# THE QUANTITATIVE DETERMINATION OF MYOGLOBIN IN FOUR BOVINE MUSCLES

BY

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1959

Submitted to the faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 1966

# THE QUANTITATIVE DETERMINATION OF MYOGLOBIN IN FOUR BOVINE MUSCLES

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## ACKNOWLEDGMENT

The author wishes to express appreciation to Dr. R. L. Henrickson, Professor of Animal Science, for assistance and guidance throughout the course of this study.

Further appreciation is also extended to Drs. J. J. Guenther and L. E. Walters for their cooperation and suggestions during the preparation of this thesis.

Special recognition is extended Dr. M. E. Mason, Professor of Biochemistry, for clarification of certain steps in the quantitative procedure employed in this study.

The author is grateful to R. W. Mandigo for assistance in running the fat and moisture determinations, and to A. W. Munson for suggestions on the statistical analysis.

The writer also wishes to express sincere appreciation to his wife, Marcia, for assistance and continuous encouragement during this program of graduate study and preparation of the thesis.

TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# INTRODUCTION

The color of lean tissue is of primary concern to all facets of the meat industry. Consumer preference studies have emphasized that color of lean is one of the basic factors influencing the acceptance of fresh meat. Thus, the retailer has become interested in methods that can be utilized for the preservation of "acceptable" meat color. It is exceedingly difficult to express the most "acceptable" color of lean by subjective methods alone. For example, the term "bright cherry red" used to describe fresh beef holds various connotations for different people. This gives rise to the problem of defining a desirable meat color for each of the various species: lamb, pork, veal and beef.

Some packers claim that ribbed carcasses and other fresh cut surfaces from pasture-fed cattle do not retain their bright color as well as those from grain-fed cattle. This presents a need for research to determine the effect of diet upon color formation and retention. In addition, the extent to which color can be controlled by the supplementation of color precursors in the diet also must be investigated.

By visual appraisal alone, there exists easily recognizable differences in the color of different muscles within the same species. There is a lack of information in accounting for these within specie muscle variations.

Before the preceding problem areas can be logically attacked, a precise quantitative method for the determination of color needs to be standardized. In addition, a precise method would serve as the initial step in the development of a procedure that would express the amounts of the heme derivatives on a quantitative basis.

These needs led to the present study with the following objectives: 1) to evaluate the selected procedure for measuring quantitative differences in myoglobin content of visually similar and dissimilar skeletal muscle and 2) the effect of moisture and fat upon these muscle differences.

# LITERATURE REVIEW

This literature review includes some of the work relative to the following areas: 1) chemistry of myoglobin, 2) function of myoglobin, 3) methods of analysis, and 4) factors affecting myoglobin concentration.

## Chemistry of Myoglobin

### Structure of myoglobin

Early knowledge of myoglobin centered around the heme structure with not much known about the globin portion of the molecule except for its mode of attachment to heme. Kendrew et al. (1960) undertook the task of elucidating the entire structure and configuration of the myoglobin molecule. Isolated sperm whale myoglobin crystals were studied by means of x-ray diffraction patterns and Fourier synthesis. They determined the tertiary structure of myoglobin and found that the globin portion contained helical configurations. Edmundson and Hirs (1961) worked on the identification of the amino acid residues in the globin portion of the molecule and were able to name 120 of the 153 amino acid residues. Edmundson (1965) has now elucidated the complete amino acid sequence of sperm whale myoglobin.

Kendrew (1963) summarized the results on the structure of myoglobin to date (Figure 1 and Table I). Myoglobin is a conjugated protein consisting of a

single peptide chain of 153 amino-acid residues associated with an iron-porphyrin complex, the heme group. It contains 1260 atoms, exclusive of hydrogen atoms; plus 400 atoms in liquid and salt solution, of which some are bound to fixed sites on the surface of the molecule. The most important forces responsible for the integrity of the whole structure comes from the van der Waals forces between nonpolar residues. To a lesser extent, the charge interactions and hydrogen bonds between neighboring polar residues on the surface of the molecule aid in maintaining the helix of the globin. There are, however, some polar interactions of the side chains which may have some significance in determining the point at which a helix is broken and gives way to an irregular segment of the chain. The bond interactions of the heme group with the globin portion of the molecule are responsible for the characteristic function of myoglobin since an isolated heme group does not exhibit the phenonmenon of reversible oxygenation. To date the oxygenation reaction of myoglobin and hemoglobin can not be explained in precise structural terms. Kendrew has also verified an earlier speculation that the fifth coordination position of the iron atom is occupied by a ring nitrogen atom of a histidine residue. On the distal side of the iron atom, occupying its sixth coordination position, is a water molecule. 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.

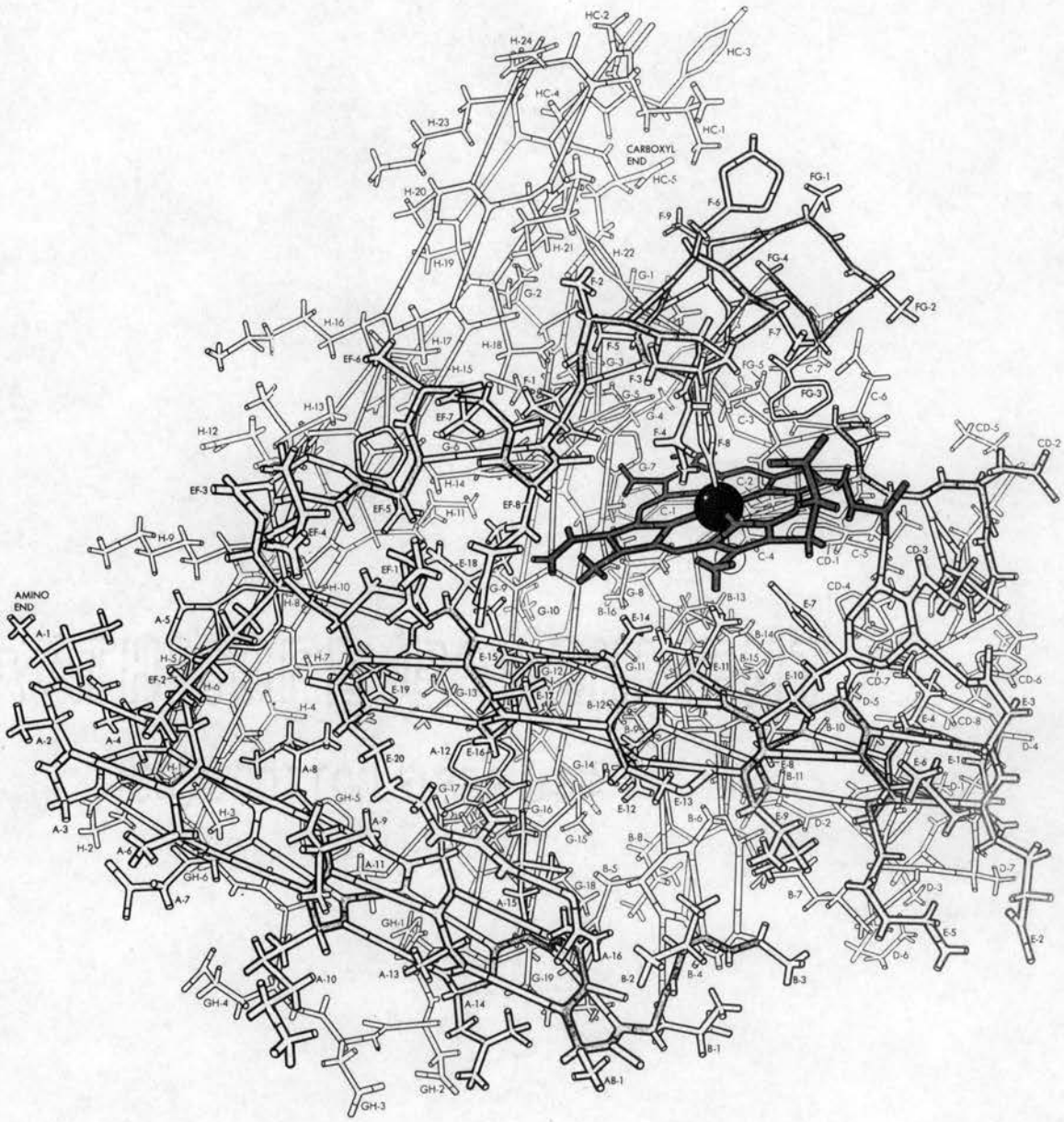



Figure 1 - Structure of Myoglobin
(Kendrew, 1961)

# 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 amino acid unit represented by each letter and number in the model of myoglobin (Figure 1) is given in the above table; the key to the abbreviations is at top left in the table. The brackets in the table indicate those amino acid units which form a helical section.

Geneticists (Kendrew, 1963) now believe that the hereditary material determines only the amino acid sequence of a protein, not its three-dimensional structure. Hence, the polypeptide chain, once synthesized, should be capable of folding itself up without being provided with additional information. Thus, one should be able to predict the three-dimensional structure of a protein from a knowledge of its amino acid sequence. Anfinsen et al. (1962) has demonstrated this capacity in vitro for one protein, ribonuclease. This phenomenon was also exhibited in the urea denatured myoglobin studied by Harrison and Blout (1965). They found that the amino acid sequence is sufficient to determine the conformation assumed by the protein in solution after removal of urea.

## Properties of myoglobin

### Electrophoretic moiety

Lewis and Schweigert (1955) found crystalline beef myoglobin isolated by ammonium sulfate fractionation to be electrophoretically heterogeneous. Perkoff et al. (1962) found structural differences between human globin and the globin from other species. Quinn et al. (1964), using the technique of starch-gel electrophoresis, detected three distinct myoglobins from bovine muscle based on differing mobilities. Further, Quinn and Pearson (1964) undertook characterization studies on these three myoglobin fractions. Results showed that all three myoglobin fractions exhibited identical wavelength positions for maximum and minimum light absorption; however, they differed in the magnitude of their respective absorbancy values. The fractions did exhibit the same autoxidation rates. Atassi (1964) studied the mobility, by electrophoresis, and amino acid

composition of myoglobin isolated from sperm whale skeletal muscle. He found that no great structural difference exists between components, but rather that these differences are due to a minor amino acid sequence difference in the globin portion of the molecule. Hence, the myoglobin of different species appear to be differentiated from each other by differences in the globin component as shown by variations in the amino acid contents.

Solubility

A problem in early research was the separation of myoglobin from hemoglobin since both are soluble in water. Morgan (1935-36) studied the solubility of myoglobin in concentrated ammonium sulfate and phosphate solutions. He modified Theorell's procedure for the isolation of myoglobin. He found, that by following the lead acetate precipitation step with the addition of primary and secondary potassium phosphate, in the solid state, to a phosphate concentration of 3M and pH of 6.6, that myoglobin could be quantitatively separated from hemoglobin.

Absorbancy

Much of the preliminary work in the determining of absorbancy constants on myoglobin stems from the findings obtained with hemoglobin. Drabkin and Austin (1932) conducted spectrophotometric studies using common hemoglobin derivatives from human, dog, and rabbit blood. They found that the molar extinction coefficients of the dog and rabbit differed slightly from that of human blood. Later, Drabkin (1945) found that the spectrophotometric constant of cyanmethemoglobin could be used interchangeably between the species since the iron content of the hemoglobins and myoglobins were remarkably similar, average $0.339 \pm 0.001$ for

hemoglobin and 0.340 ± 0.002 for myoglobin. Crandall and Drabkin (1946), and Drabkin (1947) quantitatively measured myoglobin concentration (spectrophotometrically) after conversion of myoglobin to cyanmetmyoglobin. They used the molar extinction coefficient of myoglobin in the form of cyanmetmyoglobin as $11.3 \times 10^3$ at a wavelength of 540 mu based on earlier studies by Drabkin and Austin (1935-36b) and Drabkin (1945).

Bowen (1949) investigated the absorption spectra and extinction coefficients of myoglobin. He checked the reliability of Drabkin's molar extinction coefficient by determining the iron content in six solutions of differing myoglobin concentration. There was agreement within 5% of the values obtained when analyzed as cyanmetmyoglobin.

Occurrence

Histochemical localization of myoglobin in pigeon-breast muscle has been demonstrated by the benzidine-peroxidase reaction. The red narrow fibers with their high concentration of oxidative enzymes were shown to possess most of the myoglobin in comparison with the broad white fibers which contain very little. (Chinoy, 1963). Wirsen and Larsson (1964) did not detect the presence of myoglobin in skeletal muscle of mice until after birth.

Ohyama et al. (1962) studied the formation of myoglobin in vitro. The iron chelating enzyme which catalyzes the combination of iron and protoporphyrin was prepared from duck-erythrocyte hemolyzate. This preparation in the presence of apomyoglobin caused the formation of myoglobin. They also found that the formation of myoglobin parallels that of the formation of hemoglobin from iron,

protoporphyrin and globin. These results are in keeping with the evidence of the formation of myoglobin obtained by Yoneyama et al. (1963).

Amako et al. (1963) in their study on the metabolism of myoglobin, found that myoglobin (I) is metabolized in the reticuloendothelial cells.

As further studies are conducted to elucidate the specific mechanisms and sites of formation of myoglobin, radioactive isotope experiments can be employed to determine the turnover rate of myoglobin in vivo. Such experiments would provide a logical approach to investigating the addition of color precursors in livestock diets and the subsequent effect of these precursors on myoglobin formation.

## Function of Myoglobin

The function of the heme group in hemoglobin and myoglobin is to combine reversibly with molecular oxygen. Oxygen is carried by the hemoglobin in blood from the lungs to the tissues. At this point, myoglobin, which is contained within the cells of muscle tissue, accepts oxygen from the blood. This transfer of oxygen from the blood to the cells is facilitated because myoglobin has a higher oxygen affinity than hemoglobin. Myoglobin also serves as an oxygen reservoir in the cells. Lawrie (1952) stated that, in general, high myoglobin content in muscle is associated with high enzymatic activity, particularly cytochrome oxidase. This suggests that the function of myoglobin is to assist in ensuring a constant supply of oxygen to the muscle oxidase system. Aside from the physiological function of myoglobin, it is of prime importance in the development of desirable or undesirable

color changes in fresh meat.

Schweigert (1956) has summarized the chemical reactions of myoglobin in muscle tissue (Figure 2). Myoglobin is converted to oxymyoglobin by oxygenation with molecular oxygen. Oxidation of myoglobin or reduction of oxymyoglobin to metmyoglobin occurs after prolonged exposure to oxygen, especially under the conditions of low oxygen pressure and surface dehydration. The maintenance of myoglobin in the ferrous state or the reduction of metmyoglobin to myoglobin is favored by the presence of reducing coenzymes. The supply of reducing coenzymes is dependent upon the enzymatic oxidation of available substrates, primarily glucose. The final degradation of myoglobin to free and oxidized porphyrins comes from excessive exposure to oxygen, the action of peroxide producing bacteria, irradiation with gamma rays, and the presence of light which catalyzes the oxidation reaction. As Kendrew (1963) has stated, the exact mechanisms of these reactions have not yet been explained in precise, structural terms.

## Methods of Analysis

There are several methods which have been employed for the quantitative determination of myoglobin. Whipple (1926) determined myoglobin concentration by converting myoglobin to the carbon monoxide derivative and then standardized this against a 1% solution of carboxyhemoglobin. Difficulty with the stability of the carboxymyoglobin solution was sometimes encountered and this problem was attributed to muscle autolysis.

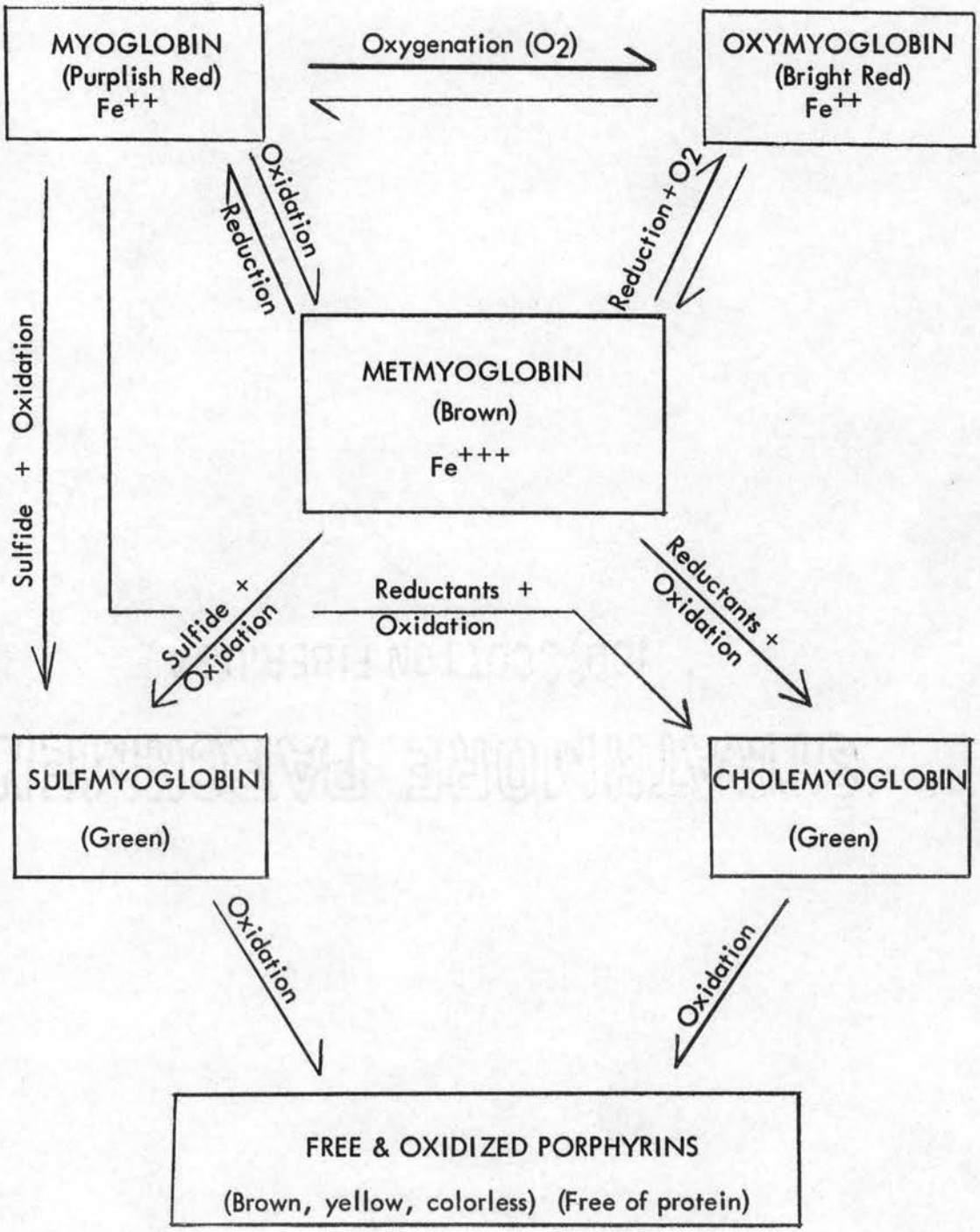



Figure 2 - Reactions of Myoglobin
(Giffee et al., 1960)

Shenk et al. (1934) were the first to determine the relative percent of myoglobin and hemoglobin when both were present in solution. Their calculations were based on an absorbancy ratio technique, based on the spectral differences between these two compounds, with the two wavelengths involved being selected as a result of a study of the maximum and minimum points in the absorption spectra of the respective oxygenated pigments. Watson (1935) used a laborious procedure for the extraction of myoglobin and hemoglobin from muscle tissue. His method is also based on the difference between the oxy-compounds, but the pigments were first reduced and then immediately reoxygenated before taking spectrophotometric readings. The problem encountered with the above procedures is that the oxy-derivatives are readily reduced to myoglobin or metmyoglobin which thereby makes uniformity of the readings a function of time. In addition, the extinction coefficient of the oxy-derivatives of myoglobin and hemoglobin are not the same at the two wavelengths selected.

DeDuve (1948) developed a quantitative pigment determination based on the difference in light absorption of the carbon monoxide compounds of myoglobin and hemoglobin. Poel (1949) also measured the concentration of myoglobin and hemoglobin when together in solution by utilizing 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 differed from the ones used by DeDuve. Bowen and Eads (1949) determined myoglobin and hemoglobin concentration in skeletal muscle of dogs by the carbon monoxide derivative method. Their work was done independently from that of Poel and results verified the

wavelengths chosen by Poel.

Drabkin (1950) calculated total pigment concentration, the sum total of hemoglobin and myoglobin, by reading optical density at a wavelength of 540 mu after conversion to the cyanmetmyoglobin and cyanmethemoglobin forms.

Ginger et al. (1954) gave a detailed procedure for quantitatively determining myoglobin concentration in beef and pork muscle by salting out the hemoglobin from myoglobin in muscle tissue extracts. Fleming et al. (1960) conducted a comparative study on the procedures used for the quantitative estimation of myoglobin and hemoglobin in beef muscle extracts. They compared the methods of Poel, Shenk, and Ginger to Drabkin's total pigment determination. They found that the carbon monoxide conversion method yielded the most favorable results.

## Factors Affecting Myoglobin Concentration

Whipple (1926) studied the variation in muscle hemoglobin, myoglobin, content of striated muscles in dogs. The leg and loin muscles were found to show a great range in their myoglobin content, which he concluded to be dependent upon exercise, and hence, a measure of latent muscular power. Myoglobin concentration was found to increase with the age of the dog. In adult dogs, an active, trained hunting dog had up to two and a half times the myoglobin concentration of a quiet house dog. In contrast, Mitchell and Hamilton (1933) found no effect on the iron and myoglobin content in beef skeletal tissues due to work. However, in one experiment they did find a reduced iron content due to light work. Their values were obtained on gross areas of the carcass rather then based

on specific muscle comparisons which may well have confounded the results by having light and dark muscles ground together from which they then removed a sample for analysis.

In the study of Shenk et al. (1934) on bovine longissimus dorsi, samples were taken from cattle fed under different conditions. It was found that cattle full-fed on pasture had a higher myoglobin content then cattle full-fed in the dry lot. However, the highest myoglobin content was obtained in cattle fed on grass alone. Their explanation for these differences were that animals receiving no supplementation are compelled to range more for their feed and therefore receive more exercise. Bull et al. (1941) measured color of the longissimus dorsi muscle by reflectance and found no significant difference in the brightness of lean between cattle fattened on pasture, full-fed grain on pasture, or cattle finished in the dry lot. Further, Bull and Rusk (1942) studied the effect of exercise on the brightness of color from selected muscles in the fore-quarter and hind-quarter in beef cattle. They found that exercise had no significant effect on brightness of color; however, exercise was found to lower feed efficiency and increase tenderness. These results do not present the total picture since myoglobin concentrations were not determined. Also, color brightness readings were not made over a period of time thereby giving no indication as to the effect of exercise or diet upon color retention time.

The data of Poel (1949) on the physiological effect of high altitude hypoxia on the myoglobin concentration in striated muscle of albino rats indicated no direct relation between changes in myoglobin concentration and adaptation to a

condition of altitude hypoxia. Skeletal muscle, in which activity is not enhanced by anoxia, eventually showed a decreased myoglobin content after prolonged exposure; whereas in cardiac muscle, the situation is reversed. These changes indicated that the myoglobin content of a muscle is determined by muscular activity rather than by anoxia, even in an anoxic environment. Bowen and Eads (1949) studied the effect of simulated altitude on the myoglobin content of dogs. Their study also indicated that the production of myoglobin is not stimulated by the hypoxia caused by 6 hours daily exposure to 18,000 feet.

The data of Drabkin (1950) suggests a tendency for an actual increase in the content of myoglobin per kilogram of body weight with increasing size of the species. However, owing to the variability of myoglobin concentration in different muscles, he could not explain myoglobin content solely as a function of body mass. Lawrie et al. (1963) compared myoglobin concentration in hogs slaughtered on a weight constant basis of 150, 200 and 250 pounds. The analysis on selected muscles in the shoulder, ham and loin all indicated increased myoglobin content with increased body weight. In addition, it was found that the psoas major and triceps showed a greater increase in myoglobin concentration between 150 and 200 pound groups while the rectus femoris and extensor carpi radialis had the greatest increase between the 200 and 250 pound groups. The research of these workers tended to dispel the concept that myoglobin concentration might be a constant throughout the life of the animal.

Lawrie (1950) found that myoglobin concentration in swine increased with age and exercise. He further found that myoglobin concentration was not affected

by plane of nutrition, but this probably resulted from the fact that animals were slaughtered at a constant weight thereby confounding diet with age. Lawrie (1953) studied the effect of enforced exercise on myoglobin concentration in the deep pectoral and gastrocnemius muscles of the rat and fowl. He found that myoglobin content increased due to enforced exercise over an extended period of time, 9 months; whereas, over a short period, even the most severe exercise elicits no such response. In another study by Lawrie (1961), the myoglobin concentration in the longissimus dorsi muscle of cattle exhibited a rapid increase to about 0.4% at 20-24 months of age with a subsequent, but much slower rise to a maximum of about 0.6% at 5-6 years of age. The variation in values obtained after 12 months of age was attributed to breed differences and castration. Wierbicki et al. (1955) also found that myoglobin concentration has some effect as an indication of age and/or sex. He found no correlation between myoglobin concentration and tenderness.

Jacobson and Fenton (1956) studied the effects of three levels of nutrition and age on the quality of beef. Their comparisons of color, total iron content and pH were made on 24 Holstein heifer carcasses with the animals being slaughtered at a specific age. The redness of color increased with the level of nutrition with the greatest effect occurring on the most active muscle sampled, the semimembranosus. The age of the animals caused varied results with the longissimus dorsi and semimembranosus muscles increasing in redness while the psoas major decreased. Iron content was found to increase with both age and level of nutrition. Also, redness of color increased with iron content. There was no

significant difference obtained in pH of the muscles sampled due to age or level of nutrition.

Niedermeier et al. (1959) in a study of the effect of diet on veal calves found that the supplementation of iron and copper increased the iron content of liver and hemoglobin content of blood. They also felt that the light pink color of the lean which is considered desirable in milk fed veal calves results from anemia. Bray et al. (1959) in further analysis of the chemical composition of the longissimus dorsi muscle on these same veal calves, found that increased iron and copper intake provided for a greater production of myoglobin. A darker colored lean was produced by increased quantities of myoglobin with a highly significant negative correlation of -0.91 between myoglobin and color.

Craig et al. (1959) studied the effect of combination of grass and grain in the ration on the color of the longissimus dorsi muscle in beef cattle. They found that dry lot fattened cattle possessed brighter color in this muscle than any of the cattle fattened on pasture or pasture plus concentrate supplement. Myoglobin concentration was not significantly different between treatments when the values were calculated on a moisture-free, fat-free basis. Since the longissimus dorsi serves as a muscle of support, actual increases in myoglobin concentration might have become apparent if selected muscles sampled from the round or fore-quarter had also been analyzed. In contrast, Janicki and Kolaczyk (1963) reported statistically significant correlations of -0.35, -0.53 and +0.44 between myoglobin and water content, myoglobin and water retention, and water content and water retention respectively as determined from 41 pork loins.

Lewis et al. (1962), in studying the effect of pre-slaughter treatments on beef cattle, measured color on the longissimus dorsi, psoas major and quadriceps femoris muscles by reflectance. All muscles were found to be darker as a result of stress, and pH was found to increase. Forrest et al. (1964) studied the influence of high iron and copper supplementation, preslaughter intramuscular epinephrine injections, and severe exercise prior to slaughter on ovine muscle characteristics. There was no difference in the myoglobin content between the treatments as measured on the longissimus dorsi, rectus abdominis and intercostal muscles. With the exception of the iron and copper supplemented lambs, all other treatments produced muscles that were significantly darker in color intensity than the control lambs.

It is apparent from the review on factors affecting myoglobin concentration that age is a definite contributor to increased myoglobin concentration. The conflicting results obtained on the contribution of exercise and plane of nutrition on myoglobin concentration may well be tied up with the methods employed for its measurement as well as the lack of sampling dissimilar muscle types.

Overexcitement or undue stress on the animal just prior to slaughter may result in masking true responses. Since a rise in pH indicates a reduction in the conversion of glycogen to lactic acid, a depletion in the glycogen reserve, it would appear that pH is a measure of the existance of favorable conditions under which the conversion of myoglobin to oxymyoglobin can occur rather than an actual difference in myoglobin concentration.

# EXPERIMENTAL PROCEDURE

## Materials

### Experiment 1

Five beef hindquarters were obtained from a meat packing plant in Oklahoma City. Since there was no way to determine the genetic or management background of these cattle, the criterion for selection was that these hindquarters be from carcasses of choice grade and in the weight range of 227 to 249 kilograms.

### Experiment 2

Seven steer beef hindquarters were obtained from the Oklahoma Agriculture Experiment Station. These carcasses were all of the choice grade and were selected because the complete management and genetic history was known (Table II).

Since it was considered essential that the analytical procedure employed be able to measure myoglobin concentration differences among muscles similar and dissimilar in visual color, the longissimus dorsi, psoas major, biceps femoris and semitendinosus muscles were selected. In addition, these muscles possess different quality attributes and represent different anatomical location and function.

TABLE II

HISTORY OF CATTLE
Experiment 2

| Animal Number | Age at Slaughter (days) | Carcass Weight (kilograms) | Carcass Grade | Feeding Treatment[a] |
|---|---|---|---|---|
| 1 | 429 | 312.5 | Choice | #3 |
| 2 | 421 | 296.6 | Choice$^-$ | #4 |
| 3 | 382 | 248.1 | Choice$^-$ | #7 |
| 4 | 412 | 277.1 | Choice$^-$ | #2 |
| 5 | 415 | 312.5 | Choice | #6 |
| 6 | 420 | 299.4 | Choice | #3 |
| 7 | 438 | 286.7 | Choice | #5 |

[a] All of the steers were of purebred Hereford breeding. They were on full-feed in the dry-lot for a period of 156 days which was preceded by a 41 day pre-data period. A milo base ration was fed with the treatment differences due to source of protein.

Treatment #2 - 1/4 of the cottonseed meal was replaced by urea.
Treatment #3 - 1/2 of the cottonseed meal was replaced by urea.
Treatment #4 - 3/4 of the cottonseed meal was replaced by urea.
Treatment #5 - the protein source was fish meal rather than cottonseed meal.
Treatment #6 - 1/4 of the fish meal was replaced by urea.
Treatment #7 - 1/2 of the fish meal was replaced by urea.

## Methods

In both experiments, the method of handling the hindquarters after arrival at the Oklahoma Station Meat Laboratory and the analytical procedures employed were the same.

### Sampling procedure

Upon arrival of the hindquarters at the meat laboratory, they were held in a 1.7°C cooler of approximately 70% relative humidity. Two inch steaks were then removed from the four muscles in question as soon as possible. The sample locations were standarized as follows:

1. Longissimus dorsi. Lateral to the first lumbar vertebra.
2. Psoas major. Lateral and ventral to the sixth and seventh lumbar vertebrae.
3. Semitendinosus. Three inches from the point of origin.
4. Biceps femoris. Anterior to the sample removed from the semitendinosus muscle.

After removal of the muscle samples from the hindquarters, they were freed of surrounding fat and connective tissue. Each sample was then separately homogenized in a Waring blender and placed in a sealed fruit jar. The sample was then stored in a freezer at a temperature of -20°C until chemical analysis was performed. No sample was held longer than two weeks prior to chemical analysis.

The samples were allowed to thaw at room temperature, approximately 25°C, while still in the sealed jar. They were again blended into a homogenous paste in

the Waring blender prior to the removal of random aliquots for chemical analysis.

### Proximate analysis

Duplicate moisture and ether extract determinations were conducted on each muscle according to the procedures outlined by the Association of Official Agricultural Chemists (1955).

## Quantitative Determination of Pigments

### Total pigment determination

Duplicate 25 gm aliquots were taken at random from the muscle homogenate. Each sample was placed in a Waring blender with 100 ml of boiled, cooled distilled water and blended for 3 minutes. The distilled water had been boiled to drive off the gas impurities and cooled to 6°C in order to maintain the slurry in the Waring blender at a moderate temperature.

The resulting slurry was centrifuged at 2000 x g at 6°C for 15 minutes. By the use of a buchner funnel and suction flask, the supernatant was filtered through Whatman #3 filter paper. The resultant residue was again extracted with an additional 100 ml of boiled, cooled distilled water for 3 minutes, then centrifuged and filtered as before. A preliminary experiment was conducted to determine the number of separate extractions necessary to remove all the pigments from the muscle sample. From this experiment, it was found that two separate 100 ml extractions proved sufficient. The two extracts were combined in a 200 ml volumetric flask which was made to volume using boiled, cooled distilled water.

Since many of the extracts were found to be turbid, they were filtered by gravity through Whatman #3 filter paper which then yielded a clear solution. One 20 ml aliquot was taken from each flask and converted to the cyanmetmyoglobin derivative by the addition of 0.005 gm potassium ferricyanide and 0.001 gm potassium cyanide. The samples were centrifuged at 2000 x g at room temperature and percent transmittance was read at a wavelength of 540 mu, using the Bausch and Lomb Spectronic 20. The concentration of total pigments was calculated from the formula: total pigments (mg/gm wet tissue) = $\frac{O.D. \times K}{\text{Sample wt.}}$; where optical density (O.D.) = 2 - log %T. The proportionality constant (K) was calculated by the formula: $K = \frac{17{,}000 \times \text{volume in liters}}{E}$. The millimolar extinction coefficient of myoglobin, 11.3 mM per liter, is represented by E. The volume in liters of the total sample solution equals 0.2 liters. 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 300.885 is obtained. The total pigment concentration values were expressed on a wet tissue, dry, fat-free; and dry, fat-free weight basis.

Myoglobin determination

One of the duplicate extracts from each muscle was selected at random for myoglobin analysis. Duplicate 50 ml aliquots were taken and placed in separate 100 ml beakers. Six ml of .5M phosphate buffer of pH 7.1 was added to each aliquot in order to bring the pH to 7.

Saturated basic lead acetate solution equal to 1/4 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. The purpose of this step was to

precipitate the foreign proteins. Temperature must be closely watched because at temperatures higher than 38°C, myoglobin also precipitates and at low temperatures the protein precipitation is incomplete.

The supernatant was filtered through Whatman #3 filter paper allowing a small portion to first pass through the filter paper into a waste suction flask. This allowed seating of the filter paper in the buchner funnel without further dilution of the extract. The remainder of the supernatant was collected in a clean, dry suction flask.

A 25 ml aliquot of this filtrate was placed in a beaker to which 11.65 gm of mono- and dibasic potassium phosphate mixture (crystals) was added to bring the phosphate concentration to 3M and the pH to 6.6. The calculations for the preparation of this solid phosphate buffer was extrapolated from the data of Green (1933). As was stated in the literature reviewed (Morgan, 1935-36), this step precipitates hemoglobin and leaves myoglobin in solution. Addition of the phosphate increased the volume to 29 ml.

This solution was then centrifuged at 2000 x g at room temperature for 15 minutes. The supernatant was filtered through Whatman #3 filter paper in the same manner as in the previous step.

To this filtrate was added potassium ferricyanide to a concentration of .6mM per liter for the purpose of converting myoglobin to metmyoglobin. Then potassium cyanide was added to a concentration of .8mM per liter which converts metmyoglobin to cyanmetmyoglobin. (Drabkin and Austin, 1935-36a).

The sample was then centrifuged for 15 minutes at 2000 x g at room temperature. Percent transmittance was read at a wavelength of 540 mu and the reading converted to optical density by the same formula as used in the total pigment determination.

The concentration of the myoglobin was then obtained from the formula: myoglobin concentration (mg/gm wet tissue) = $\frac{O.D. \times K}{\text{Sample wt.}}$. The K value was calculated from the formula:

$$K = \frac{17{,}000 \times \text{volume of aliquot in liters} \times \text{dilution factor}}{E}$$

E represents the millimolar extinction coefficient of myoglobin, 11.3 mM per liter. The molecular weight is assumed to be 17,000 mg per mM. The aliquot volume in liters equals 0.029. The dilution factor is that fraction of the total concentration present in the initial 200 ml extract represented by the concentration in the aliquot. The dilution factor equals $\frac{200}{17.857}$. Upon substitution of these values into the formula, a K value of 488.641 is obtained. The myoglobin concentration values were expressed in the same manner as those of the total pigment determination.

Statistical analysis

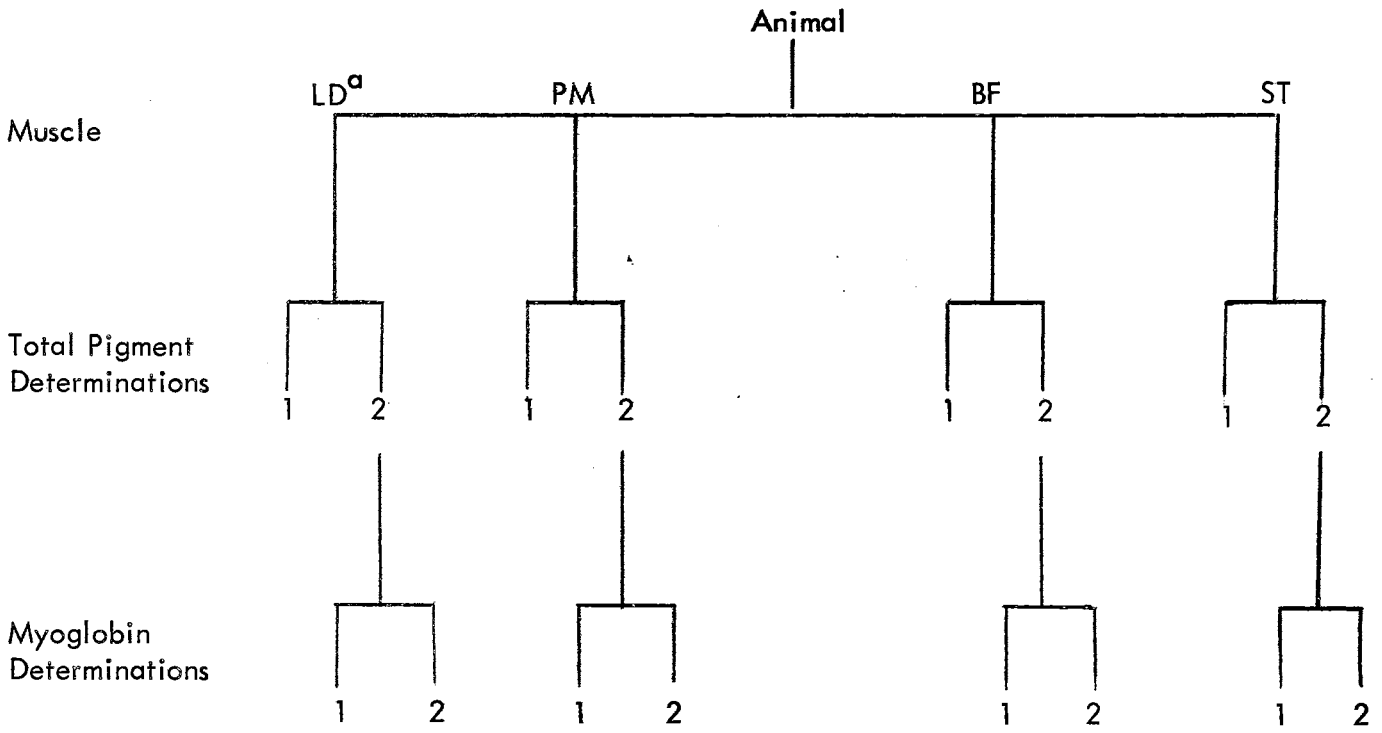
The experimental layout for the total pigment and myoglobin determinations is presented in Figure 3. A randomized complete block design was used, with treatments arranged factorially. Statistical analysis was conducted according to Steele and Torre (1960). The analysis of variance was utilized to study the components of variation within a variable (Table III).

TABLE III

COMPONENTS OF VARIANCE

| | degrees of freedom | Mean squares are an estimate of |
|---|---|---|
| Animal | a-1 | $\sigma_e^2 + ab\,\sigma_{AxM}^2 + b\,\sigma_A^2$ |
| Muscle | b-1 | $\sigma_e^2 + ab\,\sigma_{AxM}^2 + a\,\sigma_M^2$ |
| Animal x Muscle | (a-1)(b-1) | $\sigma_e^2 + ab\,\sigma_{AxM}^2$ |
| Error | ab(s-1) | $\sigma_e^2$ |

a = number of animals
b = number of muscles (This is a constant and equals 4)
s = number of determinations
$\sigma_e^2$ = error variance
$\sigma_{AxM}^2$ = animal by muscle variance
$\sigma_M^2$ = muscle variance
$\sigma_A^2$ = animal variance




[a] LD - Longissimus dorsi
PM - Psoas major
BF - Biceps femoris
ST - Semitendinosus

Figure 3 - Experimental Layout

# RESULTS AND DISCUSSION

## Experiment 1

### Procedure

Difficulty was encountered with the total pigment procedure in obtaining a clear extract. Frequently, filtration of the supernatant through Whatman #3 filter paper, by use of a buchner funnel and suction flask, resulted in a turbid filtrate. Further experimentation led to the finding that by a second filtration through Whatman #3 filter paper under gravity flow, a clear extract could be obtained. However, there was one exception observed with the semitendinosus muscle sample obtained from animal number 5. The pH of this muscle was 6.4, and the extract could not be cleared by either centrifugation or filtration. This coincided with pilot studies which concluded that muscle samples with a pH of 6.3 or above rendered a turbid supernatant upon centrifugation which was not clarified by filtration. It was possible to obtain a clear extract from such muscles by using a 0.01N acetate buffer of pH 4.5 in place of the boiled, cooled distilled water which was usually used in the extractions. (DeDuve, 1948 and Fleming et al., 1960).

After prolonged standing at room temperature, approximately 10 hours, the clear total pigment extract became cloudy even after conversion to the cyanmet-myoglobin derivatives. To alleviate this problem, optical density readings were

made on the clear solutions within three hours from the time of extraction. Owing to this difficulty of obtaining clear extracts, it was not possible to make duplicate readings for the total pigment determination in this experiment. Thus, analysis of variance was not performed on these data because the error variance could not be estimated.

In all cases, the procedure used in the myoglobin determination resulted in clear, stable solutions. Experimentation showed that these solutions were stable for 24 hours, no matter whether stored at room temperature or in the cold room. This allowed for greater flexibility in the procedure since many samples could be prepared prior to reading the percent transmittance.

Another point investigated in the myoglobin determination procedure was the effect on percent transmittance caused by the addition of potassium cyanide and potassium ferric cyanide. Two methods were used to study this question. First, the Bausch and Lomb Spectronic 20 was standardized with a water blank. This water blank was then replaced with a cuvette containing water plus potassium cyanide and potassium ferric cyanide at the same concentrations as specified in the procedure. There was no difference in the percent transmittance readings. Thus, at a wavelength of 540 mu, the light absorbed by potassium cyanide and potassium ferric cyanide was considered to be negligible. As a result, all readings were made on the sample solutions after first standardizing the instrument with a water blank. The second method sought to determine how varying amounts of potassium cyanide and potassium ferric cyanide would effect the percent transmittance readings. Cuvettes containing the sample solution, which had been

converted to the cyanmetmyoglobin derivative as prescribed by the procedure, were read. Additional potassium cyanide and potassium ferric cyanide, up to .3 gm of each, were added to each cuvette and the samples were read again. Since a slight depression in the readings occurred at the higher concentration, the samples were analyzed with the concentration of potassium cyanide and potassium ferric cyanide being maintained as prescribed in the procedure.

Pigment concentration

The analysis of variance for myoglobin concentration indicated that a highly significant ($P<.01$) animal by muscle interaction existed irrespective of the weight basis to which the myoglobin concentration values were compared (Table IV). This animal by muscle interaction measures the failure of the myoglobin concentration of each muscle to be the same for each animal, or conversely, the failure of the myoglobin concentration in the five animals to be the same for each muscle. As a result of the animal by muscle interaction, this became the correct term to test the significance of the muscle and animal effects. Both the muscle and animal effects were highly significant ($P<.01$), and again the significance level of each was independent of the manner in which the sample weight was expressed. Thus, the correction of the sample weight for fat and moisture did not eliminate the animal by muscle interaction nor did this correction reduce the level of significance of the other sources of variation.

The analysis of variance for percent fat and moisture (Table VI) also indicated a highly significant ($P<.01$) animal by muscle interaction. For percent moisture, the muscle and animal source of variance was statistically significant ($P<.05$).

TABLE IV

ANALYSIS OF VARIANCE FOR MYOGLOBIN CONCENTRATION
Experiment I

| Source | df | Wet wt. M.S. | Wet wt. F-test | Dry wt. M.S. | Dry wt. F-test | Wet, Fat-free wt. M.S. | Wet, Fat-free wt. F-test | Dry, Fat-free wt. M.S. | Dry, Fat-free wt. F-test |
|---|---|---|---|---|---|---|---|---|---|
| Total | 39 | | | | | | | | |
| Animal | 4 | 0.809 | 6.80** | 12.963 | 4.96** | 0.759 | 7.37** | 12.835 | 8.49** |
| Muscle | 3 | 3.040 | 25.55** | 38.653 | 14.82** | 3.540 | 34.37** | 58.386 | 38.62** |
| Animal x Muscle | 12 | 0.119 | 47.60** | 2.609 | 84.16** | 0.103 | 34.33** | 1.512 | 32.17** |
| Error | 20 | 0.0025 | | 0.031 | | 0.003 | | 0.047 | |

(**) P<.01

TABLE V

COMPONENTS OF VARIANCE FOR MYOGLOBIN CONCENTRATION
Experiment I

| Source | Wet wt. $\sigma_i^2$ | Wet wt. % of total variation | Dry wt. $\sigma_i^2$ | Dry wt. % of total variation | Wet, Fat-free wt. $\sigma_i^2$ | Wet, Fat-free wt. % of total variation | Dry, Fat-free wt. $\sigma_i^2$ | Dry, Fat-free wt. % of total variation |
|---|---|---|---|---|---|---|---|---|
| Total | 0.7650 | 100.00 | 9.9572 | 100.00 | 0.8594 | 100.00 | 14.3258 | 100.00 |
| Animal | 0.1725 | 22.55 | 2.5885 | 26.00 | 0.1640 | 19.08 | 2.8308 | 19.76 |
| Muscle | 0.5842 | 76.37 | 7.2088 | 72.40 | 0.6874 | 79.99 | 11.3748 | 79.40 |
| Animal x Muscle | 0.0058 | 0.76 | 0.1289 | 1.29 | 0.0050 | 0.58 | 0.0732 | 0.51 |
| Error | 0.0025 | 0.32 | 0.0310 | 0.31 | 0.0030 | 0.35 | 0.0470 | 0.33 |

TABLE VI

ANALYSIS OF VARIANCE FOR PERCENT FAT AND MOISTURE
Experiment 1

| Source | df | Percent Fat M.S. | Percent Fat F-test | Percent Moisture M.S. | Percent Moisture F-test |
|---|---|---|---|---|---|
| Total | 39 | | | | |
| Animal | 4 | 9.998 | 1.33 | 10.287 | 2.91* |
| Muscle | 3 | 37.182 | 4.96* | 20.982 | 5.94* |
| Animal x Muscle | 12 | 7.502 | 51.38** | 3.529 | 11.61** |
| Error | 20 | 0.146 | | 0.304 | |

(*) $P<.05$
(**) $P<.01$

TABLE VII

COMPONENTS OF VARIANCE FOR PERCENT FAT AND MOISTURE
Experiment 1

| Source | Percent Fat $\sigma_i^2$ | Percent Fat % of total variation | Percent Moisture $\sigma_i^2$ | Percent Moisture % of total variation |
|---|---|---|---|---|
| Total | 7.0738 | 100.00 | 5.6453 | 100.00 |
| Animal | 0.6240 | 8.82 | 1.6895 | 29.93 |
| Muscle | 5.9360 | 83.92 | 3.4906 | 61.83 |
| Animal x Muscle | 0.3678 | 5.20 | 0.1612 | 2.86 |
| Error | 0.1460 | 2.06 | 0.3040 | 5.38 |

For percent fat, only the muscle source of variance was statistically significant ($P<.05$). This is substantiated by the knowledge that different muscles contain varying amounts of fat because of the different anatomical location of the vascular system to the muscle, the vascular system being the primary factor governing fat deposition patterns. It was expected that the difference in fat content of the four muscles between animals would be minimized since the carcasses were selected from the same grade.

From the plots of myoglobin concentration for the respective muscles studied versus animal, the sites of interaction between muscle and animal becomes apparent (Figure 4). The existence of this interaction is illustrated by the points of intersection and also by the converging or diverging regions in the curves.

Since the error term associated with the analysis of variance for myoglobin concentration was so small, significance was readily obtained. This can be further understood by the relationship between the error term and the significance of the factors tested. As the error associated with a test approaches zero, the significance of the factors tested approaches infinity. As a result of this concept, the components of variance were calculated to determine the relative importance of each (Table V). From this data, the animal by muscle component of variance, wet tissue basis, accounted for only 0.76 percent of the total variation. Correction of the sample weight for both fat and moisture had the effect of reducing this component to 0.51 percent of the total variation. The largest source of variation was associated with the muscle component which accounted for 76.37 percent of the total variation on a wet tissue weight basis or 79.40 percent on a dry, fat-free

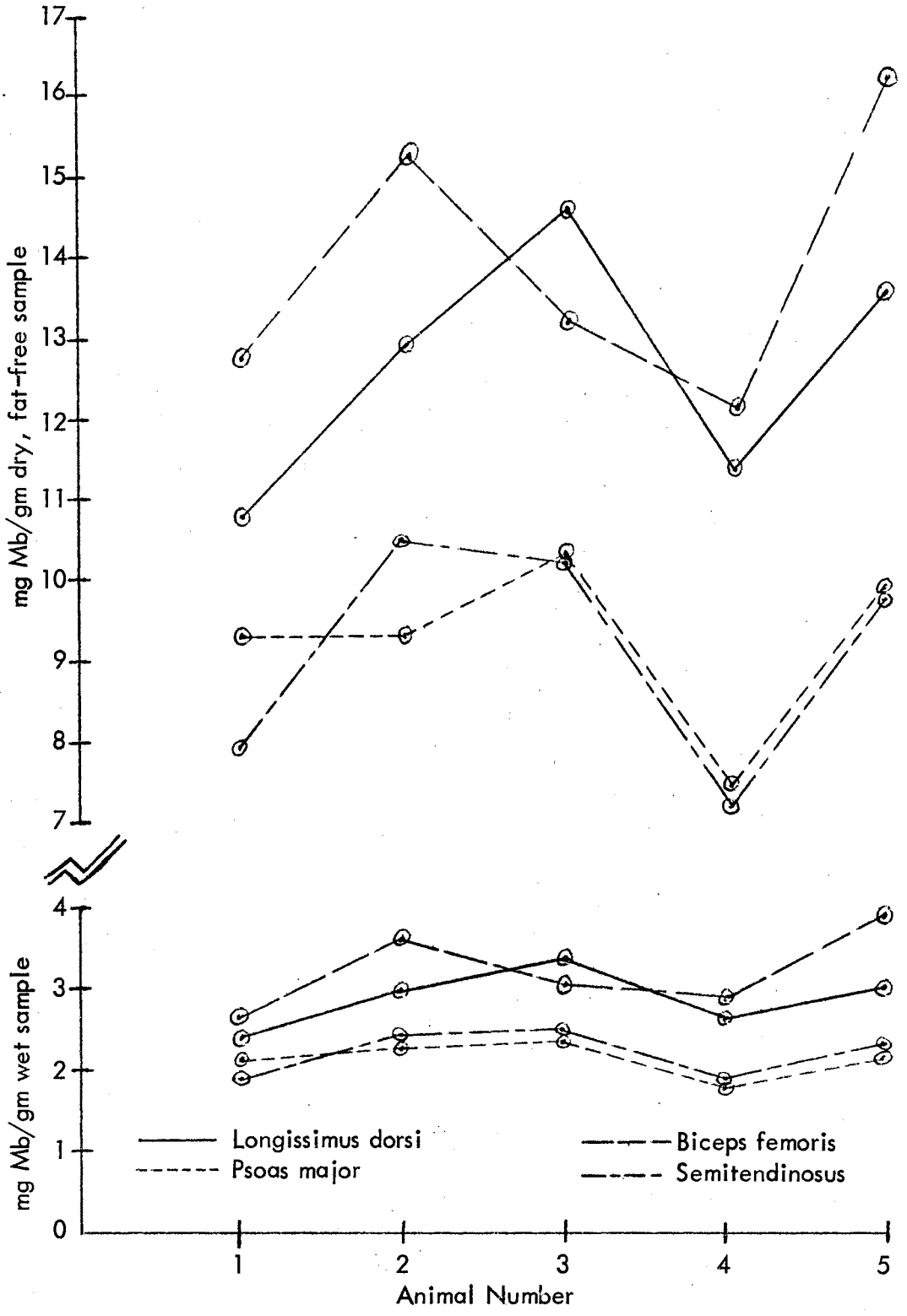



Figure 4 - Myoglobin Concentration as Influenced by Animal and Muscle Differences (Experiment 1)

tissue basis. The next largest component was the animal source of variation which represented 22.55 percent of the total variation on a wet tissue weight basis or 19.76 percent on a dry, fat-free weight basis. These same relationships exist for the components of variance associated with the fat and moisture analysis (Table VII). In contrast to the myoglobin results, the error term for fat and moisture comprises a larger percentage of the total variation, 2.06 percent and 5.38 percent respectively. This shows that greater precision was obtained with the myoglobin determination then with the proximate analysis.

From the study on the components of variance, it was felt that justification existed to point out muscle trends. The muscle means (Table VIII) indicate that myoglobin concentration is the greatest in the biceps femoris followed by the longissimus dorsi, psoas major, and semitendinosus muscles when concentration values were expressed on a fat-free or dry, fat-free tissue basis. The semitendinosus muscle supercedes the psoas major muscle in myoglobin concentration when the values were expressed on a wet tissue or a dry tissue weight basis. This shifting in values could be partially associated with the greater error in the moisture determination as opposed to the fat determination. The greatest contributor to this deviation was a result of the psoas major muscle containing an excess of twice the fat content over the semitendinosus muscle. Since adipose tissue contains no myoglobin, the uncorrected sample weight for the semitendinosus muscle would represent a greater lean tissue mass than for the psoas major.

A high correlation exists between percent moisture and percent fat, $r = -.86$ and $r = -.81$, in bovine muscle. (Henrickson et al., 1963; and

TABLE VIII

PIGMENT, MOISTURE AND FAT CONTENT OF FOUR BOVINE MUSCLES (Experiment 1)[a]

| | Longissimus Dorsi | Psoas Major | Biceps Femoris | Semitendinosus |
|---|---|---|---|---|
| Myoglobin, mg/gm tissue | | | | |
| wet | 2.90 | 2.08 | 3.20 | 2.15 |
| dry | 9.80 | 6.98 | 11.44 | 8.03 |
| wet, fat-free | 3.10 | 2.25 | 3.39 | 2.22 |
| dry, fat-free | 12.65 | 9.27 | 13.91 | 9.11 |
| Total pigment, mg/gm tissue | | | | |
| wet | 4.46 | 4.47 | 5.46 | 3.59 |
| dry | 15.16 | 14.93 | 19.53 | 13.40 |
| wet, fat-free | 4.77 | 4.84 | 5.80 | 3.71 |
| dry, fat-free | 19.49 | 19.90 | 23.75 | 15.40 |
| Moisture, % | 70.34 | 69.98 | 71.88 | 73.11 |
| Fat, % | 6.82 | 7.54 | 5.21 | 3.20 |

[a] Each number represents the mean of ten analyses.

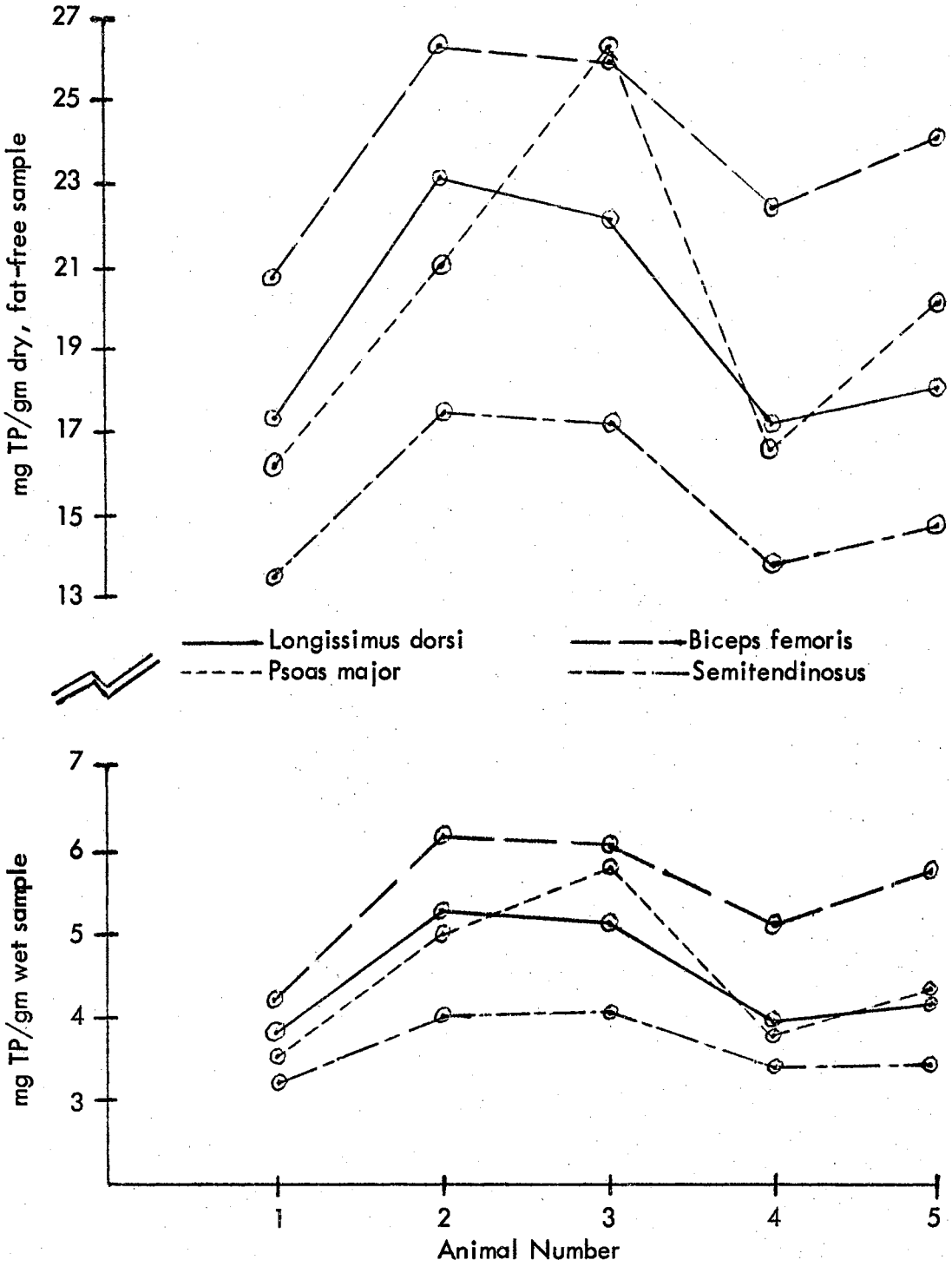



Figure 5 - Total Pigment Concentration as Influenced by Animal and Muscle Differences (Experiment 1)

Romans et al., 1965, respectively). This same converse relationship of percent fat and percent moisture can be seen in Table VIII where the muscle with the highest fat content contains the lowest moisture content, the converse also being true.

Although statistical analysis of the total pigment determination was not accomplished in this trial, the similarity of the curves is noteworthy (Figure 5). With the exception of the psoas major curve, the muscle trends are comparative to those found for myoglobin. To avoid repetition, the erratic values obtained on the psoas major muscles will be discussed as a part of the "evaluation of procedure" section of this manuscript.

## Experiment 2

### Procedure

The problems encountered with the total pigment determination were resolved during the first experiment. Therefore, in this study duplicate determinations for myoglobin and total pigment were made which allowed for a more critical evaluation of the data.

### Pigment concentration

The analysis of variance on total pigment and myoglobin concentration indicated that the same relationships existed between the sources tested commensurate with the results of Experiment 1. A highly significant ($P<.01$) animal by muscle interaction existed for both total pigment concentration and myoglobin concentration (Table IX and Table XI, respectively). This interaction term was then used to test the animal and muscle sources of variation which proved to be

highly significant ($P < .01$).

The existence of the small error term associated with each determination prompted the elucidation of the components of variance. The muscle component is the largest, representing 84.43 percent of the total variation for total pigment concentration on a wet tissue weight basis and 72.74 percent of the total variation for myoglobin concentration on a wet tissue basis (Tables X and XII, respectively). The variation due to muscle is greatest for total pigment concentration, and conversely, the component of variance caused by animal difference is of greater magnitude for myoglobin concentration. Since the total pigment content is the sum total of hemoglobin and myoglobin, the increase between muscle variance for total pigment versus the myoglobin concentration would indicate that residual blood remaining in the tissues exerts a larger influence on the different muscles than that caused by animal differences. The animal component would be of lesser importance for the total pigment determination because these steers were all slaughtered on the same day and under the same conditions. This signifies that residual blood did not differ as greatly between animals as it did between muscles. It should be re-emphasized that the animal source of variance was highly significant for the total pigment determination. This can be accounted for, in part, from the knowledge that the amount of blood drainage is not constant from animal to animal. The amount removed is dependent upon the time at which the heart quits functioning when the animal is slaughtered. Hence, the magnitude of the animal component of variance could be influenced by ineffective bleeding or undesirable environmental conditions to which the animals were subjected prior

TABLE IX

ANALYSIS OF VARIANCE FOR TOTAL PIGMENT CONCENTRATION
Experiment 2

| Source | df | Wet wt. M.S. | Wet wt. F-test | Dry wt. M.S. | Dry wt. F-test | Wet, Fat-free wt. M.S. | Wet, Fat-free wt. F-test | Dry, Fat-free wt. M.S. | Dry, Fat-free wt. F-test |
|---|---|---|---|---|---|---|---|---|---|
| Total | 55 | | | | | | | | |
| Animal | 6 | 0.956 | 10.28** | 14.626 | 9.62** | 1.025 | 9.07** | 18.403 | 5.50** |
| Muscle | 3 | 8.991 | 96.68** | 86.631 | 56.99** | 10.646 | 94.21** | 159.193 | 47.59** |
| Animal x Muscle | 18 | 0.093 | 6.64** | 1.520 | 8.89** | 0.113 | 7.06** | 3.345 | 12.67** |
| Error | 28 | 0.014 | | 0.171 | | 0.016 | | 0.264 | |

(**) P<.01

TABLE X

COMPONENTS OF VARIANCE FOR TOTAL PIGMENT CONCENTRATION
Experiment 2

| Source | Wet wt. $\sigma_i^2$ | Wet wt. % of total variation | Dry wt. $\sigma_i^2$ | Dry wt. % of total variation | Wet, Fat-free wt. $\sigma_i^2$ | Wet, Fat-free wt. % of total variation | Dry, Fat-free wt. $\sigma_i^2$ | Dry, Fat-free wt. % of total variation |
|---|---|---|---|---|---|---|---|---|
| Total | 1.5037 | 100.00 | 15.6544 | 100.00 | 1.7522 | 100.00 | 26.4025 | 100.00 |
| Animal | 0.2158 | 14.35 | 3.2765 | 20.93 | 0.2280 | 13.01 | 3.7645 | 14.26 |
| Muscle | 1.2711 | 84.53 | 12.1587 | 77.67 | 1.5047 | 85.88 | 22.2640 | 84.32 |
| Animal x Muscle | 0.0028 | 0.19 | 0.0482 | 0.31 | 0.0035 | 0.20 | 0.1100 | 0.42 |
| Error | 0.0140 | 0.93 | 0.1710 | 1.09 | 0.0160 | 0.91 | 0.2640 | 1.00 |

TABLE XI

ANALYSIS OF VARIANCE FOR MYOGLOBIN CONCENTRATION
Experiment 2

| Source | df | Wet wt. | | Dry wt. | | Wet, Fat-free wt. | | Dry, Fat-free wt. | |
|---|---|---|---|---|---|---|---|---|---|
| | | M.S. | F-test | M.S. | F-test | M.S. | F-test | M.S. | F-test |
| Total | 55 | | | | | | | | |
| Animal | 6 | 1.702 | 17.19** | 21.814 | 14.83** | 1.826 | 16.75** | 29.010 | 11.89** |
| Muscle | 3 | 7.742 | 78.20** | 83.265 | 56.60** | 8.713 | 79.94** | 129.700 | 53.18** |
| Animal x Muscle | 18 | 0.099 | 19.80** | 1.471 | 27.24** | 0.109 | 18.17** | 2.439 | 28.69** |
| Error | 28 | 0.005 | | 0.054 | | 0.006 | | 0.085 | |

(**) $P < .01$

TABLE XII

COMPONENTS OF VARIANCE FOR MYOGLOBIN CONCENTRATION
Experiment 2

| Source | Wet wt. | | Dry wt. | | Wet, Fat-free wt. | | Dry, Fat-free wt. | |
|---|---|---|---|---|---|---|---|---|
| | $\sigma_i^2$ | % of total variation | $\sigma_i^2$ | % of total variation | $\sigma_i^2$ | % of total variation | $\sigma_i^2$ | % of total variation |
| Total | 1.5010 | 100.00 | 16.8752 | 100.00 | 1.6680 | 100.00 | 24.9920 | 100.00 |
| Animal | 0.4008 | 26.70 | 5.0858 | 30.14 | 0.4292 | 25.73 | 6.6428 | 26.58 |
| Muscle | 1.0918 | 72.74 | 11.6848 | 69.24 | 1.2291 | 73.69 | 18.1801 | 72.74 |
| Animal x Muscle | 0.0034 | 0.23 | 0.0506 | 0.30 | 0.0037 | 0.22 | 0.0841 | 0.34 |
| Error | 0.0050 | 0.33 | 0.0540 | 0.32 | 0.0060 | 0.36 | 0.0850 | 0.34 |

to slaughter.

The analysis of variance for fat and moisture (Table XIII) indicated a highly significant ($P<.01$) animal by muscle interaction, which was in keeping with the results obtained in Experiment 1. For the moisture content of muscle, the animal and muscle sources of variance were also found to be highly significant ($P<.01$); for the fat content, the muscle source was highly significant ($P<.01$) with the animal source also significant ($P<.05$). In Table XIV, the relative importance of each source of variation was investigated by calculating the components of variance. From these results, it was found that the error term associated with each test constitutes a greater portion of the total variation than does the animal by muscle term. The largest contributor to the total variation was due to muscle differences followed by animal differences. Correction of the data for fat and/or moisture did not reduce the level of statistical significance found for total pigment concentration or myoglobin concentration. This would be expected since the analysis of variance on percent moisture and percent fat exhibited the same relationships between the sources of variation as was found for total pigment and myoglobin concentrations when expressed on a wet tissue basis.

Graphic representation of the total pigment and myoglobin concentrations is presented in Figures 6 and 7. The major contributor to the animal by muscle interaction for the total pigment concentration is the overlapping of values between the longissimus dorsi and psoas major muscles as denoted by the points of intersection between the two curves. For myoglobin concentration, the animal by muscle interaction is associated with the point of intersection between the

TABLE XIII

ANALYSIS OF VARIANCE FOR PERCENT FAT AND MOISTURE
Experiment 2

| Source | df | Percent Fat | | Percent Moisture | |
|---|---|---|---|---|---|
| | | M.S. | F-test | M.S. | F-test |
| Total | 55 | | | | |
| Animal | 6 | 5.768 | 3.81* | 4.678 | 4.25** |
| Muscle | 3 | 20.704 | 13.68** | 10.369 | 9.43** |
| Animal x Muscle | 18 | 1.514 | 13.40** | 1.100 | 2.98** |
| Error | 28 | 0.113 | | 0.369 | |

(*) $P<.05$
(**) $P<.01$

TABLE XIV

COMPONENTS OF VARIANCE FOR PERCENT FAT AND MOISTURE
Experiment 2

| Source | Percent Fat | | Percent Moisture | |
|---|---|---|---|---|
| | $\sigma_i^2$ | % of total variation | $\sigma_i^2$ | % of total variation |
| Total | 3.9679 | 100.00 | 2.6137 | 100.00 |
| Animal | 1.0635 | 26.80 | 0.8945 | 34.22 |
| Muscle | 2.7414 | 69.09 | 1.3241 | 50.66 |
| Animal x Muscle | 0.0500 | 1.26 | 0.0261 | 1.00 |
| Error | 0.1130 | 2.85 | 0.3690 | 14.12 |

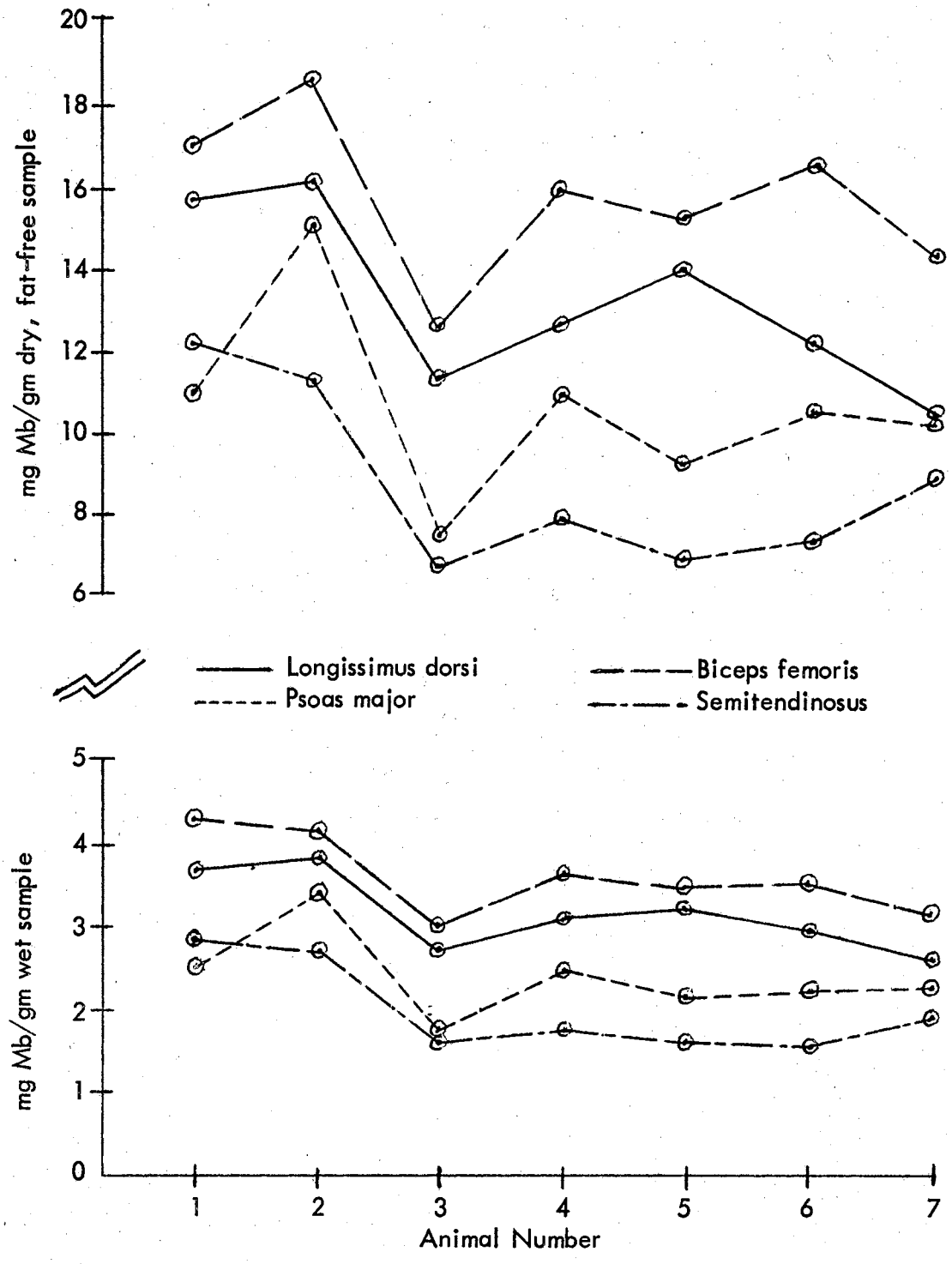



Figure 6 - Myoglobin Concentration as Influenced by Animal and Muscle Differences (Experiment 2)

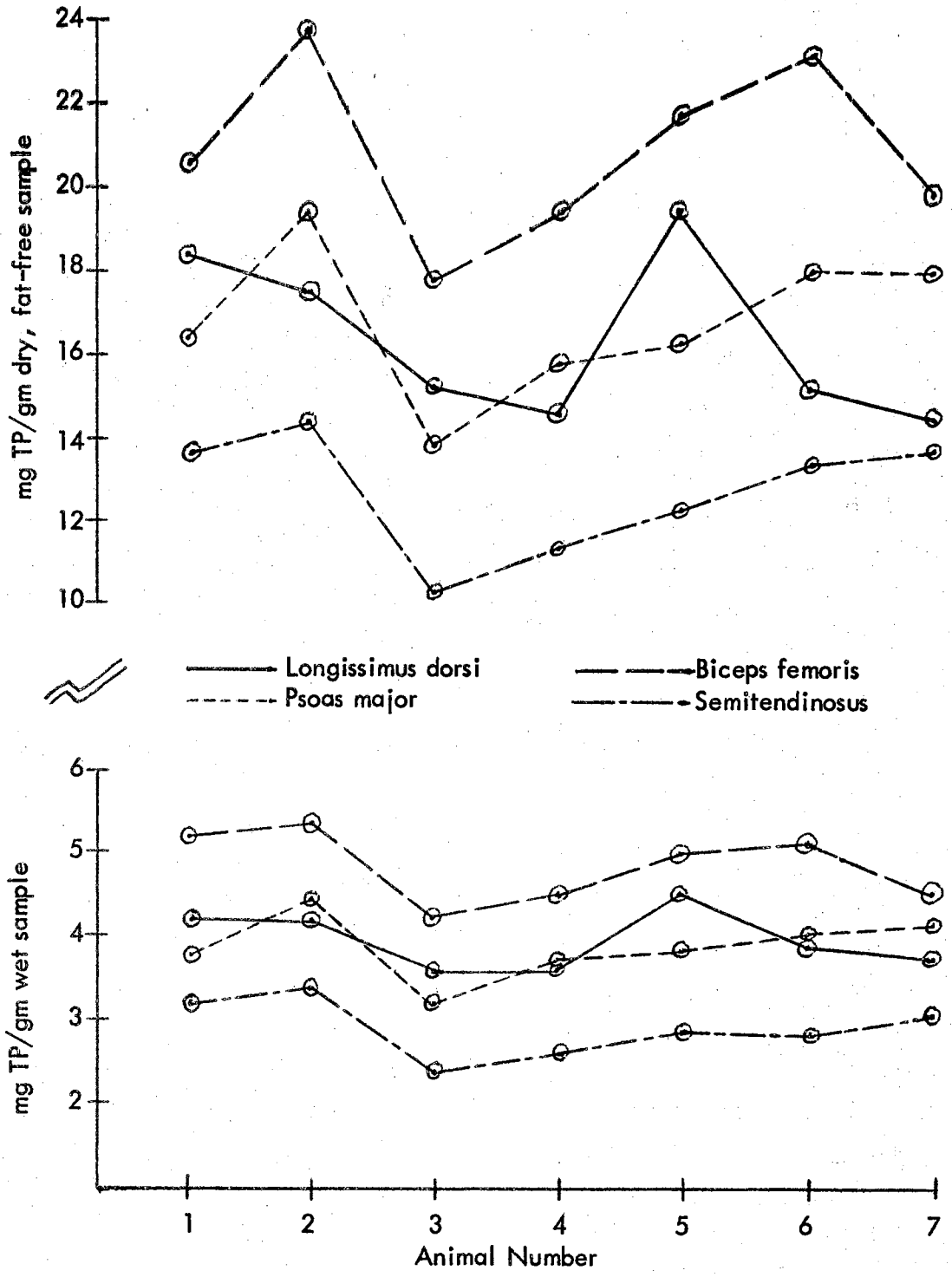



Figure 7 - Total Pigment Concentration as Influenced by Animal and Muscle Differences (Experiment 2)

TABLE XV

PIGMENT, MOISTURE AND FAT CONTENT OF FOUR BOVINE MUSCLES (Experiment 2)[a]

| | Longissimus Dorsi | Psoas Major | Biceps Femoris | Semitendinosus |
|---|---|---|---|---|
| Total pigment, mg/gm tissue | | | | |
| wet | 3.97 | 3.84 | 4.85 | 2.89 |
| dry | 13.94 | 12.91 | 16.35 | 10.34 |
| wet, fat-free | 4.15 | 4.12 | 5.18 | 3.05 |
| dry, fat-free | 16.40 | 16.84 | 20.94 | 12.69 |
| Myoglobin, mg/gm tissue | | | | |
| wet | 3.18 | 2.40 | 3.64 | 1.99 |
| dry | 11.16 | 8.10 | 12.26 | 7.13 |
| wet, fat-free | 3.32 | 2.60 | 3.89 | 2.10 |
| dry, fat-free | 13.13 | 10.57 | 15.70 | 8.72 |
| Moisture, % | 71.43 | 70.26 | 70.30 | 72.00 |
| Fat, % | 4.30 | 6.95 | 6.50 | 5.21 |

[a] Each value represents the mean for 14 analyses.

TABLE XVI

PIGMENT, MOISTURE AND FAT CONTENT AS INFLUENCED BY ANIMAL DIFFERENCES (Experiment 2)[a]

| | Animal Number | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Total pigment, mg/gm tissue | | | | | | | |
| wet | 4.14 | 4.37 | 3.37 | 3.55 | 4.04 | 3.92 | 3.81 |
| dry | 13.93 | 15.52 | 11.82 | 11.87 | 14.31 | 13.54 | 12.70 |
| wet, fat-free | 4.40 | 4.60 | 3.54 | 3.80 | 4.27 | 4.19 | 4.09 |
| dry, fat-free | 17.26 | 18.80 | 14.24 | 15.31 | 17.47 | 17.39 | 16.54 |
| Myoglobin, mg/gm tissue | | | | | | | |
| wet | 3.34 | 3.53 | 2.26 | 2.73 | 2.61 | 2.63 | 2.52 |
| dry | 11.29 | 12.56 | 7.94 | 9.13 | 9.23 | 9.09 | 8.40 |
| wet, fat-free | 3.54 | 3.71 | 2.37 | 2.93 | 2.79 | 2.81 | 2.71 |
| dry, fat-free | 13.92 | 15.18 | 9.54 | 11.76 | 11.29 | 11.60 | 10.91 |
| Moisture, % | 70.32 | 71.89 | 71.48 | 70.30 | 71.78 | 71.17 | 70.05 |
| Fat, % | 5.78 | 4.76 | 4.92 | 6.59 | 5.05 | 6.22 | 6.86 |

[a] Each value represents the mean for eight analyses.

semitendinosus and psoas major curves, and also by the diverging or converging areas in the curves.

Although the animal by muscle interaction does exist, the relative importance of this source of variance was minimized when the components of variance were calculated. As a result, justification for comparisons between muscles and animals existed. On muscle mean comparisons (Table XV), the total pigment concentration is the greatest in the biceps femoris followed in turn by the longissimus dorsi, psoas major and semitendinosus muscles. Only when the total pigment concentration was expressed on a dry, fat-free tissue basis did the psoas major concentration exceed that of the longissimus dorsi. The greater correction is due to fat and moisture of the sample weights associated with the psoas major muscle. On the other hand, the myoglobin concentration muscle means (Table XVI) do not change the order of the muscles regardless of the weight basis on which the concentrations were compared. Biceps femoris, longissimus dorsi, psoas major and semitendinosus was the descending order found in terms of myoglobin concentration. Since the difference in concentration values between the longissimus dorsi and psoas major muscles was greater in the myoglobin determination, it appears that hemoglobin contributes more to the visual color in the psoas major than in the longissimus dorsi.

In comparing animal means, the number 3 animal was found to contain the least total pigment and myoglobin. This was to be expected since the animal was a month younger than the next youngest steer. It is difficult to explain any further the differences in animal total pigment or myoglobin concentration since these values do not follow any definite pattern with respect to age or source of protein.

## Evaluation of Procedure

An estimate of the error variance of the total pigment determination was not obtainable in Experiment 1 owing to the difficulty of obtaining a clear extract. Since this problem was overcome in Experiment 2, the variance associated with the determination was found to be 0.014 which represented 0.93 percent of the total variation when the calculated values were expressed on a wet tissue weight basis. This error mean square primarily contains sampling error, which is the failure of two random samples from a muscle to be the same. A small portion of this error mean square could be attributed to determination error, the failure of two random aliquots from a sample treated alike to respond alike. The reasoning behind the above two statements is that this determination entails only one filtration after duplicate extraction of the muscle sample with boiled, cooled distilled water. In addition, pilot studies indicated that duplicate extractions removed all of the pigments from a muscle sample. These two items would tend to minimize the effect from determination error. This procedure does provide a precise method for the determination of total pigment. Since the error was so small, highly sensitive tests were obtainable with this procedure.

The myoglobin determination employed in this study proved to be a precise, highly sensitive method. The sensitivity of the procedure is dependent upon the error variance which was found to be 0.0025 in Experiment 1 and 0.005 in Experiment 2 when myoglobin concentration values were expressed on a wet tissue weight basis. The standard error then becomes $\pm$ 0.05 for Experiment 1 and $\pm$ 0.071 for Experiment 2. To gain insight into the accuracy of this method,

the percent of other pigments present in each sample was calculated by subtracting the mean myoglobin value from the mean total pigment value and then dividing this quantity by the mean total pigment value (Table XVII and Table XVIII). Since the cytochromes, flavins, and vitamin $B_{12}$ are present in muscle in such minute quantities, they probably contribute little, if anything, to the color of lean tissue. Therefore, these percentages represent mainly the amount of hemoglobin present in the samples. These values are higher then the expected range of 3-10 percent hemoglobin content for bovine skeletal muscle as reported by Shenk et al. (1934). It would appear that some of the myoglobin was also precipitated in the salting out process of myoglobin from hemoglobin which is in keeping with the findings of others. (Craig et al., 1959 and Fleming et al., 1960). However, no red color was observed in the precipitate which would be expected if the above statement is true.

Although these values appear to be high, they indicate that amounts of residual blood left in the tissues differ between the different muscles. It may be that these differences are associated with the vascular patterns which vary widely from one muscle to another. (Abramson, 1962). Lee (1958) noted that in the rabbit the red muscle, which contracts more slowly but has greater endurance, contains many tortuous capillaries. Sac-like dilatations are commonly found at the branching points of arterioles into capillaries. These dilatations may serve as energy reservoirs of blood. In white muscle, on the other hand, the arteriolar branching is regular and the capillaries follow a straight course. Although the pigmentation of the muscles used in this study do not represent the extreme

TABLE XVII

THE PERCENT OF PIGMENT IN FOUR BOVINE MUSCLES OTHER THAN MYOGLOBIN
Experiment 1

| Animal Number | Longissimus Dorsi | Psoas Major | Biceps Femoris | Semitendinosus | Animal Mean |
|---|---|---|---|---|---|
| 1 | 37.17 | 41.81 | 38.00 | 40.19 | 39.29 |
| 2 | 43.69 | 55.20 | 41.68 | 39.75 | 45.08 |
| 3 | 33.86 | 60.86 | 49.01 | 39.90 | 45.91 |
| 4 | 33.25 | 54.38 | 45.72 | 47.18 | 45.13 |
| 5 | 25.06 | 50.23 | 32.06 | 33.43 | 35.20 |
| Muscle Mean | 34.61 | 52.50 | 41.29 | 40.09 | |

TABLE XVIII

THE PERCENT OF PIGMENT IN FOUR BOVINE MUSCLES OTHER THAN MYOGLOBIN
Experiment 2

| Animal Number | Longissimus Dorsi | Psoas Major | Biceps Femoris | Semitendinosus | Animal Mean |
|---|---|---|---|---|---|
| 1 | 14.71 | 33.86 | 17.56 | 10.94 | 19.27 |
| 2 | 9.24 | 23.32 | 22.49 | 21.22 | 19.07 |
| 3 | 24.86 | 46.27 | 29.11 | 34.45 | 33.67 |
| 4 | 13.76 | 31.96 | 18.75 | 30.95 | 23.86 |
| 5 | 28.54 | 43.42 | 30.06 | 44.95 | 36.74 |
| 6 | 21.35 | 41.49 | 28.91 | 44.01 | 33.94 |
| 7 | 28.18 | 43.49 | 28.12 | 36.00 | 33.95 |
| Muscle Mean | 20.09 | 37.69 | 25.00 | 31.79 | |

differences studied by Lee, a parallelism to the above capillary patterns might exist between light and dark bovine muscle. For example, say the semitendinosus muscle had a vascular system similar to that found in white rabbit muscle. Then part of the reason the more active semitendinosus muscle contains less myoglobin and hemoglobin than the longissimus dorsi muscle would be due to the more direct blood supply. In effect, this would reduce the need for an oxygen store, myoglobin, and lessen the amount of hemoglobin trapped in the muscle when the animal is slaughtered. The actual intramuscular vascular patterns that are present in different bovine muscles have been given little attention to date. It is thus impossible to make a complete accounting attributed to vascular patterns.

Myoglobin is a heterogeneous mixture containing three distinct fractions which differ in absorptivity values at most wavelengths. (Quinn and Pearson, 1964). The quantitative procedure employed in this study has the advantage over other methods used, because the differences between myoglobin fractions disappear when analyzed in the cyanmetmyoglobin form. (Perkoff et al., 1962). Autoxidation which is frequently encountered in other procedures is eliminated by use of this procedure for determining myoglobin concentration.

In correcting data for fat and moisture content, allowance should be made for intramuscular fat since it contains no myoglobin or hemoglobin. Adjustment for fat content would further enable the detection of real differences that might not be readily apparent because fat deposition varies between muscles as well as between animals. The reverse could also be true in that the apparent differences might be minimized. Due to the previously mentioned high inverse relationship

between fat and moisture, the correction for fat also accounts for a major portion of the variation due to moisture. However, all the variation due to moisture can not be eliminated until actually determined, and the data then corrected. A matter for conjecture arises about the validity of making a total correction of the data for moisture since a certain amount is associated with the structure of myoglobin and is included in the molecular weight of the molecule. Such a correction would tend to increase values above the actual content. Although this question is primarily of academic interest, the rapid strides made over the last few years in the myoglobin and hemoglobin structures aids in the feasibility of determining that portion of the respective molecular weights actually attributed to liquid.

# SUMMARY

The primary objective of this research was to determine if quantitative differences in total pigment and myoglobin concentrations could be detected, chemically, in muscles which differed in visual color. For this purpose, a portion of the longissimus dorsi, psoas major, biceps femoris and semitendinosus muscles was removed at specific locations from choice grade steer carcasses and used as experimental material.

Initial research efforts were concerned with the identification and resolution of analytical difficulties. Five choice grade steer hindquarters of unknown origin were used in this phase of the test (Experiment 1). The major problem encountered was in obtaining a clear extract for spectrophotometric analysis. Experimentation led to the finding that a clear extract could be obtained by following the initial suction filtration with a second, gravity flow filtration. In addition, it was noted that with muscles of a high pH, a clear extract could be obtained by using a .01N acetate buffer solution for extraction in place of the boiled, cooled distilled water.

Further research was then conducted (Experiment 2) using the perfected procedure. An additional seven hindquarters from choice grade steer carcasses of known origin were used in this phase of the test. The elimination of analytical difficulties coupled with the fact that the muscle samples were from cattle of known origin permitted a more complete chemical and statistical analysis.

Precise results and highly sensitive tests were obtained with the total pigment and myoglobin procedures in Experiment 2. A highly significant ($P<.01$) animal by muscle interaction was found to exist for myoglobin concentration in both experiments. This same highly significant ($P<.01$) animal by muscle interaction existed for the total pigment concentration in Experiment 2. After calculation of the components of variance, this interaction was found to contribute very little to the total variation. The muscle component of variance constituted the major portion of the total variation followed by the animal component.

The greatest total pigment concentration was found in the biceps femoris and the least in the semitendinosus. Little difference was obtained between the longissimus dorsi and the psoas major.

Muscle trends for myoglobin concentration, in decreasing order of magnitude were found to be biceps femoris, longissimus dorsi, psoas major and semitendinosus. The difference between myoglobin concentration and total pigment concentration in the psoas major muscle was a result of hemoglobin. In this specific muscle, hemoglobin was responsible for a large portion of the tissue pigmentation.

Correction of the data for fat and/or moisture did not reduce the level of significance obtained on the variables and interaction tested. In addition, this correction did not eliminate the animal or muscle differences.

From the results of this experiment, research on the vascular patterns present in the respective muscles could provide new avenues in explaining existing myoglobin and hemoglobin concentration differences. A major factor governing

myoglobin concentration is maturity. If studies were to be made of chronological development of the vascular system, the function and necessity of myoglobin in lean tissue might be better understood.

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