diaphragm revealed the complete separation of the diastereoisomers of Lys(5-OH) along with two as yet unidentified stable components, designated 7 and 8. It was found that unknown peak 8 partially coelutes with one of the diastereoisomers of Lys(5-OH) and that another unknown ninhydrin-positive compound also present in the connective tissue fraction (F2) appeared to coelute with the same diastereoisomer of Lys(5-OH), even after epimerization, thus interfering with its quantitation (Figure 2B). As mentioned earlier, the determination of the aLys(5-OH) diastereoisomer is now being routinely used for quantitating this unique to collagen basic amino acid after epimerization (96 h). Unlike the tendinous dome portion of the bovine diaphragm, the costal diaphragm contains very small amounts of the elastin cross-links iDes and Des separating as minor peaks before and after the internal standard peak, Tyr(NO_2) (Figure 2B), and the results obtained are summarized in Table IV.

Table IV. Amino Acid Composition (Grams of Amino Acid per Kilogram of Protein) of the Intracellular Muscle Protein and Extracellular Connective Tissue Fractions (F1 and F2) Isolated from the Costal Region of the Bovine Diaphragm[a]

	F1 intracellular SDS-solubilized muscle proteins			F2 extracellular SDS-insoluble connective tissue residue		
amino acid	mean ± SEM[b]	CV[b]	F[b]	mean ± SEM[b]	CV[b]	F[b]
aspartic acid	99.89 ± 0.47	1.13	0.01[ns]	69.61 ± 0.16	0.56	0.03[ns]
threonine	48.75 ± 0.66	2.71	0.86[ns]	25.00 ± 0.43	2.71	2.72[ns]
serine	41.64 ± 0.84	2.38	6.34[ns]	40.58 ± 0.18	0.75	1.97[ns]
glutamic acid	166.36 ± 0.54	0.48	3.26[ns]	117.13 ± 0.27	0.40	1.86[ns]
proline	31.43 ± 0.33	2.35	0.30[ns]	109.81 ± 2.19	4.19	0.70[ns]
glycine	30.14 ± 0.07	0.54	0.07[ns]	181.71 ± 0.52	0.70	0.06[ns]
alanine	53.14 ± 0.18	0.68	0.93[ns]	82.00 ± 0.37	0.41	12.92[ns]
cysteine	8.59 ± 0.09	0.76	19.27*	8.21 ± 0.27	0.25	2112.62**
valine	55.22 ± 0.36	1.14	1.67[ns]	39.67 ± 0.51	2.09	2.49[ns]
methionine	27.22 ± 0.05	0.44	0.05[ns]	11.17 ± 0.41	3.32	12.81[ns]
isoleucine	52.17 ± 0.12	0.39	2.32[ns]	24.01 ± 0.31	2.77	0.69[ns]
leucine	91.24 ± 0.18	0.34	1.84[ns]	42.91 ± 0.28	0.84	5.24[ns]
tyrosine	40.24 ± 0.36	2.18	0.08[ns]	16.71 ± 0.65	3.08	17.11**
phenylalanine	45.34 ± 0.22	1.16	0.11[ns]	26.09 ± 0.35	1.05	17.98*
histidine	28.20 ± 0.11	0.87	0.15[ns]	10.99 ± 0.41	8.94	0.10[ns]
lysine	95.16 ± 0.27	0.37	5.12[ns]	42.47 ± 0.32	0.08	1075.05**
arginine	67.64 ± 0.08	0.27	0.06[ns]	81.04 ± 0.39	1.06	0.56[ns]
tryptophan	16.79 ± 0.14	0.79	10.05[ns]			
N^6-methyllysine	0.121 ± 0.008	14.31	0.82[ns]			
N^τ-methylhistidine	0.548 ± 0.008	3.41	0.27[ns]			
4-hydroxyproline				70.23 ± 0.16	0.29	5.50[ns]
5-hydroxylysine				8.85 ± 0.08	1.88	0.80[ns]
isodesmosine				0.43 ± 0.003	1.47	2.32[ns]
desmosine				0.44 ± 0.006	2.77	5.03[ns]
ornithine	0.273 ± 0.008	4.4.	4.55[ns]	0.32 ± 0.010	3.75	2.89[ns]
ammonia	12.37 ± 2.80	46.38	0.05[ns]	15.03 ± 0.18	68.67	1.37[ns]
total AA	1000.00 ± 5.09			1000.00 ± 8.28		
total AA N	170.17			190.46		
total recovery	547.80 ± 0.02			312.50 ± 0.02		
% yield (g protein/100 g total protein)	95.27			4.73		
WE,[c] g/mol	112.840			93.940		
F,[c] g/mol	115.093			94.642		
F',[d] g/mol	119.547			113.243		

[a] Prepared and quantitated by the methods of McCollister (1962) and Laurent et al. (1981). [b] Mean values and standard errors of measurements (SEM) for 4 replicates and 32 determinations. Significance: F, values from analysis of variance; **, $P < 0.01$; *, $P < 0.05$; ns, not significant; CV, coefficient of variation. [c] The WE and F constants were calculated according to Horstman (1979), using eq 1 and 2, respectively. [d] The conversion factor F' was also calculated according to eq 2 but for determining protein mass in the absence of tryptophan, cyst(e)ine, proline, and 4-hydroxyproline.

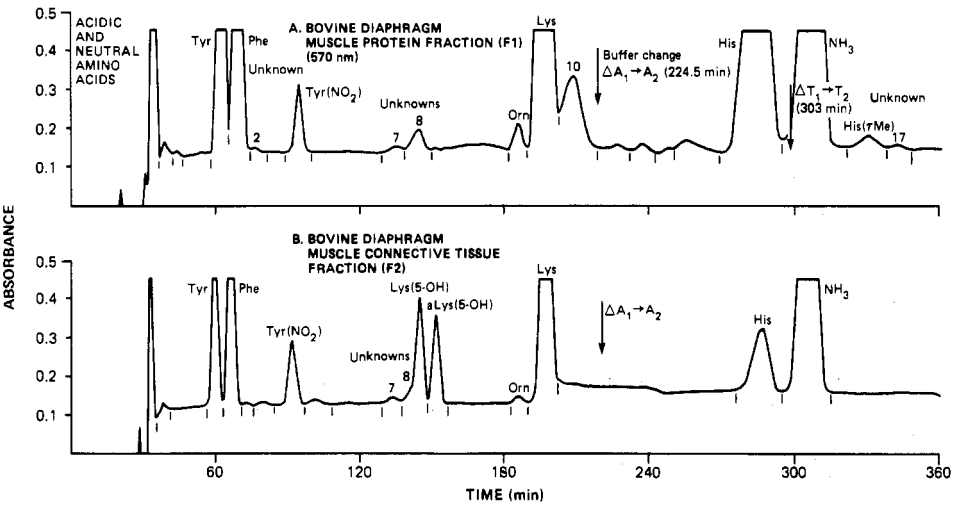

Figure 2. Typical elution patterns of the methylated basic amino acids and related compounds in the myofibrillar and connective tissue proteins from the costal diaphragm on an analytical 50 × 0.28 cm microcolumn of Dionex DC-4A resin. The buffer and temperature changes are indicated by arrows. The curves show absorbance at 570 nm. (A) Separations of all methylated lysines and histidines in the intracellular muscle protein fraction (F1). (B) Analysis of a 96-h hydrolysate of the extracellular matrix, fraction F2. Key: Tyr(NO_2), 3-nitrotyrosine; Lys(5-OH), 5-hydroxylysine; aLys(5-OH), *allo*-5-hydroxylysine; His(τMe), N^τ-methylhistidine.

The amino acid composition of the intracellular muscle protein fraction (F1) from the costal diaphragm is presented in Table IV. The relative proportions of amino acids are, on the whole, similar to those found in the dome portion of the diaphragm (Table II), with characteristic differences in their contents of proline, glycine, and alanine and with sporadic variations within the general profiles. Specifically, when compared with the dome region of the diaphragm, the aspartic acid, glutamic acid, and lysine residues are increased, whereas valine residues are de-

Table V. Composition (Number of Amino Acid Residues/1000 Amino Acid Residues) of the Extracellular Matrix Protein Fraction (F2) from the Bovine Costal Diaphragm and Purified Collagens and Elastin Standards

amino acid	bovine: costal diaphragm fraction[a] F2	bovine: semitendinosus elastin[b]	elastin std[c]	collagen std: I	collagen std: III	collagen std: IV
aspartic acid	55.6	7.4	2	43	42	46
threonine	22.8	8.2	3	18	13	29
serine	18.1	8.3	5	33	39	35
glutamic acid	83.4	17.1	12	71	71	74
proline	104.1	110.9	128	120	107	81
glycine	292.8	325.9	352	335	350	331
alanine	106.1	223.1	176	111	96	36
cysteine	7.3		1	0	2	1
valine	36.8	145.8	175	26	14	31
methionine	7.8			6	8	15
isoleucine	19.5	26.5	19	9	13	34
leucine	34.9	61.4	47	23	22	53
tyrosine	9.4	8.4	12	2	3	6
phenylalanine	16.3	29.8	23	12	8	30
histidine	7.4	0.6	1	6	6	6
lysine	30.5	5.3	4	23	30	6
arginine	47.7	5.7	5	50	46	29
3-hydroxyproline	nd			1		1
4-hydroxyproline	57.1	12.4	22	98	118	120
5-hydroxylysine	5.7			10	5	42
isodesmosine	0.088	1.2	3			
desmosine	0.099	2.1	3			
Pro(4-OH)/[Pro + Pro(4-OH)]	0.354		0.093	0.462	0.539	0.593
Lys(5-OH)/[Lys + Lys(5-OH)]	0.158			0.303	0.143	0.882
WE,[e] g/mol		84.48	85.06	91.14	90.90	95.94

[a] Means of 32 determinations (mature cows), present study. [b] Data for bovine semitendinosus muscle elastin taken from Bendall (1967). [c] Data for amorphous elastin taken from Foster (1982). [d] Average values recalculated from the data for Miller and Gay (1982), Light and Champion (1984), and Light (1985). [e] Calculated according to Horstmann (1979). Average WE values for muscle collagen and connective tissue: $\sum_{i=1}^{3} WE^{1}$ muscle collagen = [(0.615 × 91.14) + (0.335 × 90.90) + (0.05 × 95.94)] = 91.10 (see also Table I; $\sum_{i=1}^{4} WE^{11}$ muscle connective tissue = [(0.0473 × 85.06) + (0.9527 × 91.10)] = 90.814 (see also Table III).

creased in the intracellular protein fraction (F1) prepared from the costal diaphragm. The methionine and cyst(e)ine contents of fraction F1 are also reduced. As shown in Figure 2, the absence of the unique amino acids Lys(5-OH), Pro(4-OH), and Des indicates that the intracellular protein components of the costal diaphragm have been effectively separated from the extracellular matrix connective tissue proteins under the conditions described, with little or no solubilization of collagen. The content of protein-bound His(τ-Me) in the intracellular muscle protein fraction (F1) from the costal diaphragm shows a very low coefficient of variation (Table IV), and it is within the range of various skeletal muscle tissues investigated. Although the data reported in Table IV for protein-bound His(τ-Me) are in reasonable agreement with those reported by other authors (Asatoor and Armstrong, 1987; Hibbert and Lawrie, 1972; Young and Munro, 1978; Haverberg et al., 1975), some differences have been noted. These may arise from the fact that other methods were employed for these determinations. The lower His(τ-Me) content reported in Table II for the dome region of this skeletal muscle indicates that this specific site of the diaphragm had higher levels of connective tissue.

The amino acid profile of the extracellular matrix fraction F2 as presented in Tables IV and V is highly characteristic of connective tissue proteins, i.e., collagen, elastin, etc., and completely different from that of any other group of muscle proteins. Glycine is the most abundant and accounts for almost exactly 1 residue in 3. Alanine makes up 1 in 10 of all residues. Proline and Pro(4-OH) taken together account for a further 1.8 residues in 10. Thus, these four amino acids account for 5.6 residues out of every 10. The frequency of the acidic amino acids is approximately 1.4 residues in 10, while the total basic amino acids including arginine, lysine, histidine, and Lys(5-OH) account for almost 1.0 residue in 10. The high content of Pro(4-OH), together with serine and threonine and the small amounts of tyrosine and Lys(5-OH), bring the total content of hydroxylated residues to nearly 1.13 in 10 compared to 0.7 residue in 10 for the total hydrophobic amino acids. Tables IV and V show that the small amounts of Des and iDes found in the extracellular matrix (F2) of the diaphragm may have derived from the network of capillaries often separated with the SDS-insoluble membrane residue (F2). Although they may account for up to half the dry weight of the preparation (McCollester, 1962), methods to remove these capillaries by chemical means during the numerous washes are not available at present. Rowe (1986) has recently shown that skeletal muscle elastin is an integral component of epimysium and perimysium.

Actin and Myosin Components of the Diaphragm. The results presented in Tables II and IV indicate that the quantitation of protein-bound His(τ-Me), known to occur exclusively in myosin and actin (Elzinga et al., 1973; Huszar, 1984; Maita et al., 1987), can be used as an index for determining these two principal myofibrillar proteins in two anatomically defined regions of the diaphragm by eq 8. The results, expressed as grams of protein per kilogram of anhydrous, fat- and ash-free total muscle protein, are presented in Table VI. With the exception of the value for total connective tissue of the dome region of the diaphragm, the values in Table IV show deviations less than 100 ± 2.5% from the average values. These values show that, in the costal diaphragm, myosin accounts for 22.94% of the total muscle protein corresponding to 43.9% of the myofibrillar proteins and actin accounts for an estimated 11.04% of the total muscle mass or about 21.13% of the myofibrillar proteins. These results are in accord with those reported by Yates and Greaser (1983) who have shown that the rabbit Psoas muscle contains 57.71% myofibrillar protein of the total muscle mass and that the

Table VI. Myofibrillar and Connective Tissue Protein Contents (Grams per Kilogram Total Muscle Protein) of Mature Bovine Diaphragm

skeletal muscle proteins	diaphragm				skeletal muscle (psoas major) % of total muscle protein		
		costal region					
		fractions[b]					
	dome region (extracted)[a]	intracellular F1	extracellular F2	composite[h]	Yates and Greaser (1983)	Hanson and Huxley (1957)	Szent-Gyorgi et al. (1955)
			Myofibrillar[c] SDS-Soluble Proteins				
actin	82.07 ± 0.83	115.86 ± 1.66		11.04	12.69	12.00	
myosin	170.51 ± 1.72	240.77 ± 3.45		22.94	24.82	34.00	25–30
actomyosin	252.64 ± 2.55	356.63 ± 5.10		33.98	37.52	46.00	
total	388.59 ± 3.93	548.54 ± 7.85		52.26	57.71	62.00	40–48
other sol proteins	428.6	451.5		43.00		34.00	41.6
			Myofibrillar SDS-Insoluble Proteins				
connective tissue[d]	179.99 ± 10.65		548.29 ± 1.77	2.59			
collagen[e]	84.95 ± 7.07		554.75 ± 5.14	2.62			
elastin[f]	62.42 ± 0.37		27.09 ± 0.14	0.128			
total[g]	147.37		581.85	2.752			

[a] Extracted according to Bligh and Dyer (1959). [b] Separated by the combined procedures of McCollester (1962) and Laurent et al. (1981). Present distribution in the costal diaphragm: intracellular proteins, 95.27; extracellular matrix, 4.73. [c] Calculated from eq 6f, 6g, and 8a. [d] Calculated from Pro(4-OH) data (Tables II and IV) using eq 9 and the ratio of collagen and elastin determinations described below.[e,f] [e] Calculated from eq 5a with data taken from Miller and Gay (1982) and Light et al. (1984). [f] Calculated from eq 5b with data taken from Foster (1982). [g] Collagen plus elastin. [h] Expressed as percent of total muscle protein.

myofibrils contain 22% actin and 43% myosin by weight. If other published values are corrected and expressed on the basis that muscle contains 20% protein and 12% myofibrils, the results obtained agree well with those reported in Table VI. Hanson and Huxley (1957) found by quantitative extraction that the myofibril would contain 21% of actin and 43% of myosin by weight, compared to the myosin value reported by Szent-Gyorgyi et al. (1955) that accounts for 38% of the myofibrillar proteins.

When samples from the dome region of the diaphragm were analyzed by the present method (Zarkadas et al., 1987b), the quantity of actin and myosin was nearly always lower and the average result was only 25.26%, although the myofibrils appear to contain 21.12% actin and 43.89% myosin. There seem to be two possible explanations for the difference between the results obtained on the content of myosin and actin of the dome and costal regions of the bovine diaphragm. On the one hand, the dome region of the diaphragm has a very high content of connective tissue protein, accounting for an estimated 18.0% of the total muscle protein. Alternatively, the extract may contain small amounts of myosin or actin fragments of low molecular weight that escape into the chloroform layer. Quantitative amino acid analysis of the soluble extracts indicated that the protein content was very small (0.75%). These results provide additional evidence to indicate that while the content of the myofibrillar and connective tissue proteins in different anatomical regions of the diaphragm may vary, the relative amounts of actin and myosin per unit of tissue, as related to the distribution of His(τ-Me) in these two muscle proteins, remain constant. Deviations from the average figure of 57.71% reported by Yates and Greaser (1983) for the myofibrillar protein content of Psoas muscle may reflect variations in the amount of these muscle proteins present on the diaphragm and not in its distinct actin to myosin distribution. The possibility remains, of course, that there exist minor differences in the amounts of fast, slow, or mixed fast/slow classes of MHC isoforms present in the diaphragm (Green et al., 1984; Buckingham, 1985) not containing methylated histidine residues at position 755 in their amino acid sequence (Elzinga and Collins, 1977; Huszar, 1984), but there is no reliable evidence for such a possibility at present (Maita et al., 1987). Nor do the present results exclude the possibility that the contractile proteins of nonmuscle cells present in this tissue contribute to the average quantity of myofibrillar proteins found (Table VI). Such nonmuscle cells have very high actin to myosin ratios (Pollard, 1981, 1987; Pollard and Cooper, 1986). Although it appears that such nonmuscle cells, e.g., fibroblasts, etc., constitute a very small portion of these tissues, the error introduced by such muscle constituents is unknown. However, the effect of this type of error would be to increase the actin to myosin ratio in the diaphragm, but the results show that the myofibrillar actin and myosin represent 34.0% of the muscle weight, agreeing well with the 37% value reported by other methods (Hanson and Huxley, 1957; Potter, 1974; Yates and Greaser, 1983).

Quantity of Other Muscle Soluble Proteins. In addition to myofibrillar proteins, a large quantity of SDS-soluble protein is found in the diaphragm, which is on the average 42.5% of the total muscle protein (Table VI). Hanson and Huxley (1957) found that the average quantity of soluble protein (sarcoplasmic) washed out of the glycerol-extracted muscle after it has been broken up into fibrils is 28% of the total protein, but even the highest result they obtained (34%) is much lower than the figure reported in Table VI. These results, however, are in accord with the figure quoted by Szent-Gyorgi et al. (1955), 41.6%, using two different extraction procedures. Nevertheless, the discrepancy between 42% and 28% still seems too large to be explained on the basis of incomplete extraction from the fresh minced muscle used by Hanson and Huxley (1957). It seems more likely that the SDS extraction method of McCollester (1962) and Laurent et al. (1981) used in the present experiments removes, in addition to sarcoplasmic proteins (28%), a large quantity (14%) of SDS-soluble protein that originates from organelles, the Z-band, and other cell membrane structures. Further detailed studies to ascertain the nature of these rather large amounts of other unknown SDS-soluble muscle proteins are required.

Determination of the Connective Tissue Proteins. In the present study, an attempt was also made to relate the amounts of the unusual protein-bound amino acids, which occur exclusively in collagen and elastin, to the contents of connective tissue in the costal and dome regions of the diaphragm. The extracellular matrix of the costal diaphragm, which is high in collagen and elastin content, was isolated and quantitated by the combined methods of McCollester (1962) and Laurent et al. (1981). The results, summarized in Tables IV and VI, show that the

extracellular matrix of the costal diaphragm accounts for an estimated 4.73% of the total muscle protein in this skeletal muscle. The levels of Des, iDes, Lys(5-OH), and Pro(4-OH) per unit extracellular matrix protein (grams per kilogram of total protein) were determined by the sensitive methods described previously (Zarkadas et al., 1986, 1987a), but mostly by the rapid procedures (Zarkadas et al., 1986) requiring less than 1.25 h for a complete analysis. On the basis of this data, as summarized in Tables II and IV, collagen and collagen-like proteins can be estimated from the amounts of the aLys(5-OH) diastereoisomer found in the acid hydrolysates of the diaphragm and the elastin content from the amounts of Des or iDes present (Gunja-Smith, 1985; Zarkadas et al., 1986, 1987b). Connective tissue, which includes collage and elastin, can also be determined from the amounts of Pro(4-OH) present. The accuracy of such calculations, however, will depend on the purity of elastin and the various collagen isotypes on which their Des or iDes, Lys(5-OH), and Pro(4-OH) contents are based. Although the structure and amino acid composition of purified collagens and elastin from various species (Table V) have been investigated quite thoroughly at the protein and gene levels (Miller and Gay, 1982; Foster, 1982; Paz et al., 1982; Cheah, 1985), there is a paucity of compositional data on skeletal muscle collagens and elastin, and the only biochemical studies that have been done on collagens and elastin of bovine skeletal muscle are those of Bendall (1967), Light and Champion (1984), and DeMichele et al. (1985).

Light et al. (1985) have shown that the levels of six bovine skeletal muscle connective tissue in the epimysium, perimysium, and endomysium represent 1.2, 4.7, and 0.3% of the total muscle dry weight, respectively. In addition, types I and III collagens accounted (with the very small amount of type V collagen) for 95% of the recovered pepsin-soluble collagen in skeletal muscle. Type IV collagen, which is specific to basement membranes and epithelial cells, accounted for the remaining 5% (Light and Champion, 1984). Since each level of muscle organization, i.e., epimysium, perimysium, and endomysium, and the mean ratio of the various genetic forms of collagen among the six skeletal muscles investigated (Light and Champion, 1984; Light et al., 1985) appear to be relatively constant, a mean value of $n_i' = 10.00$ Lys(5-OH) residues per 1000 total amino acid residues (Table I) for skeletal muscle was calculated from the amino acid composition of types I, III, and IV collagens (Miller and Gay, 1982; Light, 1985) given in Table V. Thus, the contents of collagen in the costal and dome regions of the diaphragm were calculated by multiplying the amounts of Lys(5-OH) found in their acid hydrolysates (Tables II and IV) by the conversion factor 64.3 (eq 5a). The results summarized in Table VI show that the amount of collagen in the costal diaphragm averages 2.62% and in the dome region of this skeletal muscle averages 8.5% of the total muscle protein. These results are in reasonably good agreement with those reported by Bendall (1967), Dransfield (1977), and Light et al. (1985) for the distribution of collagen (average 4.35%; spread 1.22–15.1%) in 34 bovine skeletal muscles investigated. The difference noted in the collagen contents between the costal and the dome regions of the diaphragm, while at present is without explanation, may be attributed to the anatomical arrangement of the connective tissue proteins in each level of muscle organization, which obviously influence its function. Although part of the function of the connective tissue in various skeletal muscles is to form an elastic, stress-tolerant system, the contraction of the diaphragm during an inspiratory excursion results in a shortening of the muscle in a radial fashion so that the force of contraction is in a two-dimensional manner, which is quite different from other muscles.

If the mean Des value (3 residues/1000 total residues) for purified amorphous elastin (M_r 72000) reported by Foster (1982) is used (Table V), the amount of mature insoluble elastin in the diaphragm could be quantitated by multiplying the Des values by 62.4 (eq 5b). The values obtained for elastin in the costal and dome regions of the diaphragm averaged 0.13% (6.1% of the total connective tissue) and 6.24% (or 42.36% of the total connective tissue), respectively. Bendall (1967) has shown that the semitendinosus bovine muscle, which contained exceptionally high levels of elastin (1.82%, on a dry basis) and represented 37.0% of the total connective tissue, had rather low levels of Des and iDes. If his results, as summarized in Table V (2.1 residues of Des/1000 total residues), are used to quantitate mature elastin in the diaphragm, the amounts of elastin would be about 40% higher. The higher elastin value (1.82%) determined in semitendinosus bovine muscle compared to 0.13% elastin calculated for the costal diaphragm may be attributed to the purity of the elastin preparation and the methods of analysis used. Thus, the results presented in Table VI show that different anatomical regions of the bovine diaphragm contained different amounts of elastin. Rowe (1986) provided evidence to show that both high (semitendinosus) and low (longissimus dorsi) elastin content bovine muscles, were found to have the same anatomical organization in the two structural forms of elastin present in the epimysium and perimysium and that they are aligned with the long axis of the muscle fibers. Although the role of elastin in connective tissues is seen as one of storing the energy of deformation during a contraction cycle, the biological significance of these findings and function of elastin in this skeletal muscle remain to be elucidated.

The results summarized in Tables IV and VI show that while the extracellular matrix isolated from the costal diaphragm accounts for an estimated 4.73% of the total muscle protein, the content of total connective tissue estimated from the amounts of collagen and elastin was 2.88% of the total muscle proteins. The transcellular matrix that remains accounts for 1.85% of the muscle proteins. This suggests that, in addition to collagen and elastin, the extracellular matrix from the costal diaphragm must contain various other proteins that are relatively rich in glycine and alanine but very low in basic and acidic amino acids and have little or no Pro(4-OH). These results are in close agreement with those reported by Loewy et al. (1983) who have shown the presence of a covalently cross-linked ϵ-(γ-glutamine)–lysine bonds transcellular matrix accounting for 1% of the original muscle proteins. Based on the relative amounts of collagen and elastin given in Table VI, and the known distributions of Pro(4-OH) in both elastin (Bendall, 1967; Paz et al., 1982) and the various collagen isotypes (Table V) found in skeletal muscle and other tissues (Miller and Gay, 1982; Foster, 1982; Paz et al., 1982; Cheah, 1985; Berg, 1982; McCollester, 1962; Laurent et al., 1981), the contents of total connective tissue in the costal and dome regions of the diaphragm (Table VI) could be calculated from the amounts of Pro(4-OH). The data presented in Table V show that the total connective tissue in both the costal and dome regions of the diaphragm accounted for 2.6% and 18.01%, respectively. These results indicate that the diaphragm contained lower amounts of Pro(4-OH) than other vertebrate connective tissues and that this amino

acid shows larger variation in level than either Des or Lys(5-OH).

From the foregoing results, it may be concluded that these sensitive methods developed for determining all methylated basic amino acids, Lys(5-OH), the stable elastin cross-links Des and iDes, and related compounds in proteins and muscle tissues can be applied for the determination of the myofibrillar and connective tissue protein contents of the diaphragm and other skeletal muscle tissues. These methods of analysis should be especially valuable for studying the molecular and cellular mechanisms involved during morphogenetic and developmental processes in muscle cells and can also be applied to the study of protein methylation, hydroxylation, and elastin cross-linking formation of contractile and stroma proteins in biological systems, under both normal and pathological conditions. In addition, this method may prove valuable for industrial control and formulation of meats and meat products and could be easily applied for enforcing meat regulations.

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Registry No. Lys(5-OH), 1190-94-9; Des, 11003-57-9; iDes, 991-01-5; Tyr(NO_2), 3604-79-3; GlcN, 3416-24-8; GalN, 7535-00-4; aLys(5-OH), 18899-29-1; LysAla, 18810-04-3; N^{τ}-methylhistidine, 332-80-9; 4-hydroxyproline, 51-35-4; aspartic acid, 56-84-8; threonine, 72-19-5; serine, 56-45-1; glutamic acid, 56-86-0; proline, 147-85-3; glycine, 56-40-6; alanine, 56-41-7; cysteine, 52-90-4; valine, 72-18-4; methionine, 63-68-3; isoleucine, 73-32-5; leucine, 61-90-5; tyrosine, 60-18-4; phenylalanine, 63-91-2; histidine, 71-00-1; lysine, 56-87-1; arginine, 74-79-3; tryptophan, 73-22-3; ornithine, 70-26-8; N^{6}-methyllysine, 1188-07-4; N^{6},N^{6},N^{6}-trimethyllysine, 23284-33-5; N^{τ}-methylhistidine, 368-16-1; 3-hydroxyproline, 567-36-2.

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Determination of the Myofibrillar and Connective Tissue Protein Contents and Amino Acid Composition of Selected Composite Meat Products[1]

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The contents of myosin, actin, and collagen of six composite meat products and hamburger have been determined by the use of analytical chromatographic methods developed to quantitate the unique amino acids that occur in those proteins. The proposed chemical approach for assessing protein quality in composite meats is based on the direct determination of their myofibrillar and collagen contents from the amounts of N^{τ}-methylhistidine and 5-hydroxylysine present, respectively. When the sum of the intracellular and extracellular skeletal muscle proteins is subtracted from the total protein of a composite meat sample, the difference is an accurate assessment of the nonmuscle protein additives present. The total protein content of each sample was calculated by the summation of the amino acid content as determined by detailed amino acid analysis. Mixed-meat products vary considerably in their myofibrillar (22.7–39.8%) and other soluble muscle proteins (14.7–32.2%), connective tissue proteins (6.7–21.6%), and nonmeat protein additives (2.4–52.8%), depending upon the type and amount of specific meat cuts and nonmeat protein ingredients used to formulate them.

The protein quality and nutritive value of processed meats and poultry products vary considerably depending upon the type and amount of specific meat cuts and nonmeat ingredients used to formulate them. Such composite meat products are prepared from cheaper meat cuts, which are frequently high in connective tissue, from the major meat-yielding species. Formulations usually include a number of other nonmuscle animal and plant protein additives including milk and egg powders, gelatin, soya, and other types of oilseed protein products, wheat gluten, etc., prepared by various separation and extraction processes. The actual levels of protein used in such processed meats vary and are tailored to meet the cost and nature of such ingredients available to the processor. These mixtures are then ground, chopped or comminuted, encased to retain defined shapes or forms, and then processed into a variety of meat products [reviewed by Terrell (1982) and Rust (1982)].

There has been a continuing interest in the development of standards for labeling prepackaged meats and reliable analytical methodology useful for the precise assessment of the skeletal muscle, connective tissue, and nonmuscle protein contents of composite meat products [reviewed by Pearson (1975), Olsman and Slump (1981), Ranken (1984), McNeal (1987), and Benedict (1987)]. Several of the electrophoretic and immunological methods including the enzyme-linked immunosorbent assay (ELISA), which have been described for determining the muscle and nonmuscle protein contents of processed meats (Menzel and Glatz, 1981; Hitchcock et al., 1981; Molander, 1982; Armstrong et al., 1982; Berkowitz and Webert, 1987), are limited mainly because of the extensive denaturation, decreased solubility, structural changes, and interactions that occur in these complex protein mixtures during processing. The more promising methods appear to be those based on the chromatographic separation and determination of specific peptides derived from actin (Anderson, 1976, 1981) or soya bean proteins (Llewellyn et al., 1978) and the unique basic amino acid N^{τ}-methylhistidine [His(τ-Me)] found in myosin and actin, the two principal muscle proteins (Elzinga et al., 1973; Elzinga and Collins, 1977; Huszar, 1984; Maita et al., 1987). Thus, the determination of His(τ-Me) has been proposed as an index for quantitating the lean meat content of various muscles and meats (Perry, 1970; Hibbert and Lawrie, 1972; Rangeley and Lawrie, 1976, 1977), and numerous studies have reported the distribution and occurrence of His(τ-Me) in a variety of muscle tissues and processed meats (Haverberg et al., 1975; Rangeley and Lawrie, 1977; Poulter and Lawrie, 1980; White and Lawrie, 1985; Olsman and Slump, 1981; Jones et al., 1982, 1985, 1987). However, until now, the multicolumn systems employed for these determinations have shown wide variation in the His(τ-Me) content of various muscles and meats, and complete separation of all these unusual basic amino acids from other ninhydrin-positive compounds found in meat hydrolysates has not been achieved [reviewed by Hancock and Harding (1984) and Ashworth (1987)]. To validate the use of His(τ-Me) as an index for assessing the myofibrillar protein content of processed meats, accurate and detailed determination of His(τ-Me) in selected composite meat products is essential.

The purpose of this investigation was to quantitatively establish the levels of all methylated basic amino acids, including the diastereoisomers of 5-hydroxylysine [Lys(5-OH)] and related compounds in commercially prepared

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