connective tissue does not necessarily indicate changes in the connective tissue of other organs, the probability of a systemic action of the hormone seems likely, with several different tissues being affected. The abnormal effects observed in the hormone-fed animals seem to support the hypothesis that stilbestrol feeding may be associated with an altered connective tissue metabolism. This view seems to be substantiated further by the fact that lambs which showed increased skin thickness and whose pelts were difficult to remove invariably showed increased connective tissue in skeletal muscle as well (*1*). Acker also observed an abnormal abundance of connective tissue of a collagen-like nature under the skin of lambs which showed increased skin thickness. These observations all appear to be related to those of Anastassiadis *et al.* (*3*), who found indications that estrogen treatment increased collagen content in the skin of immature pullets.

Anastassiadis, Maw, and Common (*3*) also found that estrogen treatment brought about hypertrophy of the oviduct (*17*) accompanied by an increase in the connective tissue, particularly interfibrillar glycoprotein (mucoprotein). These results are similar to those of this investigation, where increased mucoprotein content of skeletal muscle was noted following stilbestrol treatment. It would appear that the hypertrophic edema of the secondary sex organs observed in this study was also the result of stilbestrol administration. It has been attributed to the attendant hydration which accompanies the increased mucopolysaccharide content following hormonal stimulation (*6*) and/or to the depolymerization of ground substance (mucoprotein), because of the subsequently altered capillary permeability which results (*7*). [It has been suggested that estrogens may act as depolymerizing agents of the big molecules of fundamental connective tissue components (*10*).]

Difficulty with urinary calculi (*1, 2, 19*) is presumably caused by a partial closure of the urethra effected by the hypertrophic edema of certain secondary sex organs and/or increased incidence of urinary calculi. Since urinary calculi consist of calcium-mucoprotein units (*4*), one might speculate that their increased incidence could be due to increased serum calcium (*5*) together with a heightened mucoprotein metabolism (*10*), both of which have been reported to result from estrogen treatment. As was inferred earlier, indications of an increased mucroprotein metabolism were obtained in the experiments reported here.

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MEAT TENDERNESS FACTORS

Determination of Mucoprotein in Skeletal Muscle

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In attempts to assess meat tenderness, chief attention customarily has been given the fibrillar components of connective tissue, collagen and elastin. Meanwhile, the interfibrillar component—i.e., the mucoprotein or ground substance of skeletal muscle—has been virtually ignored.

Miller and Kastelic (*13*) first focused attention upon the possible significance of this connective tissue component in skeletal muscle in relation to meat tenderness. They speculated that postmortem changes in the tenderness of meat may be caused by alterations in the mucoprotein or ground substance of the connective tissue.

Weber (*19*) reported that stromal protein constitutes about 20% of the total nitrogen of skeletal muscle. Prudent (*17*), using the method of Lowry, Gilligan, and Katersky (*10*), found that the average collagen and elastin content of bovine skeletal muscle constituted 4% of the total nitrogen. Miller and Kastelic (*13*), assaying on the basis of hydroxyproline content (*15*) of connective tissue residues, found that collagen and elastin accounted for 5 to 6% of the total nitrogen of skeletal muscle. Clearly,

A procedure for the determination of mucoprotein hexosamine as an index of total mucoprotein content in skeletal muscle involves preliminary extraction of the meat samples with a potassium chloride buffer to remove foreign hexosamine-containing protein, followed by hydrolysis of the connective tissue residue. The hydrolyzates are passed through a cation exchange column to remove interfering amino acids and sugars, and the eluted hexosamine is determined colorimetrically. The method is applicable to a variety of skeletal muscles, including beef, lamb, and veal. The relative precision, using duplicate samples, was estimated to be 1.6%. The inherent tenderness of a given cut of meat appears to be influenced by mucoprotein, as well as collagen and elastin content.

determination of connective tissue solely by the assessment of collagen and elastin leaves a sizable portion of the stromal protein fraction unaccounted for. It is interesting to note, therefore, that the 8 to 12% total nitrogen ascribed to the mucoprotein fraction by Miller and Kastelic, combined with the 5 to 6% total nitrogen value which they found for collagen and elastin, closely approximates the 20% total nitrogen figure attributed to stromal protein by Weber (*19*). This agreement supports the theory that this so-called mucoprotein fraction represents a third connective tissue component—i.e., the ground substance.

It appeared that mucoprotein content and its relationship to meat tenderness should be considered in the assessment of skeletal muscle connective tissue, along with estimations of collagen and elastin. Since no method existed in the literature for determining mucoprotein in skeletal muscle, *per se*, it was first necessary to develop a suitable method for its assay.

Miller and Kastelic (*13*) observed that the mucoprotein fraction could be solubilized either by alkali extraction or by autoclaving with water. However, either procedure resulted in degradation of the mucoprotein.

The determination of mucoprotein by repeated salt extraction with subsequent assay of the isolated material was also unsatisfactory, because the extraction was incomplete and frequently accompanied by degradation or depolymerization of the mucoprotein (*5*, *14*, *16*).

Since hexosamine is a consistent component of mucoproteins in general, its determination in biological materials other than skeletal muscle has been used to measure relative mucoprotein content (*1–3*, *6*, *7*).

Preliminary hexosamine determinations were made on acetone-dried whole bovine skeletal tissue, using various modifications of the Elson and Morgan (*8*) method. Boas (*6*) found that certain proteins, extractable at pH 8.6 and with mobilities similar to rat serum proteins, accounted for almost 50% of the hexosamine in the sample of rat connective tissue. The relative amount of plasma protein hexosamine might be even greater in skeletal muscle, since albumins and globulins constitute about 80% of the total nitrogen of this tissue (*19*). Thus hexosamine determinations on whole muscle (fresh or dried) were not valid, since the values obtained reflected hexosamine contributed by the plasma proteins (albumins and globulins) as well as that of mucoprotein origin.

Efforts were directed, therefore, toward the development of a procedure for the determination of hexosamine in the stromal protein residue. The hexosamine content of this residue (from which plasma proteins had been removed) should reflect hexosamine of mucoprotein origin only and thus should serve as a valid index of mucoprotein content in skeletal muscle.

The final procedure, outlined below, was employed in a survey of the relative mucoprotein content of various muscles from selected animals, representing beef, lamb, and veal. Collagen, elastin, and total nitrogen determinations also were made on each muscle studied.

Experimental Procedure

Meat Samples. The samples of meat used came from animals which had been fed a normal fattening ration. The representative muscles from the different animals were excised immediately after slaughter, freed of fat, and ground in a silent cutter. The comminuted meat was frozen at $-10°$ C. until the chemical determinations were made.

Hexosamine Determination. To remove hexosamine-containing plasma proteins from the skeletal muscle, the extracting solution described by Miller and Kastelic was employed (*13*). This solution consists of 0.6*M* potassium chloride buffered with 0.03*M* sodium hydrogen carbonate, pH 8.3. Exhaustive checks have shown that at this concentration and pH, potassium chloride does not extract mucoprotein from fresh bovine skeletal muscle (*11*). Samples of frozen comminuted skeletal tissue were prepared essentially according to the method also described by these investigators (*13*).

Duplicate 25-gram samples of frozen skeletal muscle tissue were placed in 1-liter wide-mouthed Erlenmeyer flasks to which 475 ml. of ice-cold buffer solution was added together with glass wool to entrap the stromal proteins during the extraction process. The tightly stoppered flasks were shaken on a horizontal shaker for 3 hours in the cold, after which the solubilized proteins were decanted from the residue using a specially designed separatory funnel.

This separatory funnel is equipped with a removable circular piece of 30-mesh stainless steel gauze, $3^1/_2$ inches in diameter. For each separation, a pad of medium grade Pyrex No. 800 glass wool, approximately $3^3/_4$ inches square, was fitted snugly under and around the mesh disk. The meat extract was decanted through this mesh–glass wool pad and discarded. Stromal proteins, entrapped in the glass wool fragments (*17*), were retained on the disk. Any small pieces of connective tissue which may have escaped through the mesh thus were entrapped by the glass wool pad underneath. After thorough washing, this glass wool pad was added to the main residue, which was carefully removed from the disk. The combined residue was placed in a 300-ml. glass-stoppered Erlenmeyer flask, covered with acetone for the extraction of water and fat, and allowed to stand overnight.

The acetone was removed by decantation. The residue was air-dried and hydrolyzed in 4*N* HCl in the stoppered flasks for 4 hours in an autoclave at 15 pounds' pressure. The cooled hydrolyzate was decanted into a volumetric flask, and the glass wool mass was washed repeatedly. The washings were added to the hydrolyzate, which was made to volume and filtered. This acid hydrolyzate is very stable and can be kept indefinitely at room temperature. A suitable aliquot of this hydrolyzate was run through a Dowex 50 cationic resin exchange column according to the method of Boas (*7*). The hexosamine then was eluted quantitatively with 2*N* HCl and determined colorimetrically.

The Boas modification (*7*) of the Elson and Morgan (*8*) method was further modified as follows for the colorimetric estimation of hexosamine:

One milliliter of 4% acetylacetone in 1*N* sodium carbonate was added to each sample (which was contained in a total volume of 4 ml.) before heating in a glass-stoppered test tube in an oil bath at 92° C. for 1 hour. Upon cooling, 4 ml. of ethyl alcohol was added with thorough shaking prior to the addition of 1 ml. of

Ehrlich's reagent, again with thorough mixing. Forty-five minutes were allowed for color development, after which the readings were taken immediately at 540 mμ on an Evelyn colorimeter. Ehrlich's reagent was prepared as described by Anastassiadis and Common (*1*). In our experience, this set of conditions was necessary in order to obtain consistently linear results.

The hydrolytic conditions described above provided maximum yields and minimum destruction of hexosamine. From the standpoint of yield, results of tests of hydrolysis times up to 24 hours showed no advantage in hydrolyzing beyond 4 hours. Approximately 95% recovery of standard glucosamine hydrochloride was obtained under these conditions. Recovery was also essentially complete when standard hexosamine was added to a meat sample, and subjected to hydrolysis and subsequent resin column treatment.

The acetylacetone reagent should be stored in the refrigerator in a tightly stoppered bottle. It is important that the solution of acetylacetone in 1*N* sodium carbonate be prepared shortly before use, and kept in a stoppered flask at room temperature until its addition to the assay tubes. The flask needs to be shaken gently at intervals to effect complete solution of the acetylacetone droplets. The test tubes must be kept tightly stoppered during the heating period in the oil bath in order to prevent volatilization of the acetylacetone with subsequently decreased color development. Sixty minutes was found to be the optimum heating time (Table I). No further increase in absorbance was observed when a longer heat treatment was used. Since different lots of acetylacetone sometimes give slightly different degrees of color development, all assays within a given series of experiments should be run using the same lot of acetylacetone.

Maximum color development occurred within 45 minutes after adding the ethyl alcohol and the color reagent to the assay tubes. Thereafter, a slow fading of the color occurred.

Other Determinations. Collagen and elastin determinations on the various muscles were made in duplicate, essentially according to the procedure outlined by Miller and Kastelic (*13*), which involves an assay for hydroxyproline using the method of Neuman and Logan (*15*). To obtain aliquots containing suitable levels of hydroxyproline, samples varying in size from 5 to 25 grams were needed, depending on the collagen and elastin content of each particular muscle studied. In all cases, however, the ratio of potassium chloride buffer volume to sample size was kept constant. The Matheson and Coleman brand *n*-propyl alcohol was the most satisfactory for use in the colorimetric hydroxyproline assay. Certain other brands of *n*-propyl alcohol appeared to contain an impurity which caused fading of the red color. Collagen nitrogen and elastin nitrogen were obtained by multiplying the corresponding hydroxyproline values by the factors 1.365 and 8.943, respectively (*12*). Total nitrogen was determined by the micro-Kjeldahl method of Johnson (*9*).

Table I. Determination of Optimum Heating Time for Reaction of 2,4-Pentanedione with Hexosamine

(100 μg. glucosamine hydrochloride in each tube)

Minutes	Absorbance
20	0.328
30	0.469
60	0.629
90	0.629

Table II. Standard Curve for Colorimetric Determination of Hexosamine

μg.	Absorbance[a]	μg.	Absorbance[a]
10	0.043	80	0.559
20	0.114	100	0.653
30	0.192	120	0.757
40	0.264	130	0.854
50	0.315	140	0.825
60	0.390	150	0.905

[a] Average values obtained from duplicate samples, using a No. 540 filter.

Table III. Precision of Hexosamine Determination

	Glucosamine Hydrochloride, μg.					Meat[a] Sample, 300 Mg.
	25	50	75	100	125	
Mean absorbance	0.126	0.323	0.496	0.644	0.797	0.234
Std. dev. (of single replicate)	0.0096	0.0044	0.0096	0.0065	0.016	0.0054
Coeff. of var., %	7.62	1.36	1.94	1.01	2.01	2.31
$\%/n\ (n = 5)$[b]	3.41	0.61	0.87	0.45	0.90	1.03
$\%/n\ (n = 2)$[b]	5.29	0.94	1.35	0.70	1.40	1.60

[a] Beef semitendinosus muscle replicates used. Analyses on appropriate aliquots of hydrolyzate from 25-gram replicates of meat sample.
[b] This computation is relevant only if the error estimated in line 2 is due solely to experimental error and not to galvanometer reading error.

Table IV. Connective Tissue Content of Representative Muscles from Various Animals

(Values represent averages of duplicate samples)

Muscle	Total N,[a] %	Collagen N,[b] %	Elastin N,[b] %	Muco-protein[c] Hexos-amine, Mg. %	Ratio, Mucoprotein Hexosamine/ Total N (×100)
Hereford steer					
Psoas major	3.00	0.83	0.60	104.	0.35
Long. dorsi	2.92	3.24	0.74	13.3	0.46
Semitendinosus	3.30	1.79	1.42	16.0	0.49
Texas longhorn steer					
Psoas major	3.45	0.80	1.03	14.1	0.41
Long. dorsi	3.40	1.66	0.50	11.1	0.33
Semitendinosus	3.28	2.08	1.43	16.1	0.49
Milking shorthorn calf					
Psoas major	3.15	1.10	2.04	16.3	0.52
Long. dorsi	3.35	1.63	2.94	17.8	0.53
Semitendinosus	3.12	2.77	3.96	21.2	0.68
California white-faced wether lamb					
Long. dorsi	3.37	0.92	0.88	9.8	0.29
Biceps femoris	2.96	1.65	5.14	14.8	0.50

[a] Fresh weight basis.
[b] Total nitrogen.
[c] Fresh weight basis.

Results and Discussion

The optimum range of the colorimetric assay was between 30 and 130 μg. (Table II). Below 30 and above 130 μg., the assay is not strictly linear. Below 30 μg., the relative precision is also considerably lessened, as shown in Table III. This table summarizes the results of a typical experiment designed to test the precision of the colorimetric assay as well as the over-all precision of the entire procedure. Five determinations were made for each concentration of standard glucosamine hydrochloride. In addition, five replicates of a meat sample were carried through the entire procedure. At glucosamine levels ranging from 50 to 125 μg., the relative precision of the colorimetric assay alone

should be 1.4% or less if the standards are assayed in duplicate (Table III). The over-all precision for the entire procedure was estimated to be 1.6%, if duplicate meat samples are used. Thus, duplicate assays should give a precision sufficient for most purposes.

The method described was used to determine relative mucoprotein content of representative muscles from different animals. The samples also were assayed for collagen and elastin, as well as total nitrogen (Table IV). Within muscles, mucoprotein hexosamine content paralleled elastin content very closely, while the correlation between collagen and mucoprotein was less consistent. The data indicate a negative correlation between the amount of the three connective tissue components and the relative tenderness of representative muscles within an animal.

The veal muscles were all considerably higher in elastin and mucoprotein (and in some instances, collagen) than corresponding muscles in the 2-year-old Hereford and Texas Longhorn steers. These results indicate that age may influence the content of these connective tissue components in skeletal muscle. Although not directly comparable, it is interesting to note that Shetlar and Masters (*18*) found the acid mucopolysaccharide content of human cartilage to be maximum in the fetus and new born, and to decrease with age.

The close correlation which appeared to exist between mucoprotein hexosamine content and the other connective tissue components is consistent with results of studies reported by Banga and Baló and others (*4*), which indicate that mucoprotein is intimately associated with both elastin and collagen fibers.

The data reported represent results from single animals and therefore do not constitute conclusive evidence for the relationships mentioned above. However, the data do show trends which should be investigated further, using more animals.

The results of this study indicate that the mucoprotein fraction of skeletal muscle is indeed worthy of consideration in the assessment of the relation of connective tissue to meat tenderness. The data suggest that the inherent tenderness of a given muscle may be influenced by its mucoprotein, as well as by collagen and elastin contents.

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INTERFACIAL ENERGIES IN MILK

Influence of Milk Proteins on Interfacial Tension between Butter Oil and Various Aqueous Phases

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THE INTERFACE between the fat and aqueous phase of milk is in a state of dynamic equilibrium (*7*). During the handling and processing of milk, there is evidence that the interfacial constituents migrate to the plasma (*4, 8, 11*) and vice versa (*6*). Unfortunately, little is known about the fat-plasma interface in milk, and any information pertaining to the composition and arrangement of the constituent components at this interface would contribute to the resolution of this problem.

To gain such information, this study utilized an indirect approach of comparing the influence of milk proteins on the interfacial tension between butter oil–water and butter oil–protein-free milk plasma. The energy-reducing activity exhibited by the proteins in the model systems used in this study indicates a preferential order of adsorption of the proteins by a milk fat surface.

Materials

Butter Oil. Freshly churned butter was washed with an equal volume of water at 50° C. and separated in a Model 619 DeLaval cream separator. This operation was repeated four times with subsequent centrifugation at 25,000 × g until an absolutely transparent oil was obtained.

Protein-Free Plasma. Two liters of distilled water in cellulose casings were dialyzed against 38 liters of skim milk for 48 hours. The dialyzate was protein-free but otherwise identical to the aqueous system of milk (*1*). The model systems which used protein-free plasma as the aqueous phase presented a native environment in which to study the interfacial behavior of milk proteins.

Protein:

Prepared Proteins. κ-CASEIN, prepared by Fox's method (*2*).

MONODISPERSED CASEIN. Calcium caseinate was centrifugally sedimented from skim milk and washed twice with protein-free plasma. Each washing consisted of completely dispersing the casein in protein-free plasma prior to centrifugation. A constant volume was maintained throughout the preparation. The protein not sedimented after the second washing was taken as monodispersed casein. All separations were completed in a gravitational field of 44,330 × g for 120 minutes.