Changes in Sulfhydryl and Disulfide Content of Chicken Muscle and the Effect of N-Ethylmaleimide

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The sulfhydryl and disulfide content of the *pectoralis major* from chickens was determined polarographically. The ratio of sulfhydryl to disulfide decreased rapidly post-mortem and reached a minimum within 2 hours, after which there was little change. Shear values were determined on excised muscles which were treated with N-ethylmaleimide, which reacts specifically with sulfhydryls. This reagent prevents complete tenderization, regardless of whether it is added before or during the period in which the muscle is in the rigor state. These results were interpreted to implicate sulfhydryl groups in the pre- and postrigor reactions which are important in the ultimate tenderization of muscle.

The post-mortem chemical and physical changes which occur in muscle tissue have been investigated intensively (Whitaker, 1959) and reviewed recently (Briskey *et al.*, 1966; de Fremery, 1966). One characteristic change is the decrease in pH of the muscle because glycogen is converted to lactic acid (de Fremery and Pool, 1963). Another change is the decrease in concentrations of both ATP and phosphocreatine. When the ATP concentration in muscle reaches a low level the muscle contracts, becomes hard, and is then considered to be in rigor (Bate-Smith and Bendall, 1956). Avian muscles require about 2 to 4 hours post-mortem to enter the rigor state, and 12 to 18 hours for postrigor tenderization (Klose *et al.*, 1959).

Chajuss and Spencer, (1962a) proposed that stabilization of the rigor state in muscle tissue was due, at least in part, to the conversion of protein sulfhydryl (—SH) groups to disulfide (—SS—) linkage forming a strained, three-dimensional network. This may be expressed in the following way:

$$2 \text{ RSH} \rightarrow \text{R—S—S—R} + 2 \text{ H}^+ + 2 \text{ } e^-$$
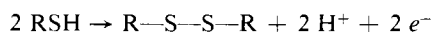
They demonstrated that the post-mortem disappearance of —SH groups paralleled the onset of rigor (Chajuss and Spencer, 1962b). The —SH content of muscle which had been aged in air decreased from an initial concentration of 9.9×10^{-6} to about 6.5×10^{-6} mole per gram after entering the rigor state. These values remained constant for 72 hours, the maximum aging period used in their studies. Chajuss and Spencer (1962b) also proposed a mechanism for postrigor tenderization which was compatible with their belief that rigor was at least partially due to oxidation of muscle sulfhydryl groups to disulfide- which would link protein molecules. They postulated that postrigor tenderization was due to sulfhydryl-disulfide exchange reactions, which could produce randomization and relaxation of the network formed at the onset of rigor. These exchanges may be described by the following reaction:

$$\text{R—S—S—R} + \text{R}'\text{—SH} \rightleftharpoons \text{R}'\text{—S—S—R} + \text{R—SH}$$
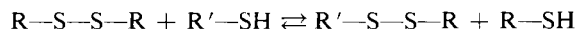
Exchange reactions of this type have been established in the relaxation of bread doughs (Frater *et al.*, 1961).

This report presents analyses of sulfhydryl and disulfide content of chicken muscle which had been aged either in air on the carcass or in water after excision. These experiments were done to determine the effect of N-ethylmaleimide (NEM), a reagent which specifically reacts with sulfhydryl groups, on the shear resistance of muscles.

EXPERIMENTAL

Sample Preparation. The mature fowl used in these experiments were sacrificed by cutting the carotid artery. For the —SH and —SS determinations, four chicken carcasses were used. For the studies with NEM, three carcasses were used. The pectoralis major muscles were removed and placed in water at 5° C. Paired muscles were used for comparisons, one muscle being the control. Other samples were aged in air on the carcass at room temperature. Samples were prepared for sulfhydryl and disulfide analysis by freeze-drying thin slices of muscle. Samples taken immediately after sacrifice were frozen in a dry ice–acetone slurry before slicing.

Sulfhydryl Analysis. A modification of the method of Kolthoff (1954) was used to determine sulfhydryl groups. Samples which had been freeze-dried were ground to a fine powder. The sample (100 mg.) was added to 20 ml. of a hydrolysis mixture containing 0.005% pepsin in 0.05M HCl. The number of titratable —SH groups reached a maximum value after digestion for 60 minutes. The 30 ml. in the titration vessel contained the following: $0.033M$ NaB_4O_7, $0.57M$ KCl, 0.066% antifoam B (Dow Corning Corp.), and 50 mg. of hydrolyzed muscle tissue. Approximately 10 ml. of chloroform were added to the electrolysis vessel to prevent contact of mercury and sample.

The titration was made with a dropping mercury electrode at a potential of −0.35 volt *vs.* a standard calomel electrode in a nitrogen atmosphere using a Sargent Model VII polarograph. The titrant was 0.01M $HgCl_2$.

Disulfide Analysis. To determine disulfide content, a 125-mg. sample of freeze-dried muscle was hydrolyzed in 20 ml. of the acidified pepsin solution. After digestion,

a 15-ml. aliquot of hydrolyzed sample was refluxed in 0.4N HCl (20 ml.) for 2 hours. This treatment quantitatively converted sulfhydryl groups to disulfide. Four milliliters of 1M sodium sulfite solution was substituted for water in the reaction vessel to make the titrated solution 0.14M in sulfite. This sulfite treatment splits disulfide bonds, producing one sulfhydryl group and one S-sulfonate group. The sulfhydryl groups were titrated as usual.

NEM Addition and Shear Resistance. The birds were sacrificed as described previously. The *pectoralis major* was removed and placed in 300 ml. of cold (5° C.) aging medium. When NEM was included, the medium contained 0.65 gram in 300 ml. of distilled water. Muscles were stored at 4° C. for 16 hours and at room temperature for an additional 2 hours. At the end of the aging period, the muscles were cooked with steam to an internal temperature of 77° C., as determined with a thermocouple. After cooling to room temperature, sections (1-sq. cm. cross section) were cut parallel to the muscle fibers. Shear measurements were made with a modified Warner-Bratzler shear apparatus (Spencer *et al.*, 1962).

RESULTS

The effect of aging in air on the sulfhydryl-disulfide content is shown in Table I. There was a definite decrease in the —SH/S—S ratio during the period of rigor. Significant amounts of sulfhydryl remain in the muscle tissue after aging. The residual sulfhydryls may be explained by the work of Ogura *et al.* (1960), who classified the —SH groups in proteinaceous tissue as fully reacting, sluggish, or masked. The preparative procedure used in the study reported here would be expected to make all —SH groups titratable, since it involved extensive hydrolysis by pepsin in acid solution.

Studies on the rate of conversion of sulfhydryl to disulfide showed that most of the changes occurred within the first 1 or 2 hours (Figure 1). The —SH/S—S ratio fell rapidly after sacrifice from an initial value of 1.71 to a value of about 1.10 in 2 hours, after which the ratio remained essentially constant. The average sulfur amino acid content of chicken muscle used in this study was 1.6 grams per 100 grams of dry tissue, which is comparable with the value of 1.3 grams per 16 grams of nitrogen obtained for young chickens by Fry and Stadelman (1960).

The effect of NEM on shear resistance was studied in three ways by varying the time of addition and aging time (Table II). In the first trial, NEM was added to one muscle of each pair at 75 minutes post-mortem. Adding NEM when the muscle was in the rigor state caused the muscle ultimately to have increased shear resistance. In the other trials NEM was added immediately after excising the muscle. Although shear values were not different statistically, there was a tendency for shear resistance to be less at the peak of rigor. After an 18-hour period there was an increased resistance to shear in comparison to the controls.
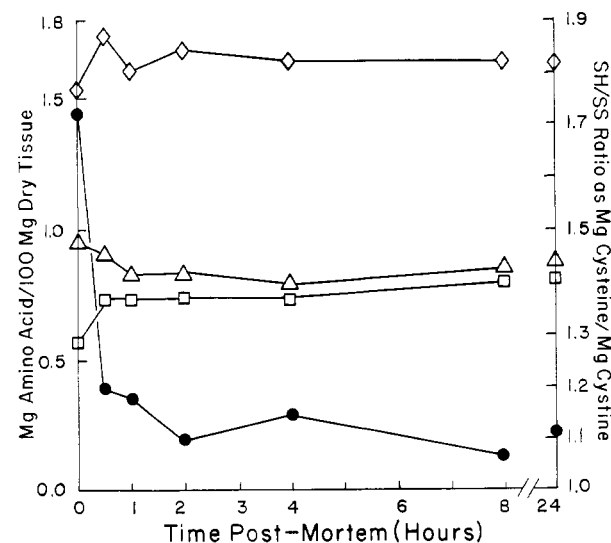
DISCUSSION

The decrease in sulfhydryl groups during the time that rigor was developing in the muscle suggested that sulfhydryls were involved in some reactions associated with rigor. The apparent effect of NEM, which reacts specifically with sulfhydryl groups, was to alter the develop-

Table II. Effect of NEM Aging Medium on Shear Resistance of Excised Chicken Muscle

Trial	Treatment	Hours, Post-Mortem Agent addition	Hours, Post-Mortem Aging time	Av. Final pH	Av. Shear Resistance, Kg./Sq. Cm.	Paired t
1	NEM			6.0	2.16	
	vs.	1.25	18			6.55[a]
	Water			5.9	0.99	
2	NEM			6.7	2.80	
	vs.	0	5			1.55
	Water			6.6	4.15	
3	NEM			6.3	1.26	
	vs.	0	18			2.39[b]
	Water			6.1	1.04	

[a] $P < 0.025$.
[b] $P < 0.05$.

Figure 1. Post-mortem changes in sulfhydryl and disulfide content of chicken muscle aged in water

● SH/SS ratio
△ Cysteine content
□ Cystine content
◇ Total S — amino acid content

Table I. Effect of Aging in Air at Room Temperature on Sulfhydryl and Disulfide Content of Chicken Muscle

Aging Time, Hours	Mg./100 Mg. of Dry Tissue Cysteine	Mg./100 Mg. of Dry Tissue Cystine	—SH/S—S[a,b]
0 (8 muscles)	0.87	0.62	1.41
18 (8 muscles)	0.67	0.74	0.90
24 (4 muscles)	0.52	0.72	0.72

[a] Mg. of cysteine per mg. of cystine.
[b] Paired $t = 5.77$, $P < 0.0005$.

ment of rigor and subsequent tenderization. The sulfhydryl groups which were combined with NEM were not identifiable. There is a possibility that sulfhydryl enzymes of the glycolytic pathway were involved. However, there was no apparent inhibition of glycolysis because there was no difference in final pH of the muscles subjected to the various treatments.

Ante-mortem injection of iodoacetate inhibits glycolysis and in some way alters the shear resistance, which is lower after short aging period but higher after a 24-hour aging period (de Fremery, 1966; de Fremery and Pool, 1963). These results were interpreted to implicate the glycolysis which occurred post-mortem as being associated with the toughness of the tissue. Further, they described rigor mortis (as determined by the disappearance of ATP) and toughness (as determined by the resistance to shear) to be distinct from each other. In contrast, the present authors have assumed that resistance to shear is dependent upon rigor mortis and interpret their results on this basis.

An exchange between sulfhydryl and disulfide groups, as suggested by Chajuss and Spencer (1962a), would be a plausible explanation for our observations. However, until identification of the sulfhydryl sites which bind NEM,

there can be no dogmatic assertion of the type of reactions which occur in the muscle after maximum toughness is observed at the height of rigor.

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