THE EFFECT OF POST - MORTEM ELECTRICAL STIMULATION ON THE MYOGLOBIN DERIVATIVES OF THREE BOVINE MUSCLES

BY

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INTRODUCTION

The color of the lean tissue has always been the universal guide for consumers' acceptance of meat. Therefore, it is the primary concern to all facets of the meat industry to direct efforts to promote and preserve the "desirable" color of meat.

However, it is extremely difficult to express what a "desirable" color of meat is because the same "bright cherry red" color used to describe fresh beef may hold different connotations for different people. This need of a more objective way to evaluate the color of muscular tissue directs the attention to one primary pigment of meat --- the myoglobin and its derivatives that give meat the "purplish red", "cherry red" or "brown" color that ultimately decides its salability on the retail counter.

Traditionally, the dressed carcass is chilled for at least 24 hours following slaughter before any further fabrications are performed. The development of rigor mortis during this period bestows on the muscular tissue a "set" configuration which facilitates the preparation of traditionally shaped cuts and an acid condition which discourages bacterial growth and promotes a "desirable" color in the cooled meat. Recently, prompted by a worldwide energy shortage, there is a growing interest in the use of electrical stimulation to accelerate the development of rigor mortis. The technical aspects of

such post-mortem electrical stimulation of the bovine carcass were carried out in a separate study. Realizing that a subjective evaluation (such as visual appraisal) of the effect of such electrical stimulation on the color of bovine muscles is inadequate, the objectives of this research are to (1) develop a quantitative method to determine the amount and proportions of myoglobin and its derivatives (2) investigate the effect of post-mortem electrical stimulation on the amount and proportions of myoglobin and its derivatives in the bovine semimembranosus, longissimus dorsi and the semitendinosus muscle.

LITERATURE REVIEW

Chemistry of Myoglobin

Structure of Myoglobin

Early study of myoglobin was centered around the heme structure. Kendrew et al. (1960) elucidated the entire structure and configuration of the myoglobin molecule using X-ray diffraction patterns and Fourier synthesis. Using sperm whale myoglobin, they revealed the tertiary structure of the molecule. Edmundson (1965) characterized the complete amino acid sequence of sperm whale myoglobin.

The general nature of the structure of myoglobin was best described by Kendrew (1960). Myoglobin is a conjugated protein consisting of a single polypeptide chain of about 153 amino acid residues associated with an iron-porphyrin complex, the heme group. It's molecular weight is about 17,500, and the molecule contains some 1,200 atoms (excluding hydrogen). Kendrew (1963) concluded that in the structure of the myoglobin molecule, some 118 amino acid residues make up eight segments of right-handed α-helix, seven to twenty four residues long. These segments are joined by two sharp corners and five nonhelical segments with another nonhelical tail of five residues at the carboxyl end of the chain. The whole molecule is folded in a complex

and unsymmetrical manner to form a flattened, roughly triangular prism with dimensions about 45 by 35 by 25 angstroms. The whole structure is extremely compact with no water inside the molecule (except for less than five single water molecules presumably trapped at the time the molecule was folded). The heme group is disposed almost normally to the surface of the molecule, one of its edges being at the surface and the rest buried deeply within the molecule. Almost all those side chains containing polar groups (lysine, arginine, glutamic acid, aspartic acid, histidine, serine, threonine, tyrosine and tryptophane) are on the surface of the molecule, with the interior of the molecule almost entirely made up of nonpolar residues. Kendrew also concluded that the integrity of the whole structure is maintained mainly by the van der Waals forces between nonpolar residues which make up the bulk of the interior of the molecule. With the iron atom lying in the center of the heme structure, four of its ligand sites are occupied by the pyrrole nitrogen atoms. The fifth coordination site of the iron atom in the tetrapyrrole prosthetic group is occupied by a histidine residue. The sixth such coordination site, on the other side of the tetrapyrrole structure, is probably occupied by a water molecule that is presumably displaced by O_2 when the myoglobin is converted to its oxygenated form. Beyond the water molecule, in a position suitable for hydrogen bond formation, is a second histidine residue. This same arrangement of two histidines also exists in hemoglobin, except that in hemoglobin there are four heme groups involved plus four polypeptide chains

which accounts for the molecular weight being approximately four times that of myoglobin (Figure 1, 2)(Table 1).

Properties of myoglobin

Electrophoretic moiety

Lewis and Schweigert (1955) found that crystalline beef myoglobin isolated by ammonium sulfate fractionation to be electrophoretically inhomogeneous. The accompanying materials were shown not to be hemoglobin. Perkoff et al. (1962) concluded that human metmyoglobin could be resolved chromatographically on diethylaminoethyl-cellulose into at least four distinct heme protein components. Perkoff also found that the two chromatographic components that accounted for 75 to 80% of the total myoglobin of normal human muscle appeared to differ from one another only in their heme prosthetic groups. Quinn and Pearson (1964) detected three distinct myoglobin fractions in bovine muscle using starch-gel electrophoresis. Characterization studies found all three myoglobin fractions to have identical wavelength positions for minimum and maximum light absorption and to exhibit the same autoxidation rates (Quinn et al., 1964). Atassi (1964) used myoglobin isolated from sperm whale skeletal muscle to study the mobility and amino acid composition by electrophoresis. He found that the myoglobin of different species appeared to be differentiated from each other by differences in the globin component as shown by variations in the amino acid contents. Rowland et al. (1968) using human myoglobin extracted from muscle obtained by biopsy, demonstrated

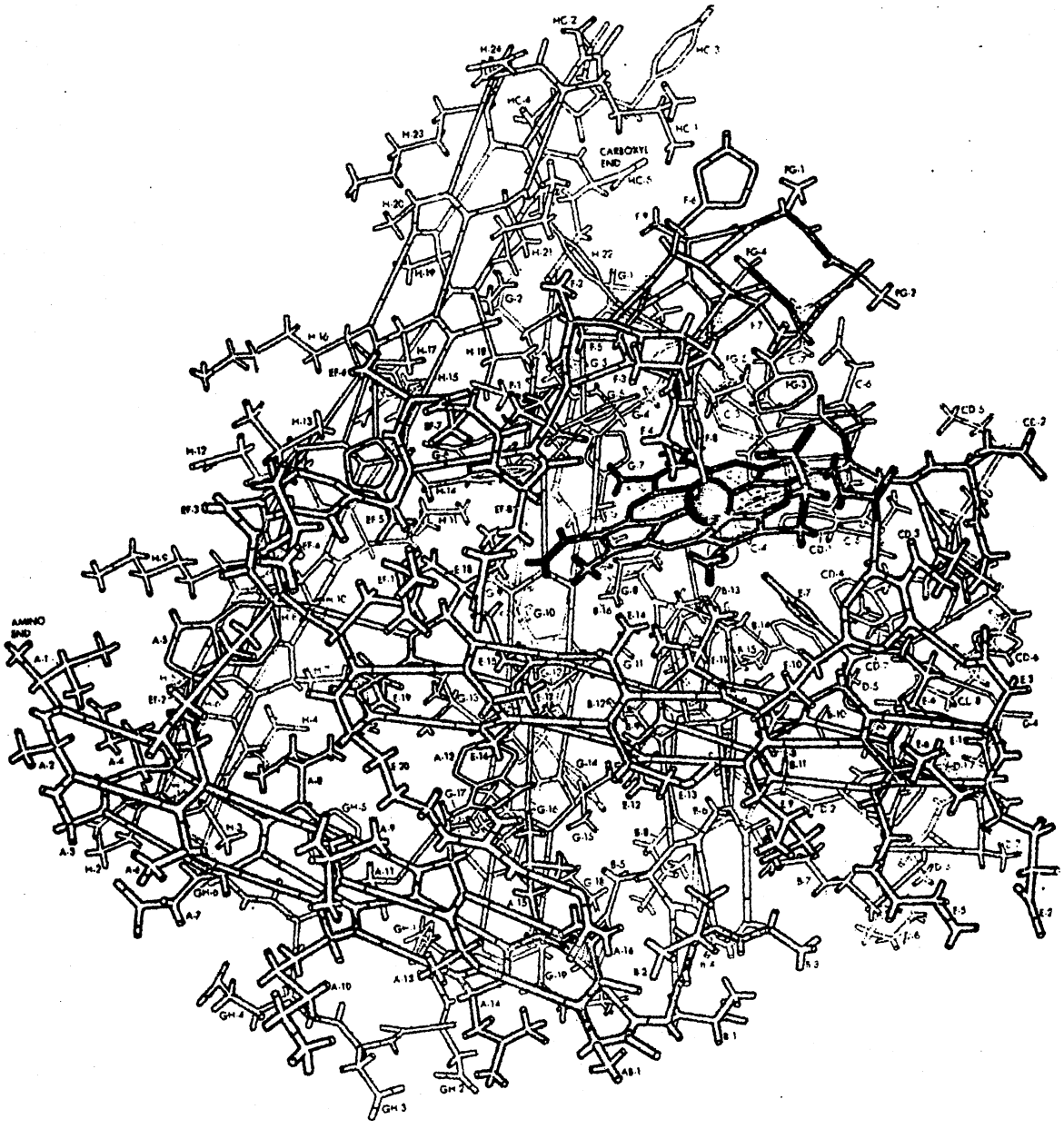


Figure 1. Structure of Myoglobin
(Kendrew, 1961)

TABLE I

AMINO ACID SEQUENCE FOR MYOGLOBIN STRUCTURE

(Kendrew, 1961)

ALANINE	ALA
ARGININE	ARG
ASPARTIC ACID OR ASPARAGINE	ASP
GLUTAMIC ACID OR GLUTAMINE	GLU
GLUTAMIC ACID	GLU. C
GLYCINE	GLY
HISTIDINE	HIS
ISOLEUCINE	ILEU
LEUCINE	LEU
LYSINE	LYS
METHIONINE	MET
PHENYLALANINE	PHE
PROLINE	PRO
SERINE	SER
THREONINE	THR
TYROSINE	TYR
VALINE	VAL

A- 1 VAL (AMINO END)
2 ALA
3 GLY
4 GLU
5 TYR
6 SER
7 GLU
8 ILEU
9 LEU
10 LYS
11 (NOT GLY)
12 TYR
13 (NOT GLY)
14 LEU
15 LEU
16 GLU

AB- 1 (NOT GLY)

B- 1 LEU
2 VAL OR THR
3 ALA
4 GLY
5 HIS
6 GLY
7 LYS
8 LEU
9 THR
10 LEU
11 ILEU
12 SER
13 LEU
14 PHE
15 LYS
16 SER

C- 1 HIS
2 PRO
3 GLU.C
4 THR
5 LEU
6 GLU
7 LYS

CD- 1 PHE
2 ASP
3 ARG
4 PHE
5 LYS
6 HIS
7 LEU
8 LYS

D- 1 THR
2 GLU.C
3 ALA
4 GLU.C
5 MET
6 LYS
7 ALA

E- 1 SER
2 GLU.C
3 ASP
4 LEU
5 LYS
6 VAL
7 HIS
8 GLY
9 ILEU
10 GLU
11 VAL
12 ASP
13 (NOT ALA, GLY)
14 ALA
15 LEU
16 GLY
17 ALA
18 ILEU
19 ASP
20 ARG

EF- 1 LYS
2 LYS
3 GLY
4 LEU
5 HIS
6 (NOT GLY)
7 (NOT GLY)
8 GLU

F- 1 GLU
2 ALA
3 PRO
4 THR
5 ALA
6 HIS
7 SER
8 HIS
9 ALA

FG- 1 (NOT GLY)
2 (NOT GLY)
3 PHE
4 (NOT ALA)
5 ILEU

G- 1 PRO
2 ILEU
3 LYS
4 TYR
5 (NOT ALA, GLY)
6 GLU
7 HIS
8 LEU
9 SER
10 (NOT GLY, ALA)
11 ALA
12 VAL OR THR
13 ILEU
14 HIS
15 VAL
16 ARG
17 ALA
18 THR
19 LYS

GH- 1 HIS
2 ASP
3 ASP
4 GLU
5 PHE
6 GLY

H- 1 ALA
2 PRO
3 ALA
4 ASP
5 GLY
6 ALA
7 MET
8 GLY
9 LYS
10 ALA
11 LEU
12 GLU.C
13 LEU
14 PHE
15 ARG
16 LYS
17 ASP C
18 ILEU
19 ALA
20 ALA
21 LYS
22 TYR
23 LYS
24 GLU.C

HC- 1 LEU
2 GLY
3 TYR
4 GLY
5 GLU C (CARBOXYL END)

The brackets in the table indicate those amino acid units which form a helical section.

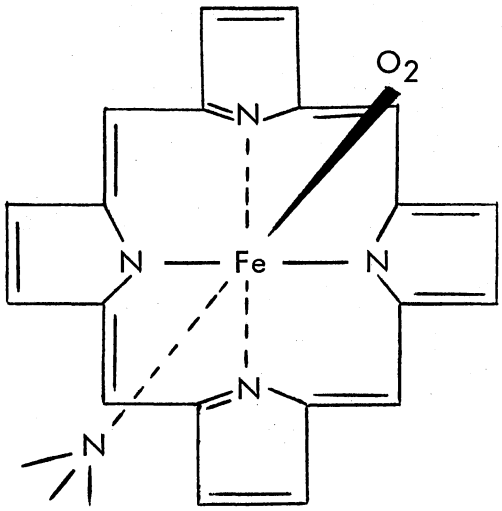


Figure 2. Attachment of O_2 to the heme structure

multiple forms of myoglobin by both column chromatography and electrophoresis in gels, with characteristically, one major form and several minor forms. He attributed the minor components to be results of changes in the state of oxidation of the heme moiety or changes in the state of aggregation of myoglobin.

Solubility

Morgan (1935) attempted to solve an early research problem for quantitatively separating myoglobin from hemoglobin. He studied the solubility of heme myoglobin in concentrated ammonium sulfate and phosphate solutions. He found that horse myocarboxyhemoglobin was readily soluble in 3M phosphate solutions, in which carboxyhemoglobin dissolved only to the extent of 1 mg per liter. Thus he was able to quantitatively separate horse myoglobin from hemoglobin by the addition of mono and di-basic potassium phosphate following the lead acetate precipitation, bringing the phosphate concentration to 3M and pH 6.6.

Absorbancy

Early research on the absorbancy of myoglobin originated from knowledge on hemoglobin. Drabkin and Austin (1932) studied the spectrophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood and found that the molar extinction coefficient of dog and rabbit blood differed slightly from the human blood. The total pigment concentration was quantitatively determined by Drabkin and Austin (1935-1936a) by measuring

the absorption of cyanmethemoglobin prepared from suitable aliquots by the addition of potassium ferricyanide to a final concentration of 0.6 mM per liter and potassium cyanide to a concentration of 0.8 mM per liter. Absorbance was read at 540 mu. Drabkin and Austin (1935-1936b) found that the molar extinction coefficient of myoglobin in the form of cyanmetmyoglobin was 11.3×10^3 at a wavelength of 540 mu. Crandall and Drabkin (1946), and Drabkin (1947) quantitatively measured myoglobin concentration using spectrophotometric technique. They converted rat myoglobin to cyanmetmyoglobin and measured at 540 mu based on earlier studies by Drabkin (1945) that the spectrophotometric constant of cyanmethemoglobin could be used interchangeably between the species since the iron content of the hemoglobins and myoglobins were amazingly similar, with an average of 0.339 ± 0.001 for hemoglobin and 0.340 ± 0.002 for myoglobin. The use of 11.3×10^3 as the molar extinction coefficient of myoglobin and the wavelength of 540 mu was later verified in studies by Bowen (1949) and Drabkin (1950). Bowen also concluded that the absorption by metmyoglobin was greatly influenced by pH.

Functions and Reactions of Myoglobin

Myoglobin is a complex protein, similar in function to the blood pigment hemoglobin in that they both serve to complex with the oxygen required for metabolic activity of the animal. Although their functions are similar, their roles are different : hemoglobin acts as an oxygen carrier in the

blood stream, whereas myoglobin is essentially a storage mechanism for oxygen in the cells. Lawrie (1952) studied horse heart myoglobin and concluded that high myoglobin content in muscle was associated with high enzymic activity, particularly of cytochrome oxidase, in the corresponding enzyme preparations. He suggested that the function of myoglobin, with its power of reversible combination with oxygen and its low loading tension, was to assist in ensuring a constant supply of oxygen to the muscle oxidase system.

Myoglobin is also an important factor in determining the color of meat. Giffee et al. (1960) and Fox (1966) summarized the reaction of myoglobin and its resulting color (Figure 3). In the presence of oxygen, myoglobin is converted to two different pigments, metmyoglobin and oxymyoglobin, the oxidized and oxygenated forms, respectively. The relative proportions of these two forms depend upon the partial pressure of oxygen. The formation of metmyoglobin being favored at low oxygen pressures. At all oxygen pressures there is a constant conversion to metmyoglobin, but as a result of enzymatic oxidation of avaliable substrates, particularly glucose, there is a continual supply of reducing coenzymes capable of reducing metmyoglobin back to myoglobin. Thus, in a fresh piece of meat, the bright red color of oxymyoglobin is on the surface where there is a plentiful supply of both oxygen and reducing substance. In the interior, the myoglobin is in the reduced state and has the typical dark purple color.

George and Stratmann (1952), Brown and Dolev (1963) used beef and

tuna myoglobin respectively to study the oxidation of myoglobin to metmyoglobin by oxygen. They found the oxidation of myoglobin to metmyoglobin by molecular oxygen at 30° C in 0.6 M phosphate buffer, pH 5.69 to be first order in unoxidized myoglobin. They also found that the oxidation of a crude preparation of beef oxymyoglobin was slower than that of purified myoglobin. Ginger et al. (1955) studied changes in beef myoglobin associated with gamma-ray radiation from a cobalt-60 source on myoglobin. They found the susceptibility of myoglobin extracts to radiation damage increased with increased purity of extract. The irradiation of myoglobin in crude extracts resulted in the formation of a green compound which absorbed light at 610 to 620 mu. It was also shown that myoglobin in meat packaged and stored in oxygen-impermeable material showed little change immediately after irradiation on storage. Myoglobin in meat packaged in oxygen-permeable material resulted in discoloration immediately after irradiation and further discoloration on storage.

Methods of Analysis

Shenk, Hall and King (1934) measured the absorption at 542, 577, and 582 mu on untreated beef muscle extracts, which were assumed to contain mixtures of oxyhemoglobin and oxymyoglobin. The spectral differences between these two compounds were used as a basis for the calculation of their respective concentrations. Watson's (1935) method was also based on the

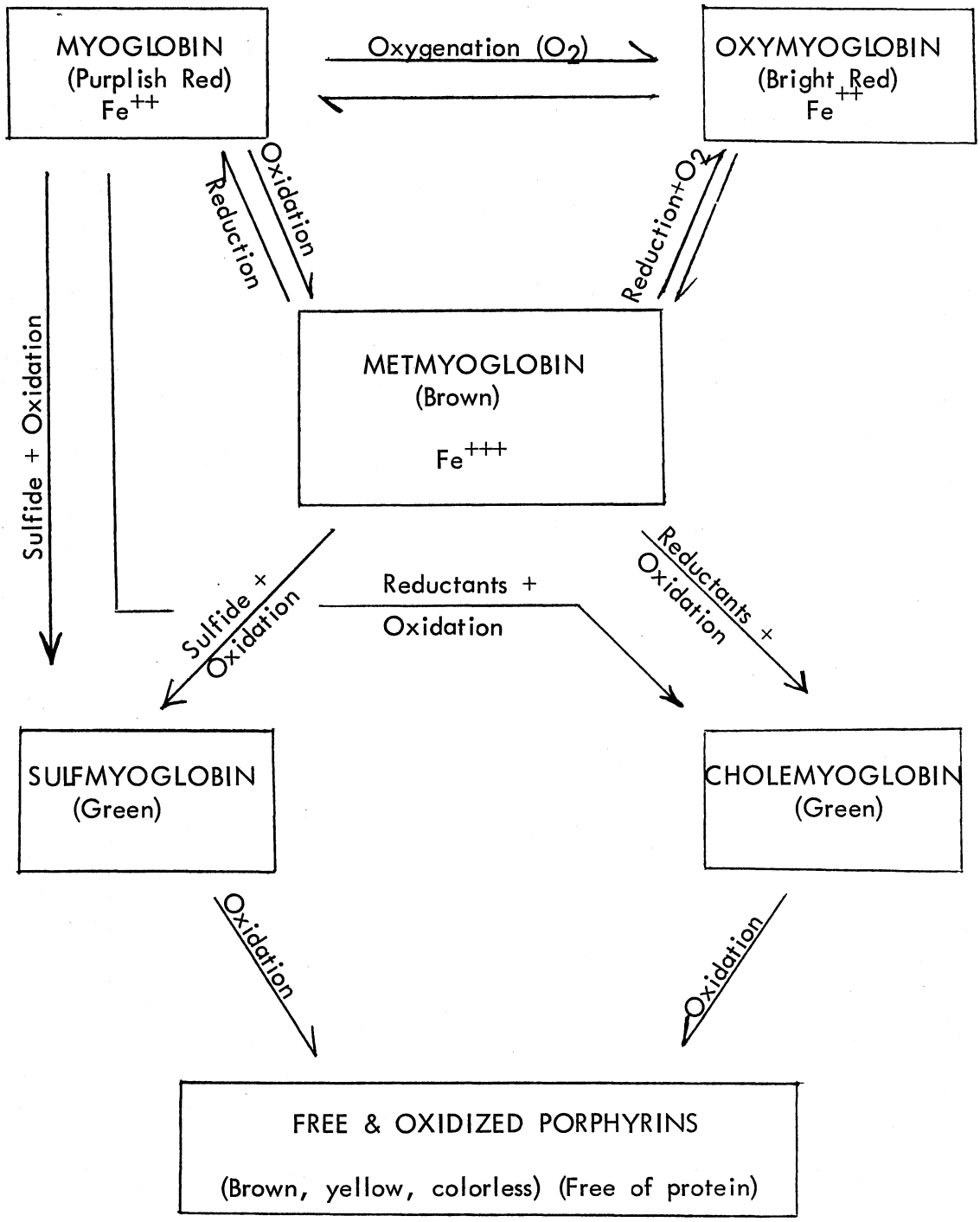


Figure 3. Reactions of Myoglobin (Giffee et al., 1960)

difference between the oxy- compounds, but his beef muscle extracts were freshly reduced and reoxygenated before the reading, which was done with the Hartridge reversion spectroscope. The position of the maximum of the α-band which varies between 577.4 and 580.8 mu was used for determining the ratio Hb/Mbb in the mixture. The total concentration of pigment was then measured by reading the absorption of the total carbon monoxide compounds at 560 and 540 mu. DeDuve (1948) quantitatively measured human muscle pigment concentration based on the differences in light absorption of the carbon monoxide compounds of myoglobin and hemoglobin. He used the carbon monoxide derivatives of each, and then calculated the molarity of each component from the densities obtained at the wavelengths 568 mu and 583 mu. Beznak (1948) studied the light absorption of sheep hemoglobin and myoglobin in the Soret region (between 400-450 mu). The difference between the extinction coefficients of oxymyoglobin and oxyhemoglobin, as compared to that of their respective reduced forms, was suggested as a possible method for estimating simultaneously hemoglobin and myoglobin in the same solution. Poel (1949) measured the concentration of hemoglobin and myoglobin when together in solution by using the carbon monoxide derivatives of each, and then calculated the molarity of each component from the densities obtained at the wavelengths of 568 mu and 538 mu, which were different from the wavelengths used by DeDuve. Bowen and Eads (1949) also determined the myoglobin content of muscle from dogs using the carbon mono-

xide derivatives method and verified the wavelengths used by Poel.

Ginger et al. (1954) developed a procedure slightly modified from that described by Morgan and Drabkin for the quantitative determination of myoglobin in beef and pork muscle. They converted the extracted myoglobin into cyanmetmyoglobin and read the absorbance of the resultant cyanmetmyoglobin at wavelength 540 mu. The concentration of myoglobin in millimoles per milliliter was obtained from the equation : $E = \frac{O.D.}{C \times l}$ where the extinction coefficient was assumed to be 11.5. Their method was later used by Fleming et al.(1960) in quantitative estimations of myoglobin and hemoglobin in beef muscle extracts and by Rickansrud and Henrickson (1967) in determining the total pigment and myoglobin concentration in bovine muscle.

Erdman and Watts (1957) studied the color change in cured meat by spectrophotometric determination of the extinction ratios of cured meat surfaces at 570/650 and 540/500 mu. Broumand et al. (1958) developed a method called the absorbancy ratio method for determining the proportions of heme derivatives in beef muscle. Their method was based on the absorbancy of a water extract of the meat sample at specified wavelengths, and the principle was described in detail by Lemberg and Legge (1949). Results of calculations by the absorbancy ratio method seemed to corroborate the validity of several phenomena which were endorsed by workers in the field of color in meat. However, successful use of the absorbancy ratio method depended upon having accurate data on the extinction coefficients of the

myoglobin derivatives.

Dean and Ball (1960), Snyder (1964, 1965), Stewart et al. (1965), Snyder and Armstrong (1967), and Franke and Solberg (1971) used reflectance spectrophotometry for the analysis of pigments at the surface of fresh meat. It was a nondestructive method of measuring the proportions of myoglobin derivatives in meat samples. Such a method was developed in which reflectance spectra were recorded on the absorbancy scale for samples of fresh beef containing predominantly myoglobin, oxymyoglobin or metmyoglobin at the surface. However, pigment below the surface could not be estimated.

Factors Affecting Myoglobin Concentration

There are a lot of factors that could affect the concentration of myoglobin in the muscle. Whipple (1926a) found that the hemoglobin content of dogs increased 4-5 times as their age increased from 2 months to 8 months. Lawrie (1950) studied the relationship of age to myoglobin content and found that in both draught horse and pig, the concentration of the pigment rose rapidly from birth, and that, after 2 years in the horse, and 1 year in the pig, it would remain fairly constant. He verified his findings in a similar research in which he also concluded that the myoglobin content of different muscle would be different (Lawrie, 1961).

Lewis et al. (1962) found that the uncooked meat color from cattle under stress prior to slaughter was significantly darker than meat from cattle

receiving no stress.

The effect of exercise was studied by Lawrie (1953). Myoglobin content from albino rat and fowl was found to be significantly higher in the "active" group than in the "immobile" group. The finding supported the result of an earlier research by himself that exercise caused demonstrable increase in myoglobin concentration in draught horse, fowl, pigeon and pigs (Lawrie, 1950). Forrest et al. (1964) studied the effect of preslaughter stress in ovine muscle. He concluded that lamb muscle tissue color was influenced by preslaughter injections of high levels of epinephrine or exhaustive exercise.

Lawrie et al. (1963) studied the effect of weight on the myoglobin content in pigs and found that the highest myoglobin content was obtained from the heaviest weight group (250 lb). The lowest myoglobin content was found in the lightest weight group (150 lb).

Variation of myoglobin content due to different muscles was investigated by Briskey et al. (1960). Using eight pork muscles, they found that there were significant differences between the individual muscles in myoglobin concentrations but no differences in myoglobin concentrations were found within the muscles. Lawrie et al. (1963) and Topel et al. (1966) used porcine muscle to study the effect of different muscles on myoglobin content and their result agreed with the findings of Briskey. Beecher et al. (1968) compared the light and dark portions of a pig's striated muscle. They found

myoglobin concentrations were more than two-fold higher in the semitendinosus dark portion than the semitendinosus light portion.

Whipple (1926b) observed that paralysis after sectioning of the sciatic nerve led to loss of myoglobin in affected muscles.

A seasonal variation has been encountered in the myoglobin content of muscle tissue of some animals. Rosenmann and Morrison (1965) reported this interesting phenomenon in the snowshoe hare of Alaska. They observed a marked increase in the myoglobin concentration of the diaphragm and gastrocnemius muscles during late winter, as compared to summer.

The effect of anoxia to exercise on myoglobin concentration was pointed out by Poel (1949). Rats exposed to a simulated altitude of 25,000 feet were studied. Skeletal muscle in which activity was not increased with anoxia actually showed a decreased myoglobin content following prolonged altitude exposure, whereas cardiac muscle, in which activity was increased by anoxia, showed an increase. Bowen and Eads (1949) also studied the effect of simulated altitude on the production of myoglobin by the hypoxia caused by 6 hours daily exposure to 18,000 feet.

Whipple and his colleagues (1928-29) studied the dietary influences on the content of myoglobin in muscle. They indicated that pups fed cooked liver had greater myoglobin stores in muscles than pups raised on bread. Jacobson and Fenton (1956) studied the effects of three levels of nutrition on

the quality of beef. They found that the intensity of the red color increased with the level of nutrition. The greatest effect was found in the most active muscle sampled, the semimembranosus. Craig et al. (1959) studied the effect of grass and grain in the ration on the color of longissimus dorsi muscle in beef cattle. They found significantly brighter color in this muscle for cattle fed in dry lot than for cattle fattened on pasture or pasture plus concentrate supplement. However, evaluation of dietary effects on myoglobin concentration of steer muscle by Craig et al. (1966) did not reveal a difference when animals were fed on pasture, grain, or cut forage and grain in dry lot. Niedermeier et al. (1959) studied the dietary effect on veal calves and found that with iron and copper supplement in the ration could increase the iron content of liver and the hemoglobin content of blood. Bray et al. (1959) further analyzed the chemical composition of the longissimus dorsi muscle of these veal calves and found that the myoglobin content increased with increased uptake of iron and copper supplement.

Other Pigments In Meat

Besides myoglobin, there are a number of muscle pigments which are of much greater importance to the living tissue but which are present in such small quantities that they probably contribute little or nothing to the total color. These include the cytochromes, red heme pigments which contain iron in similar porphyrin-protein complex structure; vitamin B_{12}, which

contains a similar porphyrin ring as the hemes and the cytochromes but which contains a cobalt atom instead of iron ; and the flavins, yellow co-enzymes involved with the cytochromes in electron transport in the cell (Giffee et al., 1960).

Post-Mortem Electrical Stimulation

Since the technical aspect of the post-mortem electrical stimulation of the bovine carcass is out of the scope of this study, this review of literature will therefore only attempt to make a brief introduction on the subject and the economics behind the electrical stimulation.

Electrical stimulation of freshly slaughtered beef carcass was first suggested by Harsham and Detherage (1951), principally as a mean of rapid tenderization of the meat, but also to hasten the onset of rigor and the fall of pH in the muscles. Carse (1973) used electrical stimulation of lamb carcasses, with the specific aim of accelerating the post-mortem changes. He employed interrupted currents at 60-250 V and at varying pulse rates, applied between the severed neck region and the ankles of the lamb. He concluded that voltage but not frequency affected the rate of post-mortem glycolysis. Bendall (1976) did a similar study and suggested that very high stimulating voltages of 1000 V or more were unnecessary to obtain optimal rates and extents of pH fall, providing electrical contact was made between a wet portion of the severed neck region and the hind-limbs. He concluded

it would be desirable to use 250 V and to deliver not less than 3000 pulses at 25 Hz.

Turning to the economic aspect, Rippen (1975) indicated that the food industries utilized 12.8% of the total energy consumed in the United States. Henrickson and McQuiston (1977) pointed out the refrigerated space usually set aside for the chilling of a 600 pounds beef carcass plus the space above and below the hanging carcass usually takes 120,400 cu. inches. Together with holding space, it would take up 240,800 cu. inches. A choice carcass weighing 600 lbs. will require 21,150 BTUs. of energy transfer to reduce it from 38.9° C to 12.8° C and 31,500 BTUs. to reduce the carcass to 0° C. The edible portion (420 lb) would require removal of only 22,050 BTUs. to lower the same edible product to 0° C. Thus saving 50 % or more of refrigeration capacity.

Electrophoresis

Electrophoresis, probably the oldest form of migratory analysis, refers to the movement of charged particles in an electric field. Methods of electrophoresis may be grouped into either of two classes : frontal (or moving boundary) electrophoresis and zonal electrophoresis.

Heftmann (1961) defined zone electrophoresis as the method of analysis in which a direct current electrical potential promotes the separation of dissolved or suspended substances by differential electrical migration from a

narrow zone in a stabilized background electrolytic solution. In simple terms, an ion in an electrolyte solution attracts ions of the opposite sign and repels ions of the same sign.

The use of cellulose acetate membrane as a supporting media for zone electrophoresis was tested by Grunbaum et al. (1960) using a newly developed apparatus for microelectrophoresis in analyzing serum protein. He found the method capable of excellent reproducibility and free of tailing and overlapping of bands, resulting in separations which were superior to those achieved with filter paper. He also cited speed as an added advantage. Grunbaum and Durrum (1963) described the microelectrophoresis technique on cellulose acetate membrane as having the ease of handling like that of paper and the breadth of application similar to that of gels. They also found that using the microelectrophoresis technique with cellulose acetate membrane as an electrophoretic support medium produced bands which were sharper, more distinct, and generally free of such secondary phenomena as trailing. However, it was concluded that fractionation in cellulose acetate did not produce as many clear fractions as were obtained in gels, probably because ultrafiltration contributed to the separation in gels. Grunbaum et al. (1963) made a quantitative analysis of normal human serum protein using the method of microelectrophoresis on cellulose acetate membrane. He reported that the normal values for serum protein fractions fell within the general range of values reported in the literature. Bartlett (1963a) reported the use

of electrophoresis utilizing cellulose acetate membrane as the supporting media could quantitatively analyze serum protein fractions by densitometry within 2 hours of initiation of the procedure. Bartlett (1963b) also applied this method in the identification and quantitation of hemoglobins. His findings showed that the quantitative values were comparable to those observed with the starch block technique. Kaplan and Savory (1965) evaluated the cellulose acetate electrophoresis system for serum protein fractionation and cited some advantages of cellulose acetate over paper as having sharp resolution of fractions in a short time ; no "trailing" of albumin ; the membrane rendered transparent easily to increase accuracy in quantitation, and the wet cellulose acetate membtane have greater tensile strength (Figure 4).

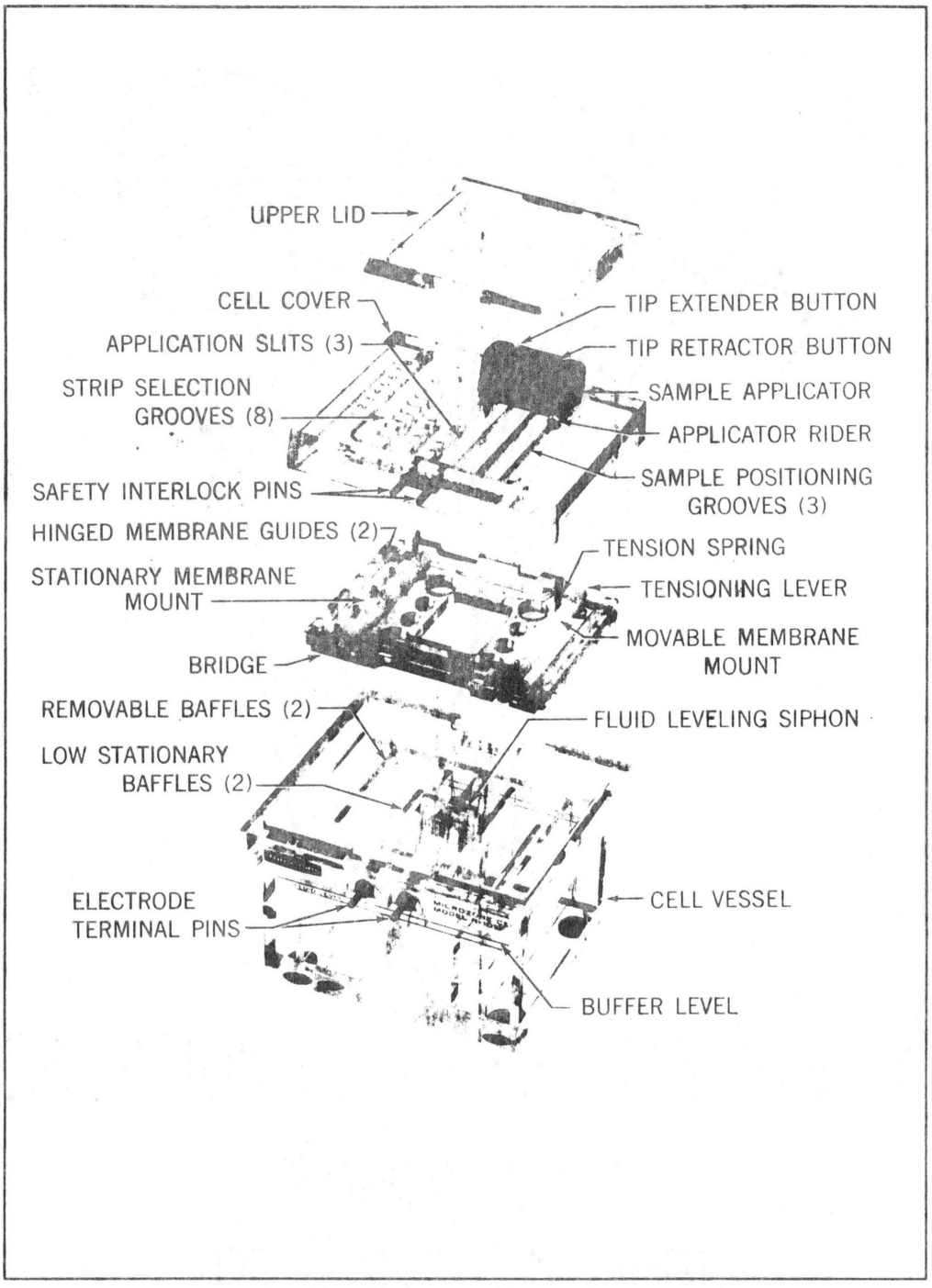


Figure 4. Electrophoresis Cell and Sample Applicator
(Beckman Instruments, Inc., 1965)

EXPERIMENTAL PROCEDURE

Electrical Stimulation

Six Hereford heifers ranging from 310 to 367 Kg (live weight) were slaughtered, dressed and split in conventional practices. The sides were then removed to a 16° C room. One side from each carcass was designated as the stimulated side and connected to a pulse generator via two leads. One lead, a set of eight stainless steel shroud pins connected by a copper wire and spaced by a teflon band, was inserted into the muscles of the round. The other lead was connected to three similar pins which were inserted into holes drilled in the third, fourth and fifth cervical vertebrae.

Stimulation started one hour after death and continued for 30 minutes. The pulse generator delivered a direct current square wave pulse, with a frequency of 400 cycles per second, and a duration of 0.5 milli-seconds. Voltage was rapidly increased to 300 volts and remained at this level throughout the stimulation period.

Sampling Procedure

Muscles were dissected from the carcass four hours after death. One

inch steaks were then removed from the semimembranosus, longissimus dorsi, and the semitendinosus muscles according to standardized locations (Figure 5).

After removal of the muscle samples, they were carefully wrapped in Loxol paper so that no air was trapped in the package. The samples were then stored in a 1.1° C cooler.

Total Pigment Determination

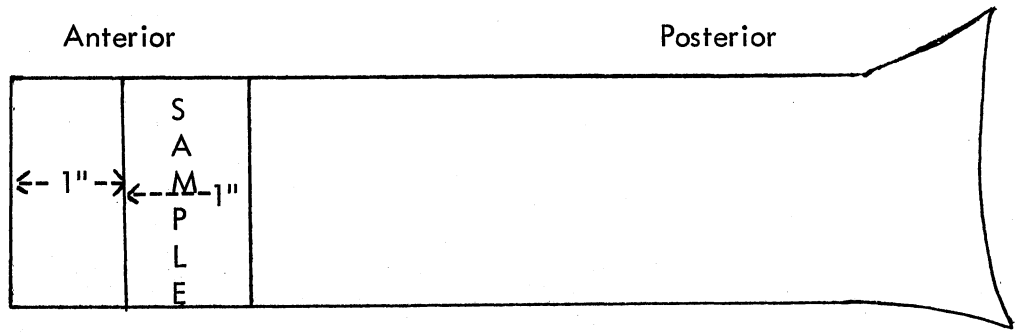
The procedure used for the total pigment and total myoglobin determination was a slight modification from the one used by Rickansrud and Henrickson (1967).

Upon removal from the cooler, the muscle sample was trimmed to remove all external fat.

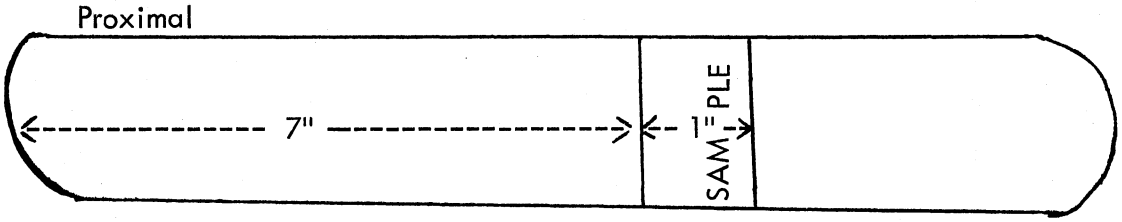
About 35 gm of muscle shavings were put into a Sorvall Omni-Mixer Homogenizer and homogenized for 1 minute at medium speed.

Exactly 25 gm of the homogenate were randomly taken from the muscle homogenate. Each sample was placed into a 400 ml beaker with 75 ml of boiled, cooled, distilled water and blended with the " Polytron " for one and one half minutes with the speed control set at " 4 ". The distilled water had been boiled to drive off the gas impurities.

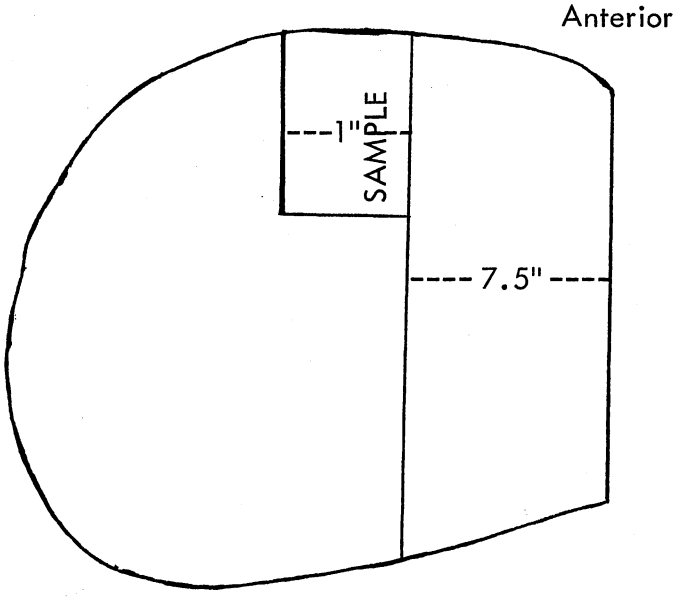
The resulting slurry was centrifuged at 2000 X G at 6° C for 15 minutes. The supernatant was filtered through Whatman # 3 filter paper using Buchner funnel and suction flask. The resultant slurry was re-extracted with



Longissimus dorsi



Semitendinosus



Semimembranosus

Figure 5. Location of Samples

an additional 75 ml of boiled, cooled, distilled water, centrifuged at 2000 X G at 6° C for 15 minutes and filtered through Whatman # 3 filter paper as before. The two extracts were combined and made to 150 ml using boiled, cooled, distilled water.

The combined extraction was then filtered through Whatman # 3 filter paper by gravity in order to yield a clear solution. One 20 ml aliquot was taken from the cleared filtrate and was converted to cyanmetmyoglobin by adding 0.005 gm of potassium ferricyanide and 0.001 gm of potassium cyanide. The sample was centrifuged at 2000 X G at room temperature and absorbance was read at a wavelength of 540 nm, using a Gilford 240 Spectrophotometer.

The concentration of total pigments was calculated from the formula :

Total Pigments (mg/gm wet tissue) = $\frac{\text{O.D. X K}}{\text{Sample Wt.}}$; where optical density (O.D.) = 2 - log % T . The proportionality constant (K) was calculated by the formula : $K = \frac{17{,}000 \text{ X volume in liters}}{E}$

E is the millimolar extinction coefficient of myoglobin and equals to 11.3 mM per liter. The volume in liters of the total sample solution equals 0.15 liter. The molecular weight of myoglobin is assumed to be 17,000 mg per mM. Upon substitution of these values into the formula, a K value of 225.6637 is obtained.

Total Myoglobin Determination

A 50 ml aliquot was taken from the cleared extract from total pigment

concentration and placed in a 100 ml beaker. A 6 ml aliquot of 0.5 M phosphate buffer at pH 7.1 was added to bring the pH to 7.

Saturated basic lead acetate solution equal to one-quarter the volume of the solution (14 ml) was added at room temperature (25° C). The solution was centrifuged at 2000 X G at 25° C for 15 minutes. This step serves to precipitate the foreign protein thus leaving only hemoglobin and myoglobin in the solution. The temperature was watched closely because at a temperature higher than 38° C, myoglobin also precipitates and at a temperature lower than 25° C, the protein precipitation is incomplete.

The supernatant was filtered through Whatman # 3 filter paper using Buchner funnel and suction flask. The suction was adjusted low enough to avoid foaming.

A 25 ml aliquot of the filtrate was placed in a 50-ml beaker with 11.65 gm of mono- and dibasic potassium phosphate mixture crystals in it, bringing the phosphate concentration to 3 M and pH 6.6. This step brings the volume to 29 ml and serves to precipitate the hemoglobin, thus leaving only myoglobin in the solution.

The resultant solution was centrifuged at 2000 X G at room temperature for 15 minutes, and the supernatant was filtered by gravity through Whatman # 3 filter paper.

A 20 ml aliquot of the filtrate was converted to cyanmetmyoglobin by adding 0.005 gm potassium ferricyanide and 0.001 gm potassium cyanide.

The absorbance was read at a wavelength of 540 nm using the Gilford 240 Spectrophotometer. The rest of the filtrate was saved for use in the electrophoresis procedure.

Total myoglobin concentration was obtained from the formula : Total Myoglobin (mg/gm wet tissue) = $\frac{\text{O.D. X K}}{\text{Sample Wt.}}$. The optical density (O.D.) = 2 - log % T. The K value was calculated from the formula : $K = \frac{17{,}000 \text{ X volume of aliquot in liters X dilution factor}}{E}$, where 17,000 is the assumed molecular weight of myoglobin ; volume of aliquot in liters equals 0.029. The dilution factor is that fraction of the total concentration present in the initial 150 ml extract represented by the concentration in the aliquot. The dilution factor thus equals $\frac{150}{17.857}$. The K value thus equals to 366.4804.

Visual Appraisal

All samples were visually appraised to detect visual differences in color between the electrical stimulated and the control samples. The appraisal was done under ordinary fluorescent lighting.

Electrophoresis

The entire electrophoresis procedure was carried out using the Microzone Electrophoresis System developed by Beckman Instruments Inc. using cellulose acetate membrane as the supporting medium.

Preparation of sample

A 3 ml aliquot of the total myoglobin extract was put on Parafilm and covered well with a small beaker.

Preparation of cell

The electrophoresis cell was filled with 40 ml of barbital buffer solution of 0.075 ionic strength and pH 8.6. The correct buffer strength and pH was obtained by dissolving one package of B-2 buffer from Beckman Instrument Inc. with 1000 ml of distilled water (Beckman , 1965). After the cell was filled with buffer, all external moisture on the cell (including the top rims, the baffle rims, and the electrode terminal pins) was removed with tissue. The cell was not removed after it was filled with buffer.

Mounting of membrane

Exactly 40 ml of buffer was put in a tray. By use of a tweezer, the cellulose acetate membrane was floated on the buffer to allow absorption of the buffer by the membrane by capillary action from below. The membrane was lifted once to dislodge any trapped air bubbles and immersed briefly. After one blotting, it was quickly suspended on the bridge. Failure to blot would result in unsatisfactory band formation. Excessive drying of the membrane would cause poor migration and resolution and partially cleared membranes (Elliott , 1966).

Application of sample

The sample applicator tip was filled by moving the tip slowly across the top surface of the myoglobin extract on the Parafilm. The applicator was positioned on the desired groove and the extract was deposited when the positioned applicator tip was extended. By use of the same groove, 20 applications were applied. After all samples were applied, the cell was covered.

Electrophoretic stage

The cell was connected to the Beckman Duostat to make the right hand electrode the anode. The migration time was 20 minutes at a setting of 250 volts. The starting current was between 3.5 to 5.8 ma.

Staining, clearing and drying of the membrane

The fixative dye, acetic acid rinse, alcohol rinse and the clearing solution were prepared in advance according to the instruction manual (Beckman 1965) and were put in their respective trays.

At the end of the 20 minutes electrophoresis run, the cell was disconnected from the Duostat. The membrane was placed in the fixative dye for 10 minutes. It was washed with acetic acid rinse until dye no longer came off. The membrane was then placed in alcohol rinse and agitated for 1 minute. A glass plate was placed in the clearing solution tray and the membrane was agitated in the clearing solution for 1 minute before being

removed. The membrane was gently squeegeed using a microscopic slide to remove excess liquid, and placed in a ventilated oven for 5 minutes at 110° C. The dried and cleared membrane was peeled from the glass plate and inserted in the protective envelope.

Scanning

The enveloped membrane was scanned using the Analytrol with the Microzone Scanning Attachment and a Variable Speed Control. The scanning speed was set at " 40 " for all membranes.

Myoglobin fraction calculation

The peaks obtained from the graphs were cut out and weighed. The total weight of the peaks from each graph was assumed to be equivalent to the total myoglobin concentration obtained from the total myoglobin calculation. The weight of each peak was then treated as a percentage of the total myoglobin concentration and the amount of each myoglobin fraction in the sample was calculated from the formula: Myoglobin Derivatives (mg/gm wet tissue) = $\frac{\text{Wt. of Mb deriv. peak}}{\text{Total wt. of peaks}}$ X Total myoglobin concentration .

Identification of myoglobin derivatives

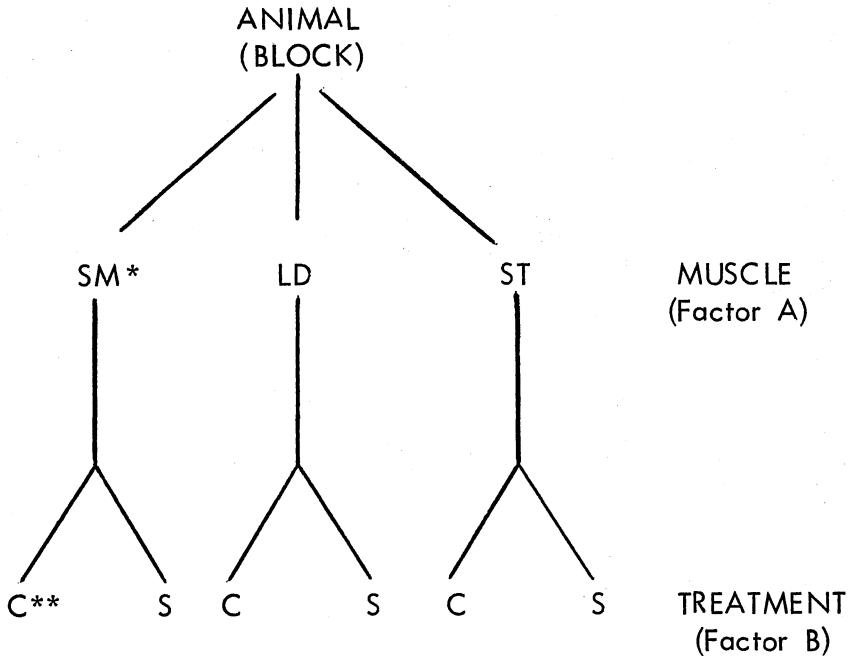
The identification of myoglobin derivatives was achieved by running commercially purchased myoglobin on electrophoresis using exactly the same migration time and procedure used for the meat samples. The migration

distance of each derivative was then checked against those obtained from the samples.

The purchased standard was myoglobin from equine skeletal muscle from the Sigma Chemical Company. A 0.02 gm of the myoglobin standard was dissolved into 20 ml of boiled, cooled, distilled water. The solution was put into small beakers labelled A, B and C. Beaker A was converted to metmyoglobin by adding a trace (less than 1 mg) of potassium ferricyanide. Beaker B was converted to myoglobin in the reduced state by adding a trace of sodium dithionite (Broumand et al.,1958). Beaker C was converted to oxymyoglobin by bubbling filtered air through the solution. The solutions in beakers A, B and C were then used in the electrophoresis using exactly the same procedure employed for the meat samples. The migration distance of each derivative was checked against those in the meat samples.

Statistical Analysis

The experiment was laid out as a split-plot design with a randomized complete block arrangement (Figure 6). The results obtained from visual appraisal was analyzed using the nonparametric Chi-square's sign test. All statistical analysis were performed as described in Steel and Torrie (1960).



* SM = Semimembranosus

LD = Longissimus dorsi

ST = Semitendinosus

** C = Control

S = Stimulated

Figure 6. Experimental Layout

RESULTS AND DISCUSSION

Procedure

The extraction procedure required about six hours to complete. As is well known, the rate of change of myoglobin and most of its derivatives, when in contact with free oxygen, varies with the temperature. Therefore, in a lengthy procedure, it becomes very important to keep the myoglobin extraction cold between filtrations. It is also of prime importance to seal the top of the myoglobin extraction container with Parafilm to avoid excessive contact with free oxygen.

Maintenance of a constant pH value is needed since myoglobin derivatives are sensitive to changes. The adding of the phosphate buffer to control the pH of the extraction was an essential step in the procedure. Although the pH of the different muscle extractions were varied, the differences were small. It was therefore found advantageous to add the same amount of phosphate buffer to all muscle extractions in order to obtain a consistent dilution factor. Adding varied amounts of the phosphate buffer to obtain a fixed pH value for all extractions was of less importance. There was no difficulty in obtaining a clear extract for the spectrophotometric measurement as was encountered by Rickansrud (1966). No matter what the pH of the

muscle extraction was, a clear solution resulted after a gravity filtration.

Total Pigment Determination

The analysis of total pigment concentration indicated a highly significant (OSL $<$ 0.01) difference in total pigment among animals. Animal 6 contained the highest total pigment content with an average of 3.01 mg/gm of wet tissue over the three muscles tested. Animal 4 had the lowest content with a 2.21 mg/gm of wet tissue (Table II). The total pigment concentration was also found to be significantly different (OSL $<$ 0.005) due to different muscles. This difference reflected a failure of the total pigment concentration to be the same for each animal as well as for each muscle in question. This result coincided with the findings of previous workers.

The semitendinosus muscle was found to contain the least total pigment with an average of only 2.26 mg/gm of wet tissue. The longissimus dorsi was averaging 2.70 mg/gm wet tissue in total pigment over the six animals while the semimembranosus contained the most total pigment with an average of 2.92 mg/gm wet tissue. The finding that the semitendinosus being the least pigmented muscle agreed with the result obtained by Rickansrud (1966). From the statistical analysis of variance, it was found that the difference in total pigment concentration due to electrical stimulation to be insignificant (OSL $>$ 0.1) (Table III). Thus electrical stimulation apparently has little or no effect on total pigment content. It was further determined that the

TABLE II

TOTAL PIGMENT
AS INFLUENCED BY ANIMAL AND MUSCLE VARIATION

ANIMAL	MUSCLE			OVERALL MEAN
	SM	LD	ST	
1	(2)* 3.30**	(2) 3.11	(2) 2.60	(6) 3.01
2	(2) 2.59	(2) 2.15	(2) 2.41	(6) 2.38
3	(2) 2.89	(2) 2.69	(2) 2.03	(6) 2.53
4	(2) 2.29	(2) 2.42	(2) 1.91	(6) 2.21
5	(2) 3.14	(2) 2.74	(2) 1.98	(6) 2.62
6	(2) 3.32	(2) 3.07	(2) 2.65	(6) 3.01
OVERALL MEAN	(12) 2.92	(12) 2.70	(12) 2.26	(36) 2.63

* = Number of treatments going into each mean

** = Mean value (mg/gm wet tissue)

TABLE III

ANALYSIS OF VARIANCE TABLE FOR TOTAL PIGMENT

SOURCE	D.F.	S.S.	M.S.	F
TOTAL	35	7.1450		
WHOLE PLOT	17	6.8576	0.4034	
ANIMAL	5	3.2038	0.6408	6.6101[a]
MUSCLE	2	2.6844	1.3422	13.8460[b]
ERROR (a)	10	0.9694	0.0969	
SUB-PLOT	18	0.2873	0.0160	
TREATMENT	1	0.0157	0.0157	1.2538[c]
MUS. X TRT.	2	0.0842	0.0421	3.3678[d]
ERROR (b)	15	0.1875	0.0125	

a = OSL $<$ 0.01

b = OSL $<$ 0.005

c = OSL $>$ 0.1

d = OSL $<$ 0.1

muscle by treatment interaction was not significantly different (OSL $<$ 0.1) as far as total pigment was concerned. That is to say, the total pigment concentration was measured to be approximately the same for the three muscles for each treatment.

The components of variance for " error b " in the analysis of variance table consisted only of the common variance due to treatment, or the within sample variation. In addition to this, the components of variance of " error a " consisted also of the variation resulted from the different muscles. It arose from the failure of each muscle treated alike to have the same response. Furthermore, the variance components from the blocking variable (the animals) included in addition to the within sample variation and variation from muscle, also the variation from animal (Table IV).

Since the total pigment content primarily measures the total amount of hemoglobin and myoglobin in the tissue, the more significance difference due to muscle than to animal may well be due to the fact that the residual blood remaining in the tissue after slaughter was drained away well enough to the point that each animal retained only the minimal amount. Thus, with hemoglobin playing a lesser role, the variation of total pigment would due mainly to the difference in myoglobin content in each animal (Table V).

Total Myoglobin Determination

It was found that the animal (animal 6) with the most total pigment

TABLE IV

TABLE FOR COMPONENTS OF VARIANCE

SOURCE OF VARIATION	D.F.	VARIANCE COMPONENTS
ANIMAL	(r - 1)	$\sigma_x^2 + b\sigma_x^2 + ab\sigma_z^2$
ERROR (a)	(r - 1)(a - 1)	$\sigma_x^2 + b\sigma_y^2$
ERROR (b)	a(b - 1) (r - 1)	σ_x^2

r = Number of animals

a = Number of muscles

b = Number of treatments

σ_x^2 = Within treatment variance

σ_y^2 = Variance due to different muscle

σ_z^2 = Variance due to different animal

TABLE V

TOTAL PIGMENT
AS INFLUENCED BY MUSCLE AND TREATMENT VARIATION

TREATMENT	MUSCLE			OVERALL MEAN
	SM	LD	ST	
CONTROL	(6)*	(6)	(6)	(18)
	2.95**	2.69	2.18	2.61
STIMULATED	(6)	(6)	(6)	(18)
	2.89	2.71	2.35	2.65
OVERALL MEAN	(12)	(12)	(12)	(36)
	2.92	2.70	2.26	2.63

* = Number of animals going into each mean

** = Mean value (mg/gm wet tissue)

also contained the highest total myoglobin concentration. The same was true as far as muscle was concerned ; semimembranosus, which contained the most total pigment also contained the highest myoglobin content (Table VI). Results from statistical analysis turned out to be similar to those in the total pigment determination. As expected, the main source of variation for total myoglobin concentration was found to be the result of different animal and different muscle (OSL $<$ 0.005) with the semimembranosus muscle having the highest total myoglobin content and the semitendinosus muscle with the least total myoglobin concentration. Values from the longissimus dorsi muscle were intermediate between the semimembranosus and the semitendinosus muscle in both total pigment and total myoglobin determinations (Table VII). Again, the electrical stimulation did not seem to cause much difference in the total myoglobin content since such effect was found to be statistically insignificant (OSL $<$ 0.1). As in the case of total pigment, the results failed to show significant difference (OSL $<$ 0.1) in total myoglobin concentration of the three muscles for each treatment or vice versa (Table VIII).

Myoglobin Derivatives Determination

The scanning of the electrophoresis membrane through the densitometer produced graphs with two peaks for all samples (Figure 7,8,9). The peak that migrated furthest was identified as oxymyoglobin since most of these peaks matched the migration distance by the oxymyoglobin standard. This

TABLE VI

TOTAL MYOGLOBIN
AS INFLUENCED BY ANIMAL AND MUSCLE VARIATION

ANIMAL	MUSCLE			OVERALL MEAN
	SM	LD	ST	
1	(2)* 3.01**	(2) 2.83	(2) 2.19	(6) 2.68
2	(2) 2.39	(2) 1.94	(2) 1.95	(6) 2.09
3	(2) 2.78	(2) 2.59	(2) 1.94	(6) 2.44
4	(2) 2.20	(2) 2.25	(2) 1.75	(6) 2.06
5	(2) 2.73	(2) 2.58	(2) 1.76	(6) 2.35
6	(2) 2.89	(2) 2.81	(2) 2.46	(6) 2.72
OVERALL MEAN	(12) 2.67	(12) 2.50	(12) 2.01	(36) 2.39

* = Number of treatments going into each mean

** = Mean value (mg/gm wet tissue)

TABLE VII

TOTAL MYOGLOBIN
AS INFLUENCED BY MUSCLE AND TREATMENT VARIATION

TREATMENT	MUSCLE			OVERALL MEAN
	SM	LD	ST	
CONTROL	(6)*	(6)	(6)	(18)
	2.69**	2.45	1.96	2.37
STIMULATED	(6)	(6)	(6)	(18)
	2.64	2.55	2.06	2.42
OVERALL MEAN	(12)	(12)	(12)	(36)
	2.67	2.50	2.01	2.39

* = Number of animals going into each mean

** = Mean value (mg/gm wet tissue)

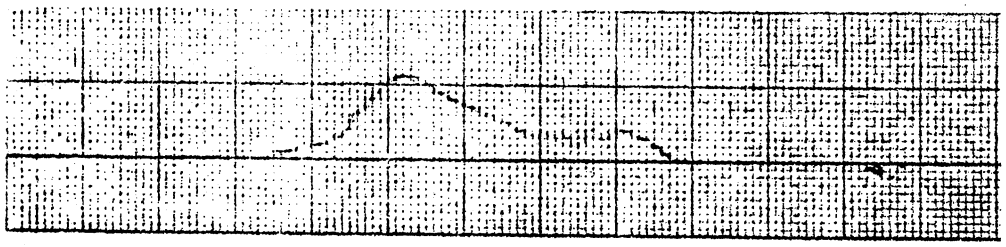
TABLE VIII

ANALYSIS OF VARIANCE TABLE FOR TOTAL MYOGLOBIN

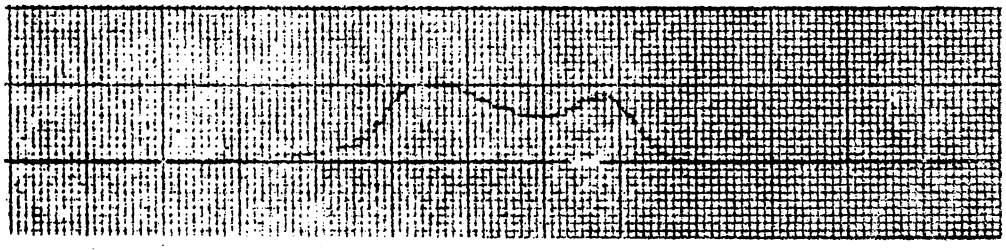
SOURCE	D.F.	S.S.	M.S.	F
TOTAL	35	5.8727		
WHOLE PLOT	17	5.7084	0.3358	
ANIMAL	5	2.3226	0.4645	8.0149[a]
MUSCLE	2	2.8062	1.4031	24.2101[a]
ERROR (a)	10	0.5796	0.0580	
SUB-PLOT	18	0.1644	0.0091	
TREATMENT	1	0.0230	0.0230	3.4143[b]
MUS. X TRT.	2	0.0404	0.0202	3.0035[b]
ERROR (b)	15	0.1010	0.0067	

a = OSL $<$ 0.005

b = OSL $<$ 0.1

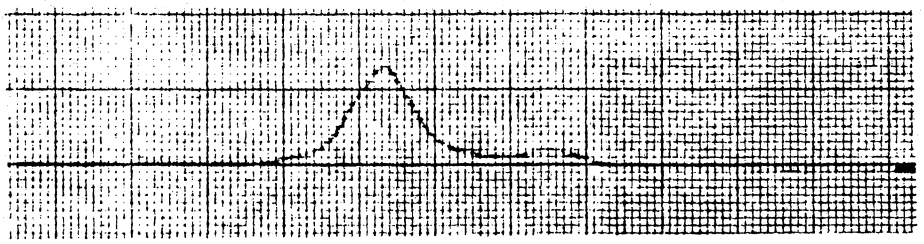


Control



Stimulated

Figure 7. Electrophoresis Graph From Semimembranosus Muscle



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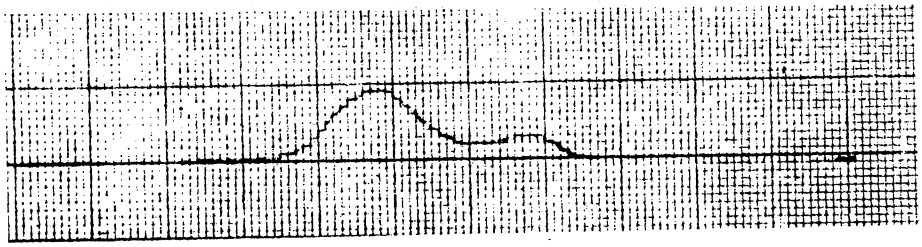
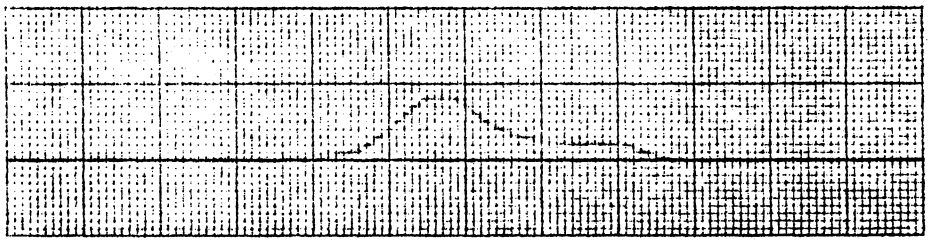
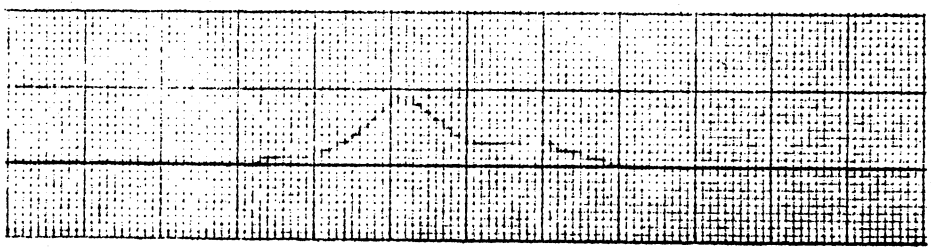


Stimulated

Figure 8. Electrophoresis Graph From Longissimus Dorsi Muscle



Control



Stimulated

Figure 9. Electrophoresis Graph From Semitendinosus Muscle

result can also be explained theoretically. On electrophoresis, the oxygen in the oxymyoglobin is ionized and carries a " 2 negative " charge. Thus, oxymyoglobin will migrate faster and further than the rest of the myoglobin derivatives to the anode on the right hand side of the membrane. The reduced myoglobin, on the other hand, carries no charge and thus will not migrate as far and as fast like the oxymyoglobin on electrophoresis. Not all of the oxymyoglobin peaks obtained from the samples were able to match exactly with the oxymyoglobin standard due to the fact that some electrophoresis membranes stretched during the squeegeeing of excess moisture on the membrane before drying. The remaining peak was identified as myoglobin in its reduced state by the same technique. Although a metmyoglobin peak was expected to show up in the graph, this two-peak result, without the metmyoglobin was understandable. This is because only very minute amount of metmyoglobin would be able to form in such a short period of time, especially underneath the surface of the meat. Therefore, the two major forms of myoglobin present would be the oxymyoglobin and myoglobin in its reduced state. This identification of the oxymyoglobin was further supported by a visual appraisal of the meat samples which will be discussed later. Since there were no distinct point of separation between the two peaks, they were separated by extrapolation as shown in Figure 10. The peaks were cut out and weighed separately, with the weight of the center piece, which was presumed to be common to both sides, added to each side. Extreme care was taken

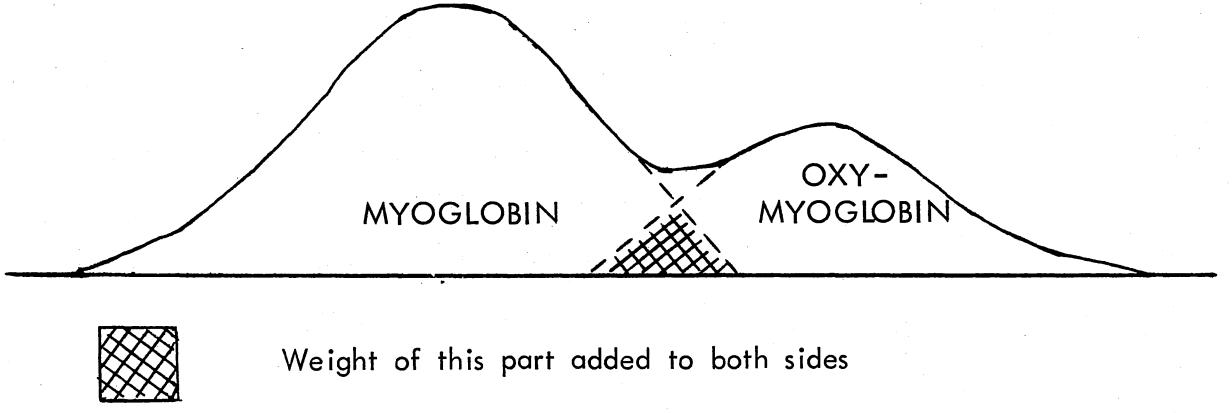


Figure 10. Extrapolation of the myoglobin derivatives

in the extrapolating procedure to eliminate bias by not looking at whether it was a graph for the control or for the stimulated. Care was also exercised in the handling of the cut-outs. They were handled with forceps and put in envelopes before being weighed.

Results indicated that the total oxymyoglobin content in the muscle was affected by neither animal and muscle differences, nor by the interaction of muscle and treatment. However, it should be noted that there was a significant difference (OSL $<$ 0.005) in the oxymyoglobin content due to the electrical stimulation (Table IX). Further analysis of those results (Table X,XI) indicated that the oxymyoglobin content was significantly higher in the electrical stimulated samples. The oxymyoglobin content of the semimembranosus and the longissimus dorsi muscle recorded the most significant differences (OSL $<$ 0.01). The finding that the semimembranosus muscle was most affected seemed to agree with the result obtained by Jacobson and Fenton (1956). They studied the effect of three levels of nutrition on the quality of beef and found that the greatest effect on the redness of beef was in the semimembranosus muscle, the most active muscle sampled. Since there was no data on exactly how much current passed through each muscle, it would be difficult to determine if the semimembranosus muscle actually lay in the path of electricity flow. The oxymyoglobin content of the semitendinosus muscle over all six animals showed a less significant difference between the stimulated and the control samples. A paired t-test was conducted in

TABLE IX

ANALYSIS OF VARIANCE TABLE FOR TOTAL OXYMYOGLOBIN

SOURCE	D.F.	S.S.	M.S.	F
TOTAL	35	1.9467		
WHOLE PLOT	17	1.2958	0.0762	
ANIMAL	5	0.5100	0.1020	1.8092[a]
MUSCLE	2	0.2221	0.1110	1.9696[a]
ERROR (a)	10	0.5638	0.0564	
SUB-PLOT	18	0.6509	0.0362	
TREATMENT	1	0.3768	0.3768	29.3651[b]
MUS. X TRT.	2	0.0816	0.0408	3.1816[c]
ERROR (b)	15	0.1925	0.0128	

a = OSL $>$ 0.1

b = OSL $<$ 0.005

c = OSL $<$ 0.1

TABLE X

TOTAL OXYMYOGLOBIN
AS INFLUENCED BY MUSCLE AND TREATMENT VARIATION

	MUSCLE			
TREATMENT	SM[a]	LD[a]	ST[b]	OVERALL MEAN
CONTROL	(6)* 0.28**	(6) 0.29	(6) 0.18	(18) 0.25
STIMULATED	(6) 0.62	(6) 0.41	(6) 0.33	(18) 0.45
OVERALL MEAN	(12) 0.45	(12) 0.35	(12) 0.25	(36) 0.35

* = Number of animals going into each mean

** = Mean value (mg/gm wet tissue)

a = OSL $<$ 0.01

b = OSL $<$ 0.025

TABLE XI

TOTAL OXYMYOGLOBIN
AS INFLUENCED BY ANIMAL AND MUSCLE VARIATION

ANIMAL	MUSCLE			OVERALL MEAN
	SM	LD	ST	
1	(2)* 0.79**	(2) 0.24	(2) 0.24	(6) 0.42
2	(2) 0.57	(2) 0.49	(2) 0.24	(6) 0.43
3	(2) 0.21	(2) 0.30	(2) 0.11	(6) 0.21
4	(2) 0.37	(2) 0.14	(2) 0.24	(6) 0.25
5	(2) 0.47	(2) 0.75	(2) 0.38	(6) 0.54
6	(2) 0.27	(2) 0.19	(2) 0.33	(6) 0.26
OVERALL MEAN	(12) 0.45	(12) 0.35	(12) 0.25	(36) 0.35

* = Number of treatments going into each mean

** = Mean value (mg/gm wet tissue)

analyzing this segment of the result. The calculated " t " from a paired " t " test was obtained by dividing the mean difference by the standard deviation of the mean. In terms of the experiment, the result showed that there were wider variations in the oxymyoglobin content between the stimulated and the control samples from the semimembranosus and longissimus dorsi muscle than the semitendinosus muscle.

Data obtained from calculation of percent oxymyoglobin indicated the stimulated samples from the semimembranosus muscle showed the highest average of 23.35 % or 0.62 mg/gm of wet tissue (Table XII). Control samples from the semitendinosus muscle had the lowest percentage with an average of 9.63 %. From muscle to muscle, the semimembranosus had an average of 16.88 % oxymyoglobin over all control and stimulated samples from the six animals followed by the longissimus dorsi muscle of 14.43 % and semitendinosus muscle with a mean of 12.73 % (Table XIII). The findings that the semimembranosus muscle had the highest oxymyoglobin content and the semitendinosus muscle had the lowest oxymyoglobin content correlated with the results discussed earlier that the semimembranosus also possessed the highest total pigment and total myoglobin content and semitendinosus possessed the least total pigment and total myoglobin concentration.

The analysis of variance for the percent oxymyoglobin showed similar results as those in the total oxymyoglobin determination with the main source of variation coming from the treatment (OSL $<$ 0.005). No statistically

TABLE XII

PERCENT OXYMYOGLOBIN
AS INFLUENCED BY MUSCLE AND TREATMENT VARIATION

TREATMENT	MUSCLE			OVERALL MEAN
	SM	LD	ST	
CONTROL	(6)*	(6)	(6)	(18)
	10.41**	12.19	9.63	10.74
STIMULATED	(6)	(6)	(6)	(18)
	23.35	16.68	15.84	18.62
OVERALL MEAN	(12)	(12)	(12)	(36)
	16.88	14.43	12.73	14.68

* = Number of animals going into each mean

** = Mean value

TABLE XIII

PERCENT OXYMYOGLOBIN
AS INFLUENCED BY ANIMAL AND MUSCLE VARIATION

ANIMAL	MUSCLE			OVERALL MEAN
	SM	LD	ST	
1	(2)* 26.29**	(2) 8.40	(2) 10.75	(6) 15.14
2	(2) 23.92	(2) 24.98	(2) 12.18	(6) 20.36
3	(2) 7.59	(2) 11.30	(2) 5.33	(6) 8.07
4	(2) 17.03	(2) 6.29	(2) 13.38	(6) 12.23
5	(2) 17.34	(2) 29.09	(2) 21.48	(6) 22.64
6	(2) 9.13	(2) 6.55	(2) 13.27	(6) 9.65
OVERALL MEAN	(12) 16.88	(12) 14.43	(12) 12.73	(36) 14.68

* = Number of treatments going into each mean

** = Mean value

significant difference was found between the stimulated and the control samples from animal, or muscle, or the muscle by treatment interaction. The error term for testing the significance of treatment effect was small and thus was sensitive to even small treatment differences. The reason for the small error term could partly be explained by the design of the experiment. The split-plot design was ideal for this experiment because variations due to the electrical stimulations were expected to be less than the variations due to the animals or the muscles. Thus after partitioning the two major variations due to animals and muscles, the remaining error term was small enough and sensitive enough to detect the effect of electrical stimulation (Table XIV).

Visual Appraisal

A nonparametric sign test was used to analyze the result from the visual appraisal. The samples were examined under ordinary fluorescent lighting. All samples were examined in pairs, comparing the control sample with the stimulated sample from the same muscle. If the stimulated sample was found to have a brighter red color, it was recorded as " + ". If the control sample was found to have a brighter cherry red color or if no difference was found in the color of the control and stimulated samples, it was recorded as " - ". It must be emphasized that this visual appraisal was not conducted by a panel and was a one man observation.

After all samples were inspected, the result was analyzed by the

TABLE XIV

ANALYSIS OF VARIANCE TABLE FOR PERCENT OXYMYOGLOBIN

SOURCE	D.F.	S.S.	M.S.	F
TOTAL	35	2858.7947		
WHOLE PLOT	17	1947.6487	114.5676	
ANIMAL	5	1024.3825	204.8765	2.5022[a]
MUSCLE	2	104.4855	52.2427	0.6381[a]
ERROR (a)	10	818.7808	81.8781	
SUB-PLOT	18	911.1460	50.6192	
TREATMENT	1	558.7094	558.7094	36.0146[b]
MUS. X TRT.	2	119.7355	59.8677	3.8591[c]
ERROR (b)	15	232.7012	15.5134	

a = OSL $>$ 0.1

b = OSL $<$ 0.005

c = OSL $<$ 0.05

formula : $\chi^2 = \frac{(|n_+ - n_-| - 1)^2}{n_+ + n_-}$ with degree of freedom = 1.

In 14 sample pairs out of the total 18, the stimulated samples showed a bright red color while the control sample had a dark purplish red color. Hence, 14 " + " were recorded. It was noteworthy that no brownish pigment was ever detected in any of the samples. This supported the finding that only two peaks showed up in the electrophoresis graph, with the metmyoglobin being in very minute amount and probably not enough to show up in the electrophoresis graph. In the remaining four pairs of samples, no visual difference was detected between the stimulated and the control samples and were thus recorded as " - ".

The null hypothesis that there was no difference in color due to electrical stimulation was tested and the observed significance level was OSL < 0.05.

Although it can not be concluded from this visual appraisal that there was an overwhelming difference in color due to electrical stimulation, it did provide some information on the trend in color change or in what way the electrically stimulated sample would be visually different from the control sample. Further research is needed before a more thorough understanding of why the stimulated samples contain more oxymyoglobin and thus have a brighter red color than the control samples can be obtained.

SUMMARY

The objectives of this research were to : (1) develop a quantitative method to determine the amount and proportions of myoglobin and its derivatives and (2) investigate the effect of post-mortem electrical stimulation on the amount and proportions of myoglobin and its derivatives in the bovine semimembranosus, longissimus dorsi and the semitendinosus muscle. For this purpose, six Hereford heifers were slaughtered and one side from each carcass was electrically stimulated at 300 volts for 30 minutes. Muscles were dissected from the carcass four hours after death.

The method developed for measuring the amount and proportions of myoglobin and its derivatives called for the extraction of myoglobin from the beef muscle for electrophoresis using cellulose acetate membrane as the supporting media. The extraction procedure for myoglobin consisted of first precipitating the foreign proteins in the beef muscle extract using lead acetate solution followed by the salting of hemoglobin using a mixture of mono- and dibasic potassium phosphate crystals. There were no difficulties in obtaining a clear solution for the spectrophotometric determination of total pigment and total myoglobin concentration. However, the electrophoresis of the myoglobin extraction produced a graph with two partially fused peaks, being identified

as oxymyoglobin and myoglobin in its reduced state. An extrapolation procedure was used to separate the peaks and calculations of the myoglobin derivatives were made.

There were no differences found in the total pigment content due to the electrical stimulation (OSL > 0.1). As expected, the total pigment content was found to be affected by differences in animal (OSL < 0.01) and by differences in muscle (OSL < 0.005). The semimembranosus muscle was found to have the highest total pigment concentration and the semitendinosus muscle with the least total pigment content.

There were also no differences found in the total myoglobin content due to the electrical stimulation (OSL < 0.1). Statistically significant differences in total myoglobin content were found as a result of different animal (OSL < 0.005) and different muscle (OSL< 0.005). Muscle by treatment effect was found to be insignificant. As in total pigment determination, the semimembranosus muscle contained the highest total myoglobin content and the semitendinosus muscle had the lowest total myoglobin concentration.

The total oxymyoglobin content was calculated from the peaks of the electrophoresis graph. The semimembranosus muscle was found to have the highest oxymyoglobin content as well as percent oxymyoglobin. The lowest being from the semitendinosus muscle. The animal and muscle differences were found not to be the contributors of the differences in oxymyoglobin content (OSL > 0.1). However, it was noteworthy that the oxymyoglobin

content was significantly higher in the electrically stimulated samples (OSL < 0.005). The percent oxymyoglobin in the sample was also calculated and the same result was obtained.

Results from visual appraisal showed detectable visual differences between the stimulated and the control samples. The electrically stimulated samples were bright red in color showing a higher oxymyoglobin content while the control samples were dark purple red in color showing a high reduced myoglobin content. This observation agreed with the calculated result that electrically stimulated samples were higher in oxymyoglobin content. No brownish pigments were detected in any of the samples which supported the finding that no metmyoglobin peak showed up in the electrophoresis graph. The reason for this phenomena that the electrically stimulated samples contained a higher oxymyoglobin content was not clear and more research in this area is needed before a thorough understanding can be obtained.

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