Influence of Orally Administered Stilbestrol on Connective Tissue of Skeletal Muscle of Lambs Fed Varying Levels of Protein

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Previous investigations have suggested that estrogen treatment affects connective tissue. The work reported here represents a study of the influence of orally administered stilbestrol on the connective tissue of the loin eye muscle of lambs. Thirty-six wether lambs were individually fed a high-energy diet which included varying amounts of stilbestrol and crude protein. Stilbestrol administration was accompanied by a highly significant linear increase in elastin and mucroprotein hexosamine content of the longissimus dorsi muscle. The data indicate that stilbestrol increases the connective tissue of lamb skeletal muscle in direct proportion to the amount of the hormone administered. The importance of using a stilbestrol level which combines maximum feed lot efficiency with minimal changes in connective tissue and other side effects is discussed.

A NUMBER of studies have shown that the administration of stilbestrol (diethylstilbestrol) improves gain and feed efficiency of lambs. Oral administration of stilbestrol is preferable to hormonal implantation, in that the lowering of carcass grade (*18*) is less marked and less severe side effects are usually observed.

Preston (*15*) noted, however, that side effects occurred to some extent on all levels of stilbestrol which were fed wether lambs. The incidence of these side effects increased with increasing levels of stilbestrol. Some of the most common effects (to some degree even at the lowest levels of stilbestrol treatment) included enlarged bulbo-urethral and seminal vesicle glands, mammary development, and ulcerated prepuces. Acker (*1, 2*) noted some difficulty with urinary calculi in wether lambs, along with increased skin thickness and increased difficulty in removing pelts. These findings and others (*3, 6, 14*) suggested that the connective tissue was being affected.

In addition to these gross observable differences between control and stilbestrol-fed lambs, there was a lowering effect of stilbestrol on carcass grade (*8*). Therefore, it seemed worthwhile to determine whether there might be accompanying changes in the relative amounts of the various connective tissue components within the skeletal muscle of these animals.

Procedure

Thirty-six California white-faced wether lambs were individually fed a high-energy diet in a 3 × 3 factorial experiment with four replicates. Treatments included 0, 0.3, and 0.6 mg. of stilbestrol per pound of ration and 9, 13, and 17% protein. The lambs were approximately 5 months old and averaged approximately 80 pounds at the onset of the experiment. Each lamb was removed from the experiment so that all lambs within each replicate gained an equal amount. Feed lot and gross carcass data (as well as moisture and fat values) were reported by Preston and Burroughs (*16*). The lambs which comprised each of the four replicates averaged the same weight at the time of slaughter, 108 pounds. On the average, the length of time the lambs remained on the experiment was 58 days.

Carcasses were held in a cooler at 35° F. for 2 days before they were cut into wholesale cuts. Both of the longissimus dorsi muscles from the nine-rib racks were removed and chopped in a silent cutter. A representative sample was wrapped and frozen for later chemical determinations.

Analyses for collagen, elastin, mucoprotein hexosamine, and potassium chloride–extractable nitrogen were conducted on 25-gram samples of the frozen comminuted meat. Collagen and elastin were determined on duplicate samples as described earlier (*13*). Mucoprotein hexosamine was determined as an index of the amount of ground substance present according to a procedure developed by McIntosh (*11*). Duplicate aliquots of the acid hydrolyzate from each sample were analyzed.

Total nitrogen was determined by macro-Kjeldahl. The micro-Kjeldahl method of Johnson (*9*) was used in the assessment of potassium chloride–extractable nitrogen (*13*). The nitrogen content of the potassium chloride–insoluble residue was obtained by difference (total

Table I. Connective Tissue Analyses of Longissimus Dorsi Muscles

(Values represent averages of four replicates, expressed as mg. per 25 grams fresh tissue)

Mg. Stilbestrol/Lb. Ration	% Protein	Collagen[a] Hydroxyproline	Collagen[a] Nitrogen[b]	Elastin[a] Hydroxyproline	Elastin[a] Nitrogen[c]	Mucoprotein Hexosamine
0	0	6.06	8.27	0.50	4.47	1.34
	13	6.88	9.39	0.32	2.86	1.38
	17	6.47	8.83	0.34	3.04	1.18
	Av.	6.47	8.83	0.39	3.46	1.30
0.3	0	6.42	8.76	0.43	3.85	1.15
	13	6.90	9.42	0.48	4.29	1.16
	17	7.28	9.94	0.47	4.20	1.39
	Av.	6.87	9.37	0.46	4.11	1.23
0.6	0	6.86	9.36	0.60	5.37	1.87
	13	6.87	9.38	0.56	5.01	1.61
	17	6.59	9.00	0.51	4.56	1.63
	Av.	6.77	9.25	0.56	4.98	1.70

[a] Values represent averages of duplicate samples from four replicates.
[b] (Hydroxyproline) × (1.365).
[c] (Hydroxyproline) × (8.943).

nitrogen − potassium chloride–extractable nitrogen).

Results

The results of the connective tissue analyses on the pooled longissimus dorsi muscles from the nine-rib racks are summarized in Tables I, II, and III.

Stilbestrol

Increased levels of stilbestrol in the ration of lambs are accompanied by consistent and highly significant increases in the elastin and mucroprotein contents of lamb muscle expressed as a percentage of the moisture-free, fat-free tissue (Table III). This trend is also apparent with elastin expressed as a percentage of the total nitrogen (Table II). When the hexosamine was expressed in proportion to the total nitrogen, however, 0.3 mg. of stilbestrol per pound of ration did not cause an increase, but there was a marked effect at the 0.6-mg. level. Statistical analysis did indicate, however, a significant linear effect of stilbestrol on hexosamine. Stilbestrol apparently caused no significant increase of the collagen content of the longissimus dorsi muscle. However, later experiments (*12*) indicated that stilbestrol caused an increase in collagen (as well as mucroprotein and elastin) content of the semitendinosus muscle of Wyoming lambs.

There was a negative correlation (−0.28) between the collagen and elastin content of the longissimus dorsi muscle, when both were expressed as a percentage of the total nitrogen. There was a positive correlation of 0.31 between elastin and hexosamine expressed on a similar basis. This would give confidence to the data in Table II, which indicate that increases in both elastin and mucoprotein hexosamine were induced by increasing the amount of stilbestrol in the ration, even though no consistent change in the collagen content occurred.

Stilbestrol caused an increase in the percentage of nitrogen insoluble in potassium chloride. This fraction represents the stromal protein, or connective tissue portion of skeletal muscle. This finding seems reasonable in view of the increased values obtained for the individual connective tissue components (Tables I to III). Correlations of 0.56 and 0.58 between potassium chloride–insoluble nitrogen and hexosamine and elastin, respectively, were obtained.

There was a marked decrease in the viscosity of the potassium chloride extracts at the 0.6-mg. level of stilbestrol feeding. This lowered viscosity is probably a reflection of the decreased nitrogen content of the extracts (Table V) at that level, which would denote a decrease in muscle protein. This in turn is probably a function of the increase in connective tissue (potassium chloride–insoluble) nitrogen. Total nitrogen, moisture, and fat (*16*) were not significantly changed by hormonal treatment.

Protein

The only consistent effect of level of protein in the ration of any of the connective tissue components was the decrease in elastin as the protein content of the ration was increased. Potassium chloride–insoluble nitrogen, expressed as a percentage of total nitrogen, decreased slightly with increased levels of protein. There was a corresponding slight increase in extractable nitrogen. Increased protein level had no significant effect on total nitrogen (Table V) or moisture and fat (*16*).

Table II. Potassium Chloride–Insoluble Nitrogen, Collagen Nitrogen, Elastin Nitrogen, and Mucroprotein Hexosamine Content of Fresh Tissue

Protein, %	Stilbestrol, Mg. per Lb. of Ration 0	0.3	0.6	Av.
	KCl-insoluble nitrogen, per cent of total nitrogen			
9	30.92	28.34	37.08	32.11
13	22.84	21.92	36.01	26.92
17	21.82	26.93	32.38	27.04
Av.	25.19	25.73	35.16[a]	28.69
	Collagen nitrogen, per cent of total nitrogen			
9	1.09	1.18	1.25	1.17
13	1.27	1.20	1.21	1.23
17	1.15	1.29	1.14	1.19
Av.	1.17	1.22	1.20	1.20
	Elastin nitrogen, per cent of total nitrogen			
9	0.57	0.50	0.70	0.59
13	0.37	0.54	0.65	0.52
17	0.38	0.54	0.58	0.50
Av.	0.44	0.53	0.64[b]	0.54
	Ratio of mucoprotein hexosamine to total nitrogen (×100)			
9	0.170	0.155	0.246	0.190
13	0.185	0.148	0.208	0.180
17	0.155	0.180	0.207	0.181
Av.	0.170	0.161	0.220[c]	0.184

[a] Linear effect significant at $P = 0.025$ or less.
[b] Linear effect significant at $P = 0.005$ or less.
[c] Linear effect significant at $P = 0.005$ or less and quadratic effect significant at $P = 0.05$ or less.

Table III. Potassium Chloride–Insoluble Nitrogen, Collagen Nitrogen, Elastin Nitrogen, and Mucroprotein Hexosamine Content of Moisture-Free Fat-Free Tissue

Expressed as per cent (grams/100 grams moisture-free, fat-free tissue)

Protein, %	Stilbestrol, Mg. per Lb. of Ration 0	0.3	0.6	Av.
	KCl-insoluble nitrogen			
9	4.58	4.22	5.39	4.73
13	3.39	3.34	5.32	4.02
17	3.18	4.00	4.79	3.99
Av.	3.72	3.85	5.17[b]	4.25
	Collagen nitrogen			
9	0.156	0.172	0.181	0.170
13	0.191	0.179	0.178	0.183
17	0.195	0.189	0.170	0.185
Av.	0.181	0.180	0.176	0.179
	Elastin nitrogen			
9	0.083	0.076	0.102	0.087
13	0.055	0.083	0.096	0.078
17	0.057	0.058	0.086	0.067
Av.	0.065	0.072	0.095[a]	0.077
	Mucoprotein hexosamine			
9	0.0251	0.0226	0.0359	0.0279
13	0.0220	0.0220	0.0307	0.0249
17	0.0226	0.0264	0.0306	0.0265
Av.	0.0232	0.0237	0.0324[b]	0.0264

[a] Linear effect significant at $P = 0.025$ or less.
[b] Linear effect significant at $P = 0.005$ or less, and quadratic effect significant at $P = 0.025$ or less.

Table IV. Analyses of Variance for Connective Tissue Components of Longissimus Dorsi Muscle

Sources of Variation	Degrees of Freedom	Mean Squares, %: % of Total Nitrogen: KCl-insol. N	Collagen	Elastin	Hexosamine	% of Moisture-Free, Fat-Free Tissue: KCl-insol. N	Collagen	Elastin	Hexosamine
Stilbestrol	2	374.1186[a]	0.0133	0.1107[b]	0.011363[c]	7.7065[a]	0.000021	0.002597[a]	0.032273[c]
Linear	1	591.8280[d]	0.0121	0.2204[c]	0.012973[c]	12.6440[d]	0.000024	0.004902[d]	0.051152[c]
Quadratic	1	156.4092	0.0145	0.0010	0.009754[a]	2.7691	0.000018	0.000292	0.013394[d]
Protein	2	105.7402	0.0138	0.0164	0.000286	2.0924	0.000394	0.000778	0.002540
Linear	1	155.6013	0.0045	0.0294	0.000337	3.2487	0.000704	0.001536	0.000925
Quadratic	1	55.8802	0.0232	0.0035	0.000235	0.9361	0.000085	0.000020	0.004156
Stilbestrol × protein	4	32.3378	0.0461	0.0146	0.001512	0.7212	0.000501	0.000301	0.002166
Replicate	3	359.0096[a]	0.0180	0.3564[c]	0.000960	10.0322[d]	0.001893	0.007661[c]	0.000105
Finish	1	882.7831[c]	0.0001	0.3640[c]	0.000420	25.8403[c]	0.000324	0.008805[c]	0.000191
Initial wt.	1	78.6473	0.0537	0.6507[c]	0.001081	2.4964	0.003885	0.013034[c]	0.000061
Replicate × stilbestrol	6	150.7813	0.0057	0.0811[d]	0.002051	3.5228	0.000417	0.001114	0.003927
Replicate × protein	6	73.0795	0.0064	0.0301	0.000693	1.6676	0.000074	0.001156	0.001733
Experimental error	8[e]	53.3269	0.0322	0.0112	0.001576	1.1302	0.000475	0.000475	0.001757
Total	31								

[a] Significant at $P = 0.05$ or less. [b] Significant at $P = 0.01$ or less. [c] Significant at $P = 0.005$ or less. [d] Significant at $P = 0.025$ or less. [e] Four missing values computed for analyses, so degrees of freedom for error and total reduced by four.

Table V. Nitrogen Analyses of Longissimus Dorsi Muscle Fractions

(Values expressed as per cent, fresh tissue)

Protein, %	Stilbestrol, Mg. per Lb. of Ration: 0	0.3	0.6	Av.
	Total nitrogen			
9	3.15	2.98	3.01	3.05
13	2.96	3.13	3.11	3.07
17	3.09	3.09	3.14	3.11
Av.	3.07	3.07	3.09	3.08
	Potassium chloride extract			
9	2.19	2.13	1.87	2.06
13	2.28	2.43	1.99	2.23
17	2.45	2.21	2.12	2.26
Av.	2.31	2.26	1.99	2.19
	Potassium chloride–insoluble residue[a]			
9	0.96	0.85	1.14	0.98
13	0.68	0.70	1.12	0.83
17	0.64	0.88	1.02	0.85
Av.	0.76	0.81	1.09	0.89

[a] Obtained by difference (potassium chloride–extractable nitrogen subtracted from total nitrogen).

Table VI. Connective Tissue Components of Longissimus Dorsi by Replicates

	% of Total N: Light	Heavy	Av.	% Moisture-Fat-Free Tissue: Light	Heavy	Av.
	Potassium Chloride–Insoluble N			Potassium Chloride–Insoluble N		
High finish	20.46	27.00	23.73	2.91	3.88	3.40
Low finish	33.95	33.32	33.64	5.05	5.13	5.09
Av.	27.20	30.16	28.68	3.98	4.50	4.24
	Collagen N			Collagen N		
High finish	1.26	1.19	1.22	0.179	0.171	0.175
Low finish	1.26	1.18	1.22	0.198	0.165	0.181
Av.	1.26	1.18	1.22	0.189	0.168	0.178
	Elastin N			Elastin N		
High finish	0.34	0.53	0.43	0.048	0.075	0.061
Low finish	0.46	0.81	0.63[a]	0.068	0.117	0.093[a]
Av.	0.40	0.67[b]	0.54	0.058	0.096[b]	0.077
	Ratio, Mucoprotein Hexosamine–Total N (×100)			Mucoprotein Hexosamine		
High finish	0.188	0.187	0.187	0.266	0.266	0.266
Low finish	0.192	0.169	0.180	0.264	0.259	0.262
Av.	0.190	0.178	0.184	0.265	0.263	0.264

[a] Average effect of finish significant at $P = 0.01$ or less.
[b] Average effect of weight significant at $P = 0.01$ or less.

Weight and Finish

Although the lambs used in this experiment were approximately the same age and weight, they were subdivided into replicates according to weight and finish: (1) light, high finish, (2) light, low finish, (3) heavy, high finish, and (4) heavy, low finish. Light animals averaged less than 82 pounds; heavy animals weighed 82 pounds or more.

Analysis of the connective tissue data from the standpoint of initial weight and finish showed certain significant differences (Table VI). Figures represent averages of the three protein levels and three stilbestrol levels fed.

Discussion

It seemed reasonable to suppose that if stilbestrol administration contributed to increased connective tissue in lean muscle, the palatability and acceptability of lamb meat might be affected also. However, organoleptic studies of legs from lambs used in other phases of this research (*7*) indicated no significant differences in flavor of lean or fat, moistness, or tenderness of cooked lamb. There was a slight decrease in tenderness when the higher level of stilbestrol was fed.

Optimum Stilbestrol Level

The data indicate that stilbestrol increases the connective tissue of lamb skeletal muscle in direct proportion to the amount of the hormone administered. Preston (*15*) concluded that 0.3 mg. of stilbestrol per pound of ration (about 1 mg. per day) is the ideal level for wether lambs from the standpoint of maximum feed lot efficiency and minimum undesirable side effects. At this level, the effect on skeletal muscle connective tissue is also at a minimum, as indicated in the results reported here. At the 0.6-mg. level, however, the skeletal muscle connective tissue is significantly increased and the seriousness of the side effects (*7*, *15*) is much more marked. At 1.2 mg., Preston reported indications of actual toxicity which offset the gain-stimulating effect of the hormone. From a feed lot standpoint, it would appear therefore that in the oral administration of stilbestrol, there is a critical level (*18*) beyond which feed lot performance is not improved and marked physiological changes occur.

Connective Tissue Metabolism

Although increased skeletal muscle

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 (mucroprotein), 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.

Acknowledgment

The authors are indebted to Rodney Preston, who supervised the experimental feeding and conducted the moisture and fat analyses which were used as a basis for some of the calculations reported herein. Grateful acknowledgment is also made to P. G. Homeyer for assistance in the experimental design and statistical analyses, and to Pearl Swanson for helpful suggestions.

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Received for review September 6, 1960. Accepted April 4, 1961. Journal Paper J-3953, Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Projects 1313 and 869.

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,