Relation between Hydroxyproline of Alkali-Insoluble Protein and Tenderness of Bovine Muscle

E. JEAN LOYD[1] and RICHARD L. HINER

Meat Quality Laboratory, Animal Husbandry Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

For many years meat analysts have tried to show the relation between composition and tenderness of meat. A method that separates alkali-insoluble protein into three fractions shows significant correlation between the total hydroxyproline content of the fractions from nine beef muscles and two measurements of their tenderness. A short method that halves the time required for the fractionation procedure shows significant correlation between the hydroxyproline content of the alkali-insoluble protein of six beef muscles and two measurements of their tenderness.

FOR MORE THAN 50 YEARS meat technologists have tried to separate the connective tissue from meat, measure it, and show its relation to tenderness. The components of connective tissue have been vaguely defined and their partition and analysis are limited by the flaws and length of existing methods. Despite these stumbling blocks, during the past 10 years, the work of biochemists and histologists has indicated that the old belief that connective tissue causes toughness in meat may have scientific basis.

Since 1950 the determination of the amino acid hydroxyproline by Neuman and Logan's method (*11*) has been accepted as the most accurate index of connective tissue content of biological materials (*5*).

Wierbicki, Kunkle, Cahill, and Deatherage (*16*) in 1956 reported hydroxyproline values ranging from 0.0296 to 0.0491% in meat for 32 *Longissimus dorsi* muscles of beef. The mean hydroxyproline content of these samples was 0.0376%. They concluded that connective tissue expressed in terms of hydroxyproline plays some part in tenderness 3 days and less significantly 13 days post mortem.

In 1955 Hiner, Anderson, and Fellers (*3*) presented histological data indicating that collagen and elastin fibers influence tenderness.

The objective of the present investigation was to show whether there is a relation between the hydroxyproline content of the alkali-insoluble protein of muscle and the tenderness of meat. The assays of hydroxyproline were made on the protein remaining undissolved after muscle samples were digested twice in 0.1*N* sodium hydroxide. A preliminary report has been made (*8*).

[1] Present address, Quality Evaluation Section, Marketing Research Division, U. S. Department of Agriculture, Plant Industry Station, Beltsville, Md.

Materials and Methods

Animals. The muscles analyzed for hydroxyproline in the collagen, elastin, and fat fractions in Table I were from animals described by Winchester, Hiner, and Scarborough (*18*). Veal animals 1347 and 3961 were bull calves from the Beltsville dairy herd. Veal 126A was an Angus bull calf. Yearlings 751 and 1197 were dairy bulls; animals 665 and K85 were barren Shorthorn heifers. All others were animals slaughtered at Beltsville for other experiments.

Measurements of Tenderness. The heated samples used for the measurement of tenderness were described by Sperring, Platt, and Hiner (*13*). Rating of tenderness by the taste panel followed the method of Alexander, Clark, and Howe (*1*).

Tenderness of the muscles was measured by the Warner-Bratzler shear developed in 1928 by Warner (*14*) and refined in 1932 by Bratzler (*2*). The tenderness of 11 muscle samples was also measured by the meat tenderness pressure method introduced by Sperring, Platt, and Hiner (*13*) in 1958.

Meat Samples for Chemical Analysis. *Longissimus dorsi* muscles (rib eye) from the 13th rib, adjacent to the 12th rib that was used for the measurement of tenderness, were aged for 10 days and frozen until the time of analysis.

Samples of *Semitendinosis* (round) and *Psoas major* (tenderloin) were taken next to the samples used for tenderness measurements. These samples were from carcasses aged 10 days.

All separable fat and connective tissue were removed from meat samples before analysis.

Method 1. Analysis of Hydroxyproline in Collagen, Elastin, and Fat Fractions. In a procedure based on that of Lowry, Gilligan, and Katersky (*7*) a slurry was prepared, weighed, and digested in alkali as outlined by Husaini, Deatherage, Kunkle, and Draudt (*6*). Eight to 10 grams of the meat-water (1 to 1) slurry were used. After the first digestion in alkali, the soluble muscle proteins were separated from the insoluble connective-tissue proteins by centrifugation and the supernatant liquid was discarded. Often this was not a clean separation. The amount of fat or fatlike material on top appeared to vary between duplicates. There was often floating material in the supernatant solution. Other workers (*9*) have questioned this separation. The lean samples of *Semitendinosis* and veal did not have a fat layer. In the present study, fat layers were transferred to test tubes for hydrolysis. The fatty material from the surface of the alkali digestion will hereafter be referred to as the fat fraction. It probably contains more than fat.

The presence of hydroxyproline in the hydrolyzates of the fat fractions was confirmed by Pasieka and Morgan's specific qualitative test (*12*).

The collagen and elastin fractions and the hydrolyzates of the collagen, elastin, and fat fractions were prepared by the Miyada and Tappel method (*10*).

The hydroxyproline content of the hydrolyzates was assayed by the Neuman and Logan method (*11*). The number of unknowns that could be assayed was limited by the autoclaving of a standard hydroxyproline solution and color development in six tubes of known hydroxyproline concentration with each set of unknowns. The standard curves, plotted from 10 sets of four dilutions each of standard hydroxyproline solution, matched well. This indicated the stability of the standard curve and in all later work a set of standards was run and a standard curve was plotted for each 10 samples analyzed.

Tryptophan does not interfere with color formation in acid hydrolyzates. Because Wierbicki and Deatherage (*15*)

found that the greatest error in hydroxyproline analysis occurred in sampling, no correction was made for tyrosine in the present study.

Method 2. A Short Procedure to Determine the Hydroxyproline Content of Meat. Exposed surfaces of the muscle were discarded. About 25 grams of muscle were minced with scissors into the bowl of a small laboratory mixer. The meat was comminuted in the mixer, beginning at slow speed and gradually increasing to high speed. The meat was mixed at high speed for only 30 seconds. Samples, 2 to 5 grams, were weighed in tared, 60-ml. heavy-duty centrifuge tubes. Samples were digested in 0.1*N* sodium hydroxide overnight at room temperature. The next morning they were centrifuged at maximum speed for 1 hour.

The fat layers of the samples being analyzed by Method 2 appeared smaller and more uniform than those of the samples analyzed by Method 1. The low-fat samples had no fat layer. Fat layers were transferred to test tubes and saved to be hydrolyzed after the collagen and elastin fractions were added. The remaining supernatant liquid was discarded.

The residue in the centrifuge tube was digested for 4 hours in fresh 0.1*N* sodium hydroxide at room temperature and then centrifuged. If any fat appeared on the surface of the supernatant, it was added to the fat from the first centrifugation. The supernatant was discarded.

With the aid of 5 ml. of 6*N* hydrochloric acid, the residue was washed into the test tube containing the fat fraction of the sample. Hydrochloric acid, 6*N* was added to make up to 10 ml. If the sample was large, more acid was added. The test tubes were sealed and autoclaved for 16 hours at 15 p.s.i. For convenience the samples were autoclaved overnight. Tubes were opened and hydroxyproline was assayed by Neuman and Logan's method (*11*). A standard hydroxyproline solution was hydrolyzed and a standard curve was plotted from each 10 samples.

Results and Discussion

The present study finds that the mean hydroxyproline content of duplicates run on five beef *Semitendinosis* muscles is 0.42 gram per 100 grams of fat, moisture-free tissue; and the mean of duplicates run on three *Semitendinosis* muscles of veal is 0.39 gram per 100 grams of fat, moisture-free tissue.

The hydroxyproline content of duplicates run on 16 *Longissimus dorsi* muscles of beef ranges from 0.037 to 0.104%, with a mean of 0.063% of fresh meat.

Table I summarizes data on three animals, highly finished and U. S. choice grade. Each sample was run in duplicate and two color assays were made on each duplicate. The means were reported because the tenderness measurements were not paired with the chemical analyses.

Table I. Hydroxyproline and Tenderness Method 1

		Hydroxyproline Content of Indicated Fractions of Alkali-Insoluble Protein, Mg./100 G. Muscle					Measurements of Tenderness	
Animal	Muscle	Collagen	Elastin	Collagen and elastin	Fat	Collagen, elastin, and fat	Mechanical shear	Taste-panel rating[a]
73	*Semitendinosis*	105.8	3.3	109	0.7	110	9.72	5.80
	Longissimus dorsi	19.9	1.6	22	38.6	60	9.72	5.80
	Psoas major	12.7	1.1	14	16.3	30	6.86	6.00
84	*Semitendinosis*	89.7	2.6	92	6.5	99	13.89	5.40
	Longissimus dorsi	4.1	0.7	5	51.4	56	5.47	6.20
	Psoas major	11.4	1.1	12	14.3	27	3.12	7.00
86	*Semitendinosis*	55.3	2.4	58	10.1	68	8.75	5.40
	Longissimus doris	9.3	0.8	10	34.7	45	7.61	6.00
	Psoas major	2.99	0.7	4	14.4	16	1.58	6.80

[a] 1. Very tough. 7. Very tender.

Table II. Correlation between Hydroxyproline Content of Alkali-Insoluble Protein and Tenderness of Beef Muscles

Hydraxyproline Samples		Value of r: Mechanical shear	Value of r: Taste-panel rating
	Method 1		
Total collagen + elastin	9	0.814[a]	−0.695[b]
Total collagen + elastin + fat	9	0.879[a]	−0.823[a]
	Method 2		
Total	6[c]	0.960[a]	−0.903[b]
Total	13[d]	0.964[a]	−0.811[a]

[a] Significant at 1% level. [b] Significant at 5% level. [c] These 6 samples, 2 each of *Semitendinosis*, *Longissimus dorsi*, and *Psoas major*, were analyzed separately to weigh the correlation equally by type of muscle. [d] Group includes samples of [c] plus 7 *Longissimus dorsi* muscles.

In Table I figures for the two total columns of *Semitendinosis* muscle agree. The ether-extract fat content of these muscles ranged from 4.36 to 7.74%. The figures for the two total columns of *Longissimus dorsi* and *Psoas major* muscles do not agree. The ether-extract fat content of *Longissimus dorsi* muscles ranged from 11.51 to 19.20%. The ether-extract fat content of *Psoas major* muscles ranged from 10.10 to 13.65%. In the muscles with lower fat content there is less tendency for the hydroxyproline-bearing protein to be lost in the fat fraction. In the fatter muscles most of the hydroxyproline was recovered from the fat fraction. In the *Longissimus dorsi*, on the average, 71% of the hydroxyproline was found in the fat fraction, while in the *Psoas major*, 58% of the hydroxyproline was found in the fat fraction.

In Table II, the correlation between tenderness as measured by mechanical shear and taste panel and the sum of the hydroxyproline contents of two fractions is compared with the correlation between tenderness and the sum of the hydroxyproline contents of three fractions. The correlation between hydroxyproline and mechanical shear is improved by the addition of the fat fraction, but both correlations are significant. The correlation between hydroxyproline and taste-panel rating is significant for the total of collagen and elastin fractions, but it becomes highly significant with the addition of the fat fraction. This raises the coefficient of determination ($r^2 \times 100$) from 66.28 to 76.88% in the relation of hydroxyproline to mechanical shear; and from 48.46 to 67.74% in the relation of hydroxyproline to taste-panel rating.

Table III. Hydroxyproline and Tenderness, Method 2[a]

Animal	Hydroxyproline, Mg./100 G. Meat	Measure of Tenderness: Mechanical shear, lb.	Measure of Tenderness: Taste-panel[b] rating
665[c]	160	34.78	2.20
K85[c]	99	24.03	2.40
665[d]	104	18.06	4.80
K85[d]	79	14.75	4.80
665[e]	37	10.49	6.80
K85[e]	36	7.58	6.80
31[c]	84	11.89	6.20
104[c]	70	16.11	4.40
95[c]	65	13.28	5.80
1347[c]	63	11.96	5.20
96[c]	61	13.78	5.80
89[c]	59	13.86	6.60
90[c]	56	11.28	7.00

[a] First 6 muscles analyzed separately to weigh the correlation equally by type of muscle.
[b] 1. Very tough. 7. Very tender.
[c] *Semitendinosis*. [d] *Longissimus dorsi*.
[e] *Psoas major*.

Analysis of variance showed that the difference due to the addition of the fat fraction to the hydroxypyroline total gives the following values of *F*: 5.1, not significant for *Semitendinosis* muscles; 71.2, significant at the 5% level for *Longissimus dorsi* muscles; and 154.0, significant at the 1% level for *Psoas major* muscles.

Table III summarizes data from the analyses of 13 muscles by the short Method 2. These hydroxyproline totals include the hydroxyproline from the fat fraction. The first six muscles were analyzed separately to weigh the correlation equally by type of muscle. Table II shows that the first six muscles give a highly significant correlation between hydroxyproline content and mechanical shear and the coefficient of determination was 92.16%. The correlation between hydroxyproline content and taste panel rating was significant and the coefficient of determination was 81.54%. When the seven *Longissimus dorsi* muscles are included, the correlation on the total 13 muscles is highly significant. There is little change in the correlation between hydroxyproline content and mechanical shear. With the taste-panel rating the correlation is slightly less significant and the coefficient of determination drops from 81.54 to 65.77%.

Tenderness measurements and hydroxyproline assay, including the fat fraction, were made on 26 muscles. These included *Longissimus dorsi*, 16; *Semitendinosis*, 5; and *Psoas major*, 5. Method 1 was used for 13 of the analyses; Method 2 was used for 11. Both methods were used for two analyses. Because two methods were used, it is statistically unsound to consider this entire group of data as a single experiment. Even with this variable, the data show highly significant correlation between hydroxyproline content and tenderness measurements. For hydroxyproline and mechanical shear the coefficient of correlation is 0.840; for hydroxyproline and taste-panel rating, −0.734.

Tenderness measurements of 11 samples were made by the meat-tenderness pressure method. The correlation coefficient of 0.59 is not significant between hydroxyproline content and tenderness-pressure readings on raw meat. This may be due to the fact that the readings made with the press on the raw samples were made at various times post mortem. The correlation coefficient of 0.894 for hydroxyproline content and tenderness-pressure readings on cooked samples is highly significant. The readings on cooked samples were all made 10 days post mortem.

Only two sets of duplicates were run by the two methods on the same muscles. On *Longissimus dorsi* from animal 90, Method 1 yielded 56 and 63 mg. of hydroxyproline per 100 grams of meat. Method 2 yielded 56 and 57 mg. per 100 grams. On the *Longissimus dorsi* from animal 95, Method 1 yielded 64 and 67 mg. of hydroxyproline per 100 grams of meat; Method 2 yielded 65 and 65 mg. per 100 grams. These results indicate that short Method 2 may be more precise than Method 1.

The procedure of Method 1 requires 5 or 6 days. Each sample is ground three times, blended once, weighed twice, centrifuged five times, and autoclaved three times. Method 2 requires 3 days. Each sample is comminuted once, weighed once, centrifuged twice, and autoclaved once. It is possible for the analyst to carry on about twice as many analyses simultaneously with Method 2 as with Method 1. More significant correlations of hydroxyproline content with tenderness, in samples analyzed by Method 2, indicate the simplified procedure may prevent losses through manipulation and give more accurate results. Both methods recover hydroxyproline associated with the fat. Fourteen sets of duplicates analyzed by Method 2 show that the average deviation from the mean was ±3.72%. Tenderness data were available on 13 of these muscles.

Table IV gives the hydroxyproline content of muscles from animals of different ages. The short Method 2 was used for the analysis of most of these samples. The analyses of veal indicate that the hydroxyproline content of veal is not significantly greater than that of beef. Wilson and Bray (*17*) reported that veal contained 50% more connective tissue than beef. Recovery and analysis of hydroxyproline from the fat fraction of beef brought the hydroxyproline content of beef to the same range as that of veal. The veal muscles reported in Table IV contained less than 1% of intramuscular fat. These facts indicate that veal, containing almost no fat, is analyzed accurately by almost any method, while the higher fat content of beef interferes with accurate analysis.

The muscles from the two yearling bulls reported in Table IV had high hydroxyproline contents, but not as high as those of the barren heifers. Analysis of a larger number of samples from animals with great age differences is needed to establish an age and hydroxyproline-content relationship. The relation between age and tenderness in beef was reported in 1950 (*4*).

Table IV. Effect of Age on Hydroxyproline Content

Animal	Age, Months	Hydroxyproline, Mg./100 G. Muscle: Semitendinosis	Longissimus dorsi	Psoas major
126A	2	103	42[a]	34
1347	2	98	63	31
3961	2	104[a]	70[a]	30
751	12	113[a]	60[a]	...
1197	12	118	74[a]	...
665	132	160	104	37
K85	96	99	79	36

[a] These analyses followed Method 1. All others followed Method 2.

Literature Cited

(1) Alexander, L. M., Clark, N. G., Howe, P. E., "Methods of Cooking and Testing Meat for Palatability," Bur. Human Nutrition and Home Economics and Bur. Animal Ind., U. S. Dept. Agr., 1933.
(2) Bratzler, L. J., "Physical Research in Meat," final rept. U. S. Dept. Agr. and Kansas State Experiment Station, 1933.
(3) Hiner, R. L., Anderson, E. E., Fellers, C. R., *Food Technol.* **9,** 80 (1955).
(4) Hiner, R. L., Hankins, O. G., *J. Animal Sci.* **9,** 347 (1950).
(5) Husaini, S. A., Deatherage, F. E., Kunkle, L. E., *Food Technol.* **4,** 313 (1950).
(6) Husaini, S. A., Deatherage, F. E., Kunkle, L. E., Draudt, H. N., *Ibid.*, **4,** 366 (1950).
(7) Lowry, O. H., Gilligan, D. R., Katersky, E. M., *J. Biol. Chem.*, **139,** 795 (1941).
(8) Loyd, E. J., Hiner, R. L., *J. Animal Sci.* **17,** 1157 (1958).
(9) Miller, M., Kastelic, J., J. Agr. Food Chem. **4,** 337 (1956).
(10) Miyada, D. S., Tappel, A. L., *Food Research* **21,** 217 (1956).
(11) Neuman, R. E., Logan, N. A., *J. Biol. Chem.* **184,** 299 (1950).
(12) Pasieka, A. E., Morgan, J. F., *Proc. Soc. Exptl. Biol. Med.* **92,** 96 (1956).
(13) Sperring, D. D., Platt, W. T., Hiner, R. L., *Food Technol.* **13,** 155 (1959).
(14) Warner, K. F., "Progress Report of the Mechanical Test for Tenderness of Meat," *Proc. Am. Soc. Animal Production* **114** (1928).
(15) Wierbicki, E., Deatherage, F. E., J. Agr. Food Chem. **2,** 878 (1954).
(16) Wierbicki, E., Kunkle, L. E., Cahill, V. R., Deatherage, F. E., *Food Technol.* **10,** 80 (1956).
(17) Wilson, G. B., Bray, R. W., Phillips, P. H., *J. Animal Sci.* **13,** 826 (1954).
(18) Winchester, C. F., Hiner, R. L., Scarborough, V. C., *Ibid.*, **16,** 426 (1957).

Received for review November 14, 1958. Accepted July 6, 1959. Presented in part at the 50th Annual Meeting, American Society of Animal Production, Chicago, Ill., November 28-29, 1958.